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Influences of Disrupted Circadian Rhythms on Stroke Outcome

Jennifer A. Liu

Dissertation submitted to the West Virginia University Health Science Center Neuroscience Program at West Virginia University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Neuroscience

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Keywords: circadian rhythm, circadian disruption, light at night, stroke, ischemic stroke, survival, immune function, vascular dementia

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ABSTRACT

Influences of Disrupted Circadian Rhythms on Stroke Outcome

Jennifer A. Liu

The circadian system is composed of a subset of temporal oscillators that function through a transcriptional and post-translational molecular and functional negative feedback loop cycling approximately every 24 hours. The central clock located in the suprachiasmatic nucleus is responsible for entrainment using light as the key timekeeper (zeitgeber); it is responsible for synchronizing and optimizing physiological behavior and function to the environment. Exogenous information, such as day length and light-dark cycles, provide critical temporal cues for adjusting to environmental conditions. Proper alignment to natural light dark cycles and circadian rhythms is optimal for vital health, fitness, and survival in organisms. Although considered previously innocuous, exposure to nighttime lighting is becoming increasingly prevalent due to development and urbanization. This can disrupt time of day variations to environmental adaptations and current literature has demonstrated that light at night (LAN) substantially disrupts physiological function and behavior, dysregulates circadian organization of immune function, metabolism. Further, this is associated with increased cardiometabolic disease, cancer, and other significant health risks. Exposure to LAN is becoming more pervasive to modern life and environments, where over 80% globally, and up to 99% of the United States and European populations are exposed to nighttime lighting. Circadian disruption has been linked to poor disease outcome in cases such as stroke, sepsis, and global ischemia, and critically ill and compromised patients may be especially at risk and vulnerable to the detrimental impacts of nighttime light.

During acute injury, critically ill hospital patients require modern intensive care, which often results in nighttime lighting exposure. Circadian disruption through dim lighting exposure has been demonstrated to dysregulate pro-inflammatory cytokine production and neuroinflammation, and immune function. This is a concern given that chronic neuroinflammation can potentiate increased secondary damage in patients experiencing injury or exacerbate the effects of aging. However, there are critical gaps of knowledge that delineate the molecular and cellular mechanisms that are identifying how circadian disruption directly affects physiology. Because previous literature has identified neuroinflammation as a key aspect of physiological changes after exposure to light at night, a strategy for delineating how nighttime lighting exposure can change physiology after injury and in aged populations would involve focusing on immune system changes that would likely contribute to injury progression, and physiological aging and cognitive impairments. In this dissertation, I propose that disruptions to circadian rhythms through exposure to dim white light at night (dLAN) disrupts the circadian clock, resulting in increased health risks after ischemic injury, changes to immunological function, and greater susceptibility to cognitive impairments in aged mice. The central hypothesis tested in this dissertation is that **exposure to light at night changes immune physiology and parameters**

that adversely affects injury progression in a murine model of stroke, physiology, and cognitive aging. The studies presented in this dissertation characterize the role of light at night affecting ischemic injury and infarct progression across two murine models of stroke. Further in **Chapter 2**, a cellular mechanism was identified. driving changes in immune response through shifts in microglial population phenotypes that drives increased infarct progression from nighttime lighting. The effect of nighttime lighting on immune activation and stroke outcome was prevented by using alternative spectral lighting that minimally activates intrinsically photosensitive retinal ganglion cells (ipRGCs) **(Chapter 3)**. Next, the role of dLAN was investigated in aged populations that resulted in sex-specific alterations in physiology, immune response, and lifespan in **Chapter 4**. Lastly, I identified the role of disrupted circadian rhythms in aged populations to identify that chronic phase advances increase cognitive impairment and alters a component of vascular structure in aged populations in **Chapter 5**. Taken together, these studies indicate that exposure to ecologically relevant levels of dim broad spectrum (white) LAN can result in changes to physiology that adversely affect the development of neuronal damage and ischemic injury producing poor functional outcomes. Further, these results suggest that disruptions to circadian rhythms in aged populations could be an additional risk factor for cognitive impairments and dementia, and LAN accelerates physiological aging. Together, these studies provide evidence that characterizes and further emphasizes the harmful consequences of disruptions to circadian rhythms and nighttime lighting exposure. These data can provide foundational research required to identify potential chronotherapeutic targets for pharmacological intervention or to improve thrombolytic therapy intervention in ischemic strokes that occur across time of day.

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I would like to first acknowledge and thank my advisors, Drs. Randy Nelson and Courtney DeVries for their mentorship, advisement, and all of the opportunities they have provided for me, that has shaped me into becoming a more confident and successful researcher. Randy has been a supportive and truly dedicated mentor who has allowed me to pursue my research interests and ideas, scientific resources, taught me to conduct hypothesis driven research. Platt's Strong Inference will be forever ingrained into my brain for the remainder of my research career.

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List of symbols, abbreviations, and nomenclature

2-4 dinitro-1-fluorobenzene (DNFB)

2,3,5-triphenyltetrazolium (TTC)

adenosine diphosphate (ADP)

adenosine triphosphate (ATP)

Alzheimer's disease (AD)

ATP-binding cassette (ABC)

ATP-binding cassette sub-family B member 1 (ABCB1)

Blood pressure (BP)

Blood-brain barrier (BBB)

Blue-filtered dim light at night (Bf-dLAN)

Brain and nuscle ARNT-like protein 1 (*Bmal1*)

C-Jun N-terminal kinases (JNKs)

Calcium (Ca^{2+})

Casein kinase II (CKII)

Chronic jet lag (CJL)

Circadian locomotor output cycles kaput (*Clock*)

Colony stimulating factor receptor 1 (CSFR1)

Cognitive impairment (CI)

Complementary Deoxyribonucleic acid (cDNA)

Conditioned stimulus (CS)

Crytochrome (*Cry*)

Delayed-type hypersensitivity test (DTH)

Delta-sleep inducing peptide (DSIP)

Deoxyribonucleic acid (DNA)

Dim light at night (dLAN)

Dopaminergic amacrine (DA)

Endothelial NOS (NOS3)

Endothelium-derived hyperpolarizing factor (EDHF)

Endothelium-derived relaxing factor (EDRF)

Glucose transporter member 5 (Glut5)

Glutamate and pituitary adenylyl cyclase-activating protein (PACAP)

Glutathione reductase (Gsr)

Helper T cells (TH1)

Heme oxygenase 1 (Hmox1)

Hypothalamic-pituitary-gonadal axis (HPG)

Hypothalamic-pituitary-adrenal (HPA)

Inducible NOS (NOS2)

Intergeniculate leaflet (IGL)

Interleukin-10 (IL-10)

Interleukin-1β (IL-1β)

Interleukin-4 (IL-4)

Interleukin-6 (IL-6)

Intracerebral artery (ICA)

Intrinsically photosensitive retinal ganglion cells (iPRGCs)

Light at Night (LAN)

Light dark (LD)

Light-emitting diodes (LED)

Lipopolysaccharide (LPS)

Lipoprotein lipase (Lpl)

Melanopsin (OPN4)

Middle cerebral artery occlusion (MCAO)

Mild cognitive impairments (MCI)

N-methyl-D-aspartate (NMDA)

NADPH oxidase 2 (Nox2)

National Institute of Aging (NIA)

National Institute of Health (NIH)

Natural Killer cells (NK)

Neuronal NOS (NOS1)

Nicotinamide adenine dinucleotide phosphate (NADPH)

Nitric oxide (NO)

Nitric oxide synthase (NOS)

Nuclear factor κB (NFκB)

Olivary pretectal nucleus (OPN)

Period (Per)

Permanent middle cerebral artery occlusion (pMCAO)

Permeability-glycoprotein (Pgp)

PI3-kinase (PI3K)

Plasmin-alpha-2-antiplasmin (PAP)

Plasminogen-activator inhibitor 1 (PAI-1)

Plexxikon 5622 (PLX 5622)

Polymerase chain reaction (PCR)

Prostacyclin (PGI2)

Prostaglandin D2 (PGD2)

Quantitative polymerase chain reaction (qt-PCR)

Red light at night (rLAN)

Retinohypothalamic tract (RHT)

Retinoic acid-related orphan receptor response element (RORE)

Ribonucleic acid (RNA)

Suprachiasmatic nucleus (SCN)

Thrombin-antithrombin (TAT)

Tissue growth factor β1 (TGFβ1)

Tissue plasminogen activator (tPA)

Transient middle cerebral artery occlusion (tMCAO)

Tumor necrosis factor alpha (TNF-α)

Unconditioned stimulus (US)

Vascular contributions to cognitive dementia (VCID)

Ventrolateral preoptic nucleus (VLPO)

Zeitgeber Time (ZT)

A-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)

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CHAPTER 1

DISRUPTIONS TO CIRCADIAN RHYTHMS, LIGHT AT NIGHT, AND THE RISK FOR ADVERSE STROKE OUTCOMES

[1] Liu, et al. *Frontiers Neurosci.*

Note: Part of this chapter includes text and figures obtained from publications highlighted above.

Introduction

Endogenous biological rhythms have evolutionarily developed from the rotation of earth around its axis resulting in consistent brightly illuminated bright solar days and dark night cycles that occurs approximately over a 24 h period. This becomes set precisely to 24 h by external environmental cues, or *zeitgebers* (time-giver) including light information that synchronizes rhythms to the external environment. This is important, given that organisms' physiology and behavior can optimally couple to light dark cycles to adapt and predict changes to the external environment and maintain internal physiology. With the introduction of electrical lighting over the past decade, natural light dark cycles have been obscured, which is problematic because they are important in maintaining rhythms which, when disrupted, can disrupt the molecular clock and alter physiology and behavior. LAN affects up to 80% of the world population and ~99% of people in the United States and Europe. Further, epidemiological evidence from shift workers who experience prolonged exposure to light at night suggests increased risk for adverse health outcomes, including metabolic dysfunction, cardiovascular and cerebrovascular disease, cognitive impairments, neurodegeneration, and risk for stroke. Several aspects of physiology and behavior that are involved and contribute to cerebral ischemic injury and aging are governed by the circadian system. Stroke, caused by a sudden interruption to the blood supply of the brain, has a wide range of detrimental consequences that have contributed to its recognition as the leading cause of disability and second most common cause of death worldwide. It has serious social and economic consequences for both the individual and society. This disease requires hospitalization and intensive critical care throughout the day and night; it is common to expose patients to dimly illuminated hospital rooms during the day and often constant artificial lighting at night during recovery. Lighting practices are considered necessary to monitor patients, but

because previous research has identified that nighttime lighting exposure disrupts physiology, this may pose a potentially elevated risk and susceptibility to sustained injury and slow recovery for patients at risk. Further, aged populations are predisposed to physiological changes and impairment, and disability that can be vulnerable to disrupted physiology from disruptions to circadian rhythms. The work presented within this dissertation will (1) focus on identifying the role of disrupted circadian rhythms through exposure to light at night and other forms of circadian disruption, including chronic jet lag, and (2) characterize its effect on physiology after ischemic outcome and risk for cognitive impairment in aged murine populations. Here, I propose that exposure to light at night is associated with changes to physiology that adversely affect injury progression in a murine model of stroke and physiology and cognitive impairments in aged populations. In this introductory chapter, I will provide an introduction to the circadian system with a focus on models of disruptions to circadian rhythms, including nighttime lighting, shift work, and phase shifts. Additionally within this chapter, I will discuss the relationship between the circadian system and ischemic stroke and injury, by describing how disrupted circadian rhythms are associated with an increased risk for stroke and play a role in stroke outcome. **Chapter 2** will characterize the role of nighttime illumination in the acute infarct development and progression after ischemic injury which will identify a cellular mechanism altering brain-immune interactions resulting in changes to stroke outcome. **Chapter 3** will address and further identify the role of differential lighting intensities and wavelengths from melanopsin-containing retinal ganglion cells in ameliorating the effects associated with nighttime lighting on ischemic injury. Next, I will shift to investigate and characterize how circadian disruption alters physiology in aged populations that are susceptible to modifiable risk factors for dementia and stroke. To address this, **Chapter 4** will investigate how nighttime lighting affects

physiology, immune response, and survival in aged murine populations. Finally, **Chapter 5** evaluates how exposure to other forms of circadian disruption from exposure to shifted phase advances as a model of chronic jet lag can disrupt and result in cognitive impairments, suggesting that chronic circadian disruption increases the risk and development of dementias and components of vasculature.

Circadian Rhythms and the Mammalian Clock

Circadian rhythms are endogenous daily fluctuations in physiology and behavior driven through biological oscillators or clocks, and have been documented in most organisms, from bacteria to vertebrates (Bhadra et al., 2017). Importantly, these biological clocks synchronize internal physiology within an individual and are optimized to promote biological adaptations and survival by adapting to the 24-hour solar day (Daan and Aschoff, 1982). For example, coordinating activity to specific light or dark phases can avoid predation. Circadian rhythms are driven and regulated through a transcriptional-translational feedback loop of core clock genes lasting approximately 24 hours and are synchronized through exposure to the external light-dark cycle as a result of Earth's daily rotation. These external factors that can influence and synchronize the circadian rhythms to the environment are known as zeitgebers (German word for "time-giver"). Zeitgeber time (ZT) is often used in circadian research to standardize time of day, and refers to the unit time based on the duration of time from initiation of the zeitgeber; for example, the onset of lights is designated ZT 0, whereas the onset of darkness 12 h later would be designated ZT 12. Thus, diurnal animals would be most active between ZT 0 and ZT12, whereas nocturnal organisms would be most active between ZT 12 and ZT 24 (Jürgen Aschoff, 1981). Another fundamental component of circadian rhythms is the ability to persist in the absence of any external or environmental cues. Other external cues, including feeding, physical activity, and

social cues, have the capacity to entrain circadian rhythms as well, but generally less effectively than light (Stephan, 2002; Lewis et al., 2018; Aschoff et al., 1971). Because light is a potent zeitgeber that synchronizes internal rhythms, changes such as prolonged exposure to lighting conditions outside of standard light dark cycles, or increased levels of light during the dark period, can contribute to changes in internal rhythms and the molecular clock that are not synchronized to the external environment. This can result in changes to physiology and behavior that can be associated with increased risk for poor health outcomes. In order to investigate how disruptions to circadian rhythms alter disease, it is important to identify the molecular mechanisms driving the circadian clock.

Mechanism of the Molecular Circadian Clock

The mechanism underlying cellular circadian clocks arises from an a transcriptional-translational autoregulatory feedback loop from a distinct set of genes including the Circadian Locomotor Output Cycles Kaput (*Clock*), Brain and Muscle ARNT-like Protein 1 (*Bmal1*), Period (*Per*), and Cytochrome (*Cry*). *Clock* and *Bmal1* encode for proteins containing bHlh-PAS domains, which form the positive arm of the feedback circuit by heterodimerizing to initiate transcription through binding to E-boxes (5'-CACGTG-3') and (5'-CACGTT-3') in the promoter of target genes. In turn, Per and Cry dimerize, and accumulate in the cytoplasm during the circadian day. These proteins will form a complex which then acts as the negative limb of the feedback loop to inhibit *Clock:Bmal1* transcriptional activity for the cycle to repeat, and degradation of Per and Cry proteins are responsible for terminating repression and restarting transcription. This process occurs and takes approximately 24 h to complete a full loop. A second transcriptional feedback loop is initiated by the Clock:Bmal1 dimer which involves E-box mediated transcription of orphan nuclear-receptor genes *Rev-Erbα/β* and *RORα*/β (Guillaumond et al., 2005; Preitner et al.,

2002; Sato et al., 2004). Rev-Erb has been implicated in normal period regulation (Cho et al., 2012), which competes with ROR proteins for Retinoic acid-related Orphan receptor Response Element (RORE) binding sites which are located within the promoter of *Bmal1*. ROR proteins are responsible for initiating *Bmal1* transcription, whereas Rev-Erb inhibits it (Preitner et al., 2002).

Suprachiasmatic nucleus and additional molecular mechanisms entraining the circadian clock

Photoentrainment occurs via light signals to intrinsically photosensitive retinal ganglion cells (ipRGCs) from the retina which depolarize and project through a monosynaptic neuronal pathway known as the retinohypothalamic tract (RHT) to the suprachiasmatic nucleus (SCN). The SCN serves as the master circadian clock in mammals and is responsible for coordinating internal circadian synchronization; it sits at the top of a hierarchy of clocks of various tissue and cell types that display independent circadian gene expression patterns that are entrained by neural and humoral signals from the SCN (Yamazaki et al. 2000, Balsalobre et al. 1998, Tosini et al. 1996). IpRGC's synapse with dopaminergic amacrine cells (DA cells) on the retina. Excitatory signals to DA cells from ipRGC's which conversely, respond with inhibitory signals which are mediated through GABA in conjunction with dopamine (Do and Yau, 2010). Glutamate and pituitary adenylyl cyclase-activating protein (PACAP) is released by iPRGC which excites SCN neurons with PACAP enhancing this response (Butcher et al. 2005). This evoked activation results in the activation of signaling pathways including chromatin remodeling, induction of immediate early genes, and clock genes. The phototransduction process occurs through G-protein coupled receptors, opsins, with light-induced isomerization from 11-

cis-retinal to all-trans-retinal to change conformation of the opsin GPCR to induce signal transduction cascades resulting in closure of cyclic GMP-gated cation channels which in turn hyperpolarizes the photoreceptor (Arshavsky et al. 2002).

IpRGC's further project to other regions including the intergeniculate leaflet (IGL) in the thalamus responsible for playing a role in circadian entrainment, and produces neuropeptide Y release (Smale & Boverhof 1999), the olivary pretectal nucleus (OPN), which is a subset of neurons responsible for pupillary light reflex located in the midbrain, the amygdala, and to the ventrolateral preoptic nucleus (VLPO), which is a group of sleep-active neurons which is hypothesized to inhibit ascending monoaminergic arousal systems during sleep located in the hypothalamus (Do and Yau, 2010). The raphe nuclei is another region that is directly modulated by ipRGC's with the lateral habenula projecting serotoninergic efferent neurons to the SCN (Hay-Schmidt et al. 2003). The pineal gland is controlled and regulated by the suprachiasmatic nuclei, stimulating melatonin biosynthesis during the inactive or dark cycle (also referred to as scotophase). These non-image forming ipRGCs also function to regulate pineal melatonin secretion, the sleep/wake cycle, and pupillary constriction (Fu et al., 2005; (Do and Yau, 2010; Gamlin et al., 2007)

The function of mammalian cellular clock proteins are additionally regulated through posttranslational modifications through various kinases, methylation, polyadenylation, histone modifications, and non-coding RNAs (Uchida et al. 2010; Hirayama et al. 2005; Mendoza-Viveros et al. 2017). Phosphorylation was first discovered to be of major importance in vertebrate circadian clock regulation through the *tau* mutation found in the Syrian Hamster. This

caused a short-period phenotype, where a lowered rate of CKI-dependent phosphorylation of Per2 occurred (Lowrey et al. 2000). Other post-translational regulators including Casein kinase II (CKII), PI3-kinase (PI3K), and c-Jun N-terminal kinases (JNKs) have been identified as regulators for the cellular clock and is integral for overall circadian control. Stability, subcellular localization, transcriptional activity, and interaction with proteins and signaling pathways have additionally been distinguished as under circadian control (Okamoto-Uchida et al. 2019). Although the circadian system is entrained primarily through light, other zeitgebers, including feeding and social cues, have the capacity to entrain rhythms as well.

Within the SCN, *Per* paralogs function as the state variable such that variations in the levels of proteins account for the phase of the clock (Kim et al. 2018). Acute exposure to light at any point during the dark period alters *Per1* expression in the core of the SCN (Shearman et al., 1997; Shigeyoshi et al., 1997) resulting in physiological and behavioral phase advances or delays. Exposure to light during the early dark phase results in increased *Per2* expression in the shell of the SCN whereas light exposure late in the dark phase increases *Per1* expression in the shell of the SCN, leading to a phase delay or phase advance in activity rhythms respectively (Yan & Silver, 2004). This activation of *Per* paralogs occurs through CREB/MAPK signaling acting on cAMP-response elements (CRE) in *Per* promoters (Travnickova-Bendova et al., 2002).

Sources of Circadian Disruption

Daily rhythms of natural bright days and dark nights have shaped the evolution of internal biological clocks almost since the emergence of life, from physiological pathways to behavior. During the past century, widespread adoption of electric lighting and shifts in daily activity in the form of night shift work and social jet lag schedules has drastically affected circadian

organization. Despite the seemingly innocuous nature of prolonged and inconsistent exposure to light in the evening, phase shifts from shift work, or social/leisure activities, several aspects of behavior and physiology have been affected (Navara & Nelson, 2007). Next, this section will discuss several sources of circadian disruption.

Environmental Lighting

The light-dark exposure patterns of humans have vastly changed over the past 100 years with the progression of civilization and industrialization, resulting in exposure to artificial light at night, or alternatively known as light pollution. Sources of illumination include street, architectural, and vehicle lighting, producing radiance from towns and cities via sky glow. Furthermore, light pollution introduces intensities and spectrums of light that differ significantly from naturally occurring starlight and moonlight (Gaston et al., 2014). Approximately 5 lux of light exposure during the night is consistent with current light pollution levels in urban areas (Gaston et al., 2012) and sleeping environments (Obayashi et al. 2017). In comparison, the full moon is generally 0.05-0.2 lux of light at night (Kyba, Mohar, et al., 2017). The light spectrum also varies depending on the light source used, ranging from high pressure sodium bulbs with broad bands of mainly yellow-orange wavelengths to high intensity discharge and light-emitting diodes (LEDs) producing narrow bandwidths with a strong peak of blue wavelengths in the spectrum, creating the appearance of whiter lighting (Falchi et al., 2011). This shift from incandescent bulbs to LEDs occurred during the early 21st century based on reduced energy consumption and cost from the more energy efficient bulbs. IpRGCs are most sensitive to short wavelength (i.e., blue/violet light; ~459 nm) light (Wahl et al., 2019), and exposure to as little as 1.9 $\mu W\ cm^{-2}$ of light at night suppresses nightly melatonin production in humans (Thapan et al., 2001).

Suppression of melatonin induced by lighting exposure during the night has been replicated in other mammalian species (Benshoff et al., 1987; Klein and Weller, 1972; Rollag et al., 1980).

This is a concern given that a recent study using light pollution propagation software and satellite data reports that 83% of the world's population reside under light-polluted skies (>µcd/m^2), with that statistic jumping up to 99% in the United States and Europe (Falchi et al., 2016). Furthermore, light pollution levels have increased at a rate of 2.2% per year (Kyba et al., 2017). Given the importance of light as a zeitgeber for humans and other species, nocturnal light exposure likely has serious consequences for health and wellbeing. Exposure to light during the dark phase can phase advance or delay the circadian clock (Schwartz et al., 2011), depending on the duration, timing, and intensity of the lighting source. Low levels of artificial light at night (5 lux) disrupt circadian rhythmicity with a reduction in Per1 and Per2 expression in the SCN, concurrent with altered expression of *Bmal1, Per1, Per2*, and *Cry1* in the liver of mice (Fonken et al., 2013).

Night Shift Work, Travel, and Social Jet Lag

Coinciding with the increase in light pollution as a result of industrialization, current society has deviated from "9-5" workdays; the majority of the working population is engaged in "non-standard" or irregular hours, encompassing on-call work, weekend work, compressed weeks, telework, split-shifts, or more classically thought of as night shift work (Costa et al., 2004). Indeed, 15-30% of adults are engaged in shift work according to American and European surveys (Boivin & Boudreau, 2014). Social jet lag, defined as the difference in lighting exposure and sleep time between work days and free days, is another form of circadian disruption that has become especially prevalent in adolescent and young adult populations (Crowley et al., 2007;

Valdez et al., 1996; Wittmann et al., 2006). During social, travel, jet lag, or shift work, desynchronization of central and peripheral oscillators occurs. Misalignments of circadian rhythms are responsible for "jet lag" syndrome characterized through fatigue, disturbed sleep rhythms, reduced alertness and ability to perform cognitive tasks, and headaches (Choy & Salbu, 2011).

Molecular Clock's Influence on Cardiovascular, Cerebrovascular, and Immune Parameters Influencing Stroke Risk

Circadian regulation of cardiovascular and cerebrovascular physiology is well documented (Kostenko and Petrova, 2018; Thosar et al., 2018), and includes heart rate and heart rate variability (Massin et al., 2000), sympathetic tone (Panza et al., 1991), blood pressure (Coca, 1994), and cerebral blood flow (Conroy et al., 2005; Hodkinson et al., 2014). Coordinated circadian patterns of these key physiological measures are crucial for meeting the increased physical demands of the active part of the day and the reduced demands of sleep. Aberrations in temporal rhythms can both directly and indirectly enhance cardiovascular and cerebrovascular disease (Bøggild and Knutsson, 1999), by enhancing sympathetic drive (Morris et al., 2012) increasing obesity (Shi et al., 2013), inducing insulin resistance and metabolic disorders (Marcheva et al., 2013), promoting premature aging (S. A. Brown et al., 2011), and inflammatory pathologies (Salavaty, 2015; Savvidis and Koutsilieris, 2012). These physiological perturbations can interact with cardiovascular phenotypes and prothrombotic states to play a significant role in stroke recurrence, functional outcome (Arboix, 2015), and outcome with pharmacological intervention with tissue plasminogen activator (tPA) (Seet et al., 2014). Cardiovascular tissue displays robust circadian oscillations in cells including vascular smooth muscle, fibroblasts, cardiomyocytes, and cardiac progenitor-like cells, all of which regulate

physiological functions including endothelial function, blood pressure, and heart rate (Paschos and FitzGerald, 2010).

At the cellular level, the core clock genes, including *Bmal1, Clock, Per, Cry,* and *Rev-Erb*, play an important and vital role in maintaining physiological homeostasis. In addition to studies evaluating the role of time of day, genetic approaches can be used in basic science models to assess the role of specific genes within the molecular circadian clock. Notably, *Bmal1, Clock, 1/2 Per* double knock-out, and *Cry1/2* double knock-out mice are completely arrhythmic (van der Horst et al., 1999; Bunger et al., 2000; Bae et al., 2001; DeBruyne et al., 2007), whereas single knockouts of *Cry* and *Per* have altered periods (van der Horst et al., 1999; Bae et al., 2001). Please see (Cermakian et al., 2001) for a more extensive review of the circadian phenotypes of genetically altered models.

The next section will discuss how aberrations to circadian expression rhythms from environmental and genetic factors will alter cardiovascular, cerebrovascular, and immune parameters that increase stroke risk and contribute to the pathogenesis, damage, and outcome of cerebral ischemia (Brown et al., 2009; Ramsey et al., 2020).

Coagulation, Hemostasis, and Fibrinolysis

In response to vascular injury, platelets are activated upon coming in contact with subendothelial matrix proteins. Platelet activation is responsible for clot formation in the procoagulant pathway, while several inhibitors function to inhibit clot propagation and avoid thrombus propagation (Palta et al., 2014). Several pro-inflammatory mediators including chemokines, adhesion molecules, vasoactive mediators, growth factors, and surface ligands are released upon platelet activation (Morrell et al., 2007). Indeed, this system relies on the intricacy of two systems in

order to maintain fluidity and circulation of blood. In the context of cerebral ischemia, platelets play a crucial role in the pathogenesis of this condition; time of day alterations in factors involved in hemostasis and fibrinolysis contribute to a hyperfibrinolytic state, in turn increasing risk for stroke during morning time points.

In a study evaluating platelet aggregation in response to adenosine diphosphate (ADP) and adrenalin, platelet adhesiveness was measured using blood samples from healthy subjects (5 males and 5 females) over six time points (0800, 1200, 1600, 2000, 0000, and 0400 h); prothrombin peaked at 1600 h, suggesting a transient morning hypercoagulable state (Haus et al., 1990). Increased platelet activation along with fibrinogen, plasminogen-activator inhibitor 1 (PAI-1), plasmin-alpha-2-antiplasmin (PAP), and thrombin-antithrombin (TAT) complexes has been observed in healthy human volunteers (20-50 years old) during the morning (0800 h), with increasing concentrations of endogenous tPA, d-dimers, and PT prolongation in the afternoon (Budkowska et al., 2019). Clotting times were significantly reduced during the active period (night) in nocturnal rats, with increased prothrombin (II), factor VII, and factor X during the inactive period (day) (Soulban and Labrecque, 1989).

Coagulation and fibrinolysis has been further investigated in *Clock* mutant and *Cry1/2* knockout (arrhythmic) mice; euglobulin clot lysis time was reduced in *Clock* mutants and significantly increased in *Cry1/2*-deficient mice without time-of-day differences (0900 and 2100 h). Further characterization showed reduced PAI-1 fluctuation in *Clock* mutant mice while *Cry1/2*-deficient mice held similar levels at both 0900 and 2100 h (Ohkura et al., 2006). Mice with deficiencies in *Bmal1*, showed hypercoagulable states, increased arterial and venous thrombogenicity, that lead to progressive dysfunction in endothelial cells and subsequent increased platelets and factor VII with age (Hemmeryckx et al., 2019). In common with these findings, vascular occlusion time

was increased in this mutant mouse (Westgate et al., 2008). *Bmal* knockout (arrhythmic) mice display progressing prothrombotic state characterized by reduced prothrombin times, increased platelet count, a reduction in nitric oxide/thrombomodulin expression from endothelial cells (Hemmeryckx et al., 2011). *Clock* is also involved in the regulation of hemostasis. Mutations in *Clock* increased the total and active PAI-1 levels along with reduced tPA in plasma (Westgate et al., 2008) . Taken together, these results suggest that core clock genes are involved in regulating expression of key components in hemostasis and the fibrinolytic system, leading to an increased risk for the development of prothrombotic phenotypes and thus, an increased risk for cerebrovascular events.

Vascular Function and the Blood-Brain Barrier

Cerebrovasculature plays important role in brain development and regulation of homeostasis. Endothelial cells comprise the inner monolayer of blood vessels that forms a barrier between the artery wall and circulating blood is responsible for regulating vascular tone, regulation of hemostatic properties on the vascular surface, and determining blood-tissue/brain permeability (Paschos and FitzGerald, 2010). The blood-brain barrier (BBB), is a tightly regulated system composed of transporters including permeability-glycoprotein (Pgp) and ATP-binding cassette sub-family B member 1 (ABCB1). Movement across the BBB occurs via transmembrane efflux pumps (Abbott et al., 2010) that transport molecules through endocytosis or through pores, carrier-mediated transport systems, or through direct permeation through the plasma membrane via lipophilic molecules (Banks and Kastin, 1992). These efflux pumps are regulated by ATP-binding cassette (ABC) transporters or paracellular aqueous diffusion which are inhibited by tight junctions.

The BBB plays an integral role in the neuronal damage that evolves from disrupted blood flow. As a result of impaired blood supply, essential nutrients such as glucose and oxygen fail to adequately reach the ischemic core and surrounding region known as the penumbra. Endothelial ion transporters, including the Na-H and Na-K-Cl cotransporter, become dysregulated upon neuronal injury, resulting in an imbalance of ionic gradients and cytotoxic edema that contribute to secondary neuronal damage (Pinheiro et al., 2016). Additionally, increased paracellular permeability (Keaney and Campbell, 2015), and increased immune cell trafficking, including infiltrating leukocytes, can exacerbate inflammatory responses, leading to increased neuronal damage and injury (Huang et al., 2006). Furthermore, disruptions to the BBB following thrombolytic intervention can be an early predictor for hemorrhagic transformation (Kastrup et al., 2008).

Evidence of an endogenous circadian rhythm in blood brain barrier permeability has been characterized in Drosophila where rhodamine B and daunorubicin concentrations were significantly increased in the brain during the rest phase (ZT 12) compared to their active phase through increased efflux activity via pgp-like transporters during the day (Zhang et al., 2018). This oscillation was abolished in *Per* deficient flies (altered period) (Zhang et al. 2018). Further, several molecules, including tumor necrosis factor alpha (TNFα), leptin, β-amyloid, delta-sleep inducing peptide (DSIP), and prostaglandin D2 (PGD2) display changes of rhythmic entry into murine CNS (Cuddapah et al., 2019). Other aspects of the blood brain barrier including integrity are also under circadian control. *Bmal1* deficient mice display hyperpermeability and downregulation of platelet-derived growth factor receptor β (PDGFRβ), suggesting a significant role for *Bmal1* in pericyte regulation for blood-brain barrier maintenance (Nakazato et al., 2017).

Secondly, vascular function and tone also demonstrate robust core clock rhythms, best characterized through blood pressure peaks which are observed prior to the onset of the active cycle (light phase in diurnal species) followed by mid-morning peaks and decreases during the evening (inactive phase) (Millar-Craig et al., 1978). Additionally, a time-of-day dependent vasoreactivity exists (Durgan et al., 2017), further supporting the notion of circadian regulation of the cardiovascular system. *Bmal1* knockout mice display lower blood pressure and abolished rhythmicity (Curtis et al., 2007), whereas *Per1* has been implicated in aldosterone (Richards et al., 2013) and sodium regulation (Stow et al., 2012), both of which play a role in blood pressure maintenance. Selective deletion of Bmal1 in cardiomyocytes results in decreased heart rate from altered Na^+ and K^+ channels (Schroder et al., 2013), while deletion in smooth muscle cells advances systolic and diastolic blood pressure (Xie et al. 2015). Furthermore, *Bmal1* deficient mice exhibit acute vascular dysfunction, vascular remodeling, and injury (Anea et al., 2009).

Nitric oxide (NO) and redox signaling involved in vascular contraction is another process that displays robust circadian oscillations involved in the vasoconstriction process. In the context of cerebral ischemia, NO synthases have both neuroprotective and damaging effects during brain hypoxia. Inducible NOS (NOS2) and neuronal NOS (NOS1) are involved in neurotoxicity, leading to excitotoxicity cascades, inflammation, apoptosis, and deterioration to the primary brain injury through the release of free radicals and production of nitrates, which directly damage mitochondrial enzymes and genetic material (Sims and Anderson, 2002; Chen et al., 2017). Endothelial NOS (NOS3) has a neuroprotective effect through regulation of cerebral blood flow, preventing neuronal injury, and inhibiting platelet and leukocyte adhesion (Liu et al., 2014). A study examining time of day variation in contractility response in mesenteric arteries isolated from Wistar rats reported increased NOS3 expression during the active period, which coincided

with reduced vasoconstriction in response to phenylephrine and increased vasodilation response to acetylcholine during the active period (Rodrigo and Denniff, 2016). Further, NOS activity and cytosolic protein content was evaluated in Sprague-Dawley rats at 0300, 0900, 1500, and 2100 h in various regions of the brain including cerebellum, brainstem, hypothalamus, and hippocampus and reported that a significant upregulation during the dark (active) period (Ayers et al., 1996). Both endothelial nitric oxide synthase and NADPH oxidases (Nox), two factors involved in producing vasodilators (EDRF, EDHF, and PGI2) exhibit circadian variations in vasculature (Denniff et al., 2014). In addition to this time of day variation, disruption of circadian rhythms from exposure to dim light at night (dLAN; 1-2 lux) resulted in increased NOS3 protein expression in the arteries after 2 weeks of exposure in male Wistar rats (Molcan et al., 2019). Furthermore, NOS3 knockout mice display increased infarct sizes compared to wild type mice post middle cerebral artery occlusion (MCAO) (Huang et al., 1996). As mentioned above, NOS has significant neurotoxic impacts, and acts as a neuroprotective agent through mediation of vasodilation, plays a role in platelet aggregation inhibition, and contributes to endothelial leukocyte adhesion (Huang, 2007), and the studies above provide evidence and further support that disruptions to circadian rhythms support and increase neurotoxic effects and dampened neuroprotective aspects of NOS. In conclusion, several aspects of the vascular system are critically involved in acute injury and recovery from cerebral ischemia and are tightly regulated by core clock genes. Time of day alterations in function exist, suggesting a potential avenue for targeted therapeutic intervention. Thus, the BBB has reduced trafficking during the active phase compared to other time points during the day, along with an increased morning surge in blood pressure contributing to a heightened prothrombotic state, increasing risk for ischemia. Additionally, morning time points and studies evaluating circadian disruption have increased

NOS, that can contribute towards neurotoxicity and secondary ischemic damage. Disruptions circadian rhythmicity in core clock gene expression patterns has vast implications contributing to increased stroke risk and impaired recovery.

Neuroimmune System and Peripheral Immune Response During Stroke

Components of the immune system are critical in pathophysiology of cerebral ischemia and are also regulated by the circadian clock. Acute cerebral hypoxia caused by thrombosis during ischemia triggers inflammatory cascades, and immune activation can cause secondary damage within the penumbra of the ischemic infarct (Kamel and Iadecola, 2012). Molecular clocks have been discovered in immune cells including macrophages/monocytes, T cells, Natural Killer (NK) cells, dendritic cells, and B cells (Ella et al., 2016), along with a circadian regulation in recruitment and infiltration of immune cells including neutrophils, monocytes/macrophages, and T cells, which infiltrate the injury site after the integrity of the BBB is compromised (Scheiermann et al., 2013). Rhythmic daily oscillations in both circulating innate and adaptive immune cells, and in cytokine and chemokines occur in the healthy brain (Labrecque and Cermakian, 2015), and circadian clock disruptions dysregulate immune response (Comas et al., 2017) and alters circulating proinflammatory cytokines, complement factors, and oxidative stress (Shivshankar et al., 2020).

In addition to the highly organized and regulated immune system, there are specific immune cell populations that exist in the CNS that are vital in maintaining normal brain homeostasis and function. Glial cells including microglia and astrocytes are non-neuronal populations in the central nervous system that function to maintain homeostasis, provide support, and protection, which are both involved in the innate and adaptive immune system (Herculano-Houzel, 2014;

Zuchero and Barres, 2015). Inflammation is a key component and contributor in the pathophysiology of ischemia, existing in every stage of the ischemic cascade (Iadecola and Anrather, 2011). Upon neuronal and cell injury from ischemic infarction, glial populations function as the primary proponents in the early peri-infarct environment, however, have been implicated in both beneficial and a detrimental impact on the ischemic core (Xu et al., 2020).

Microglia are a glial cell that plays an active role in immune surveillance in the central nervous system and are heavily involved in the initiation of the innate and adaptive immune response (van Rossum and Hanisch, 2004). This cell population accounts for up to 15% of all cells found within the brain (Lawson et al., 1992). This population plays a crucial role in triggering innate immune response by releasing pro and anti-inflammatory cytokines, chemokines, nitric oxide, prostaglandins, growth factors, and superoxide species that can modulate secondary injury and recovery (Loane and Byrnes, 2010). Microglia also recruit leukocytes, myeloid dendritic cells, monocytes/macrophages, and neutrophils to the site of ischemic damage during the earlier stages of stroke (Iadecola and Anrather, 2011; Jian et al., 2019). There are several types of cytokines that are produced that play important roles in immune signaling. Tumor-necrosis factor α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), are pro-inflammatory markers that promote inflammation, while interleukin-4 (IL-4), interleukin-10 (IL-10), and tissue growth factor β1 (TGFβ1) are anti-inflammatory cytokines that inhibit inflammation. This activation process has a central role in initiating a neuroinflammatory response post stroke which can cause pathological progression of damage in the ischemic penumbra, however, aspects of the neuroinflammatory response are also critical for tissue repair (Yenari et al., 2010). Microglia are under circadian clock control, with robust hippocampal TNFα, IL1-β and IL6 expression, peaking during the light or inactive phase in male Sprague-Dawley rats, with increased cytokine expression in

response to an immune stimulation with lipopolysaccharide (LPS) during the light/inactive phase (Fonken et al., 2015). Microglial activation, and interleukin expression specifically, is regulated by *Bmal1* expression (Takano et al., 2009), along with a circadian variation in markers involved in oxidation, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (Nox2), and inflammatory TNF-α, IL-1β and IL6 in microglia increased during the light or inactive phase in nocturnal mice. Conversely, glutathione reductase (Gsr), heme oxygenase 1 (Hmox1), glucose transporter member 5 (Glut5) and lipoprotein lipase (Lpl) were increased during the dark, or active period, suggesting that microglia have increased nutrient uptake during the active period. Wang et al. continued using *Bmal1* knockout mice and reported altered clock gene expression (Cry2 and Per2) along with decreased Nrd1, Dbp, IL1-β, and Nox2 expression and increased Gsr and Lpl expression, indicating that *Bmal1* regulates immune response and cellular metabolism in microglia (Wang et al., 2020). Other circadian clock proteins, such as Rev-erbα have also been implicated as a mediator of microglial activation and neuroinflammation, where the authors observed a time of day difference in microglial immunoreactivity in the hippocampus (Griffin et al., 2019). Further examination with Rev-erbα knockout mice, indicated increased basal nuclear factor κB (NFκB) and enhanced hippocampal neuroinflammatory reactivity after inflammatory challenge (Griffin et al., 2019) providing further evidence of the role and importance of circadian clock regulation on immune function. These results also highlight the detrimental effects that result from altered circadian rhythms, and the downstream dysregulation of this highly conserved system.

Astrocytes are another specialized glial cell population integrated into both the vascular and neuronal system; and are critical for maintenance and survival of neurons during normal brain function (Stubblefield and Lechleiter, 2019). In the context of cerebral ischemia, astrocytes are

especially important in regulation and maintenance of glutamate during acute injury, which causes excitotoxicity and perturbates calcium influx resulting in cell death (Brancaccio et al., 2017). Processes under astrocyte control including glutamatergic signaling (Beaulé et al., 2009) are regulated through the molecular clock, and in addition, extracellular glutamate displays rhythmicity, peaking during the mid-late day (inactive period) in mice. Disruptions to core clock genes including *Per2, Clock*, and *NPAS2* display significant reductions in glutamate uptake, mRNA expression of Glast, an astrocyte-specific glutamate transporter, and protein expression (Sofroniew, 2015). Astrocytes are also involved in the recruitment of inflammatory cells to the site of injury, through the formation of a glial scar termed reactive astrogliosis which involves activation and proliferation of astrocytes to limit extending damage (Lananna et al., 2018). Astrocytic activation is regulated by *Bmal1*, shown through activation and increased inflammatory gene expression using an astrocyte specific *Bmal1* knockout (Ali et al., 2020). Other studies using *Bmal1* knockouts in astrocyte cultures have reported reduced actin-binding protein cortactin and impaired actin stress fiber formation (Ali et al., 2020)

Other immune cell populations that are involved in regulating the progression of ischemic injury are neutrophils, macrophages, and T-cells (Jian et al., 2019). As mentioned, the immune system is a tightly regulated system which is important to maintain health and survival. Appropriate inflammatory balance is especially important during ischemic injury, where there is major neuronal death and without proper neuroprotective measures, progressing injury can have detrimental effects on functional outcome and survival. Neutrophils are among the first immune cell populations to respond to ischemic injury; they are important for their phagocytotic properties to remove necrotic debris, but can result in neuroedema, disruption to the blood brain barrier, and collateral tissue damage (Jickling et al., 2015). Neutrophils have been well

characterized to display robust oscillating rhythms in blood (Casanova-Acebes et al., 2013), variations in *Bmal1* expression (Gibbs et al., 2014), and time-of-day regulated infiltration into bone marrow, lung, liver, and spleen tissue (Casanova-Acebes et al., 2018). Macrophages and monocytes are other immune cells in the mononuclear phagocyte system that have robust cell-autonomous rhythms (Scheiermann et al., 2013). Clock genes such as Rev-erb repress distal enhancers resulting in the repression of macrophages (Lam et al., 2013), whereas *Bmal1* which controls rhythmic trafficking of inflammatory monocytes to sites of inflammation (Nguyen et al., 2013). In the context of stroke, macrophages and monocytes function similar to neutrophils and are especially important for their phagocytic abilities during ischemia. T-cells are one of the major white blood cell types involved in the adaptive immune system and migrate to the site of ischemic injury after the initial inflammatory cascade within 24 hours (Jian et al., 2019) which is dependent on adhesion molecule expression in the endothelium (Arumugam et al., 2005). This infiltration is important, due to the high mortality rate associated with infection during the acute phase of stroke recovery (Shi et al., 2018). Helper T cells (TH1) can play a neuroprotective role during recovery through anti-inflammatory cytokines (IL-4, IL-5, IL-10, IL-13), however, cytotoxic T cells can also contribute towards secondary injury from cytotoxic granules (Arumugam et al., 2005). Circadian variations in T cell proliferation in the lymph nodes exist, where T cell proliferation was significantly greater during the dark or active period in mice compared to the light, which was abolished in *Clock* mutant mice (Marie-Pierre Hardy et al., 2021). Based on the crucial role that timing plays in stroke intervention, circulating immune factors and immune response is most active during the morning. Upon neuronal injury, heightened immune activation is observed during the morning increasing the risk for neurotoxicity and secondary damage compared to the evening. In addition, disruptions to

circadian rhythms and core clock gene function through exposure to light at night disrupts immune function and increases neuroinflammation (Walker et al. 2020), that has been corroborated in an ischemic murine model in which three days of exposure to dLAN post stroke increases pro-inflammatory response (TNF-α, IL-6, and IL-1β) that can further amplify secondary damage of the infarct. Disruptions to circadian rhythms can lead to a prothrombotic state, in which patients during the morning time point are especially at risk for poor outcome, further highlighting the importance of time of day when considering therapeutic strategies.

Clinical Studies, Disruptions to Circadian Rhythms, and Stroke

During the late 20th century hospital analyses began to investigate the correlation between circadian variations in ischemic onset and outcome, highlighting the increased morning frequency of this cerebrovascular event. A meta-analysis of 31 publications (11,816 strokes) revealed a 49% increase of occurrence between 0600-1200 h in all types of strokes, and 79% increase over normalized risk compared to the other 18 h of the day (Elliot 1998), with the lowest incidence of stroke reported between 0000-0600 h (Pardiwalla et al. 1993). Additionally, mortality rates for strokes that occur during the morning were significantly higher, even when adjusted for sex, severity, and age (Marler et al., 1989). Analysis of stroke bank data revealed a significant increase in the number of strokes in awake patients from 1000-1200 h, compared to any other 2-hour interval throughout the day (Lago et al., 1998). Another study of 1223 patients reported increased frequency of acute ischemia between 0600 to 1200 h; lacunar, thrombotic, and embolic strokes displayed increased frequency between 1801-0000 h (MC et al., 2014). Ischemic stroke patients who experience “Wake-up Strokes”, characterized by patients who were asleep for greater than 3 hours, with noted stroke symptoms upon waking between 0100 and 1100 h had increased diastolic blood pressure, a longer hospital stay. Further, this study indicated

that patients were also at an increased risk for in-hospital mortality (Brown et al., 2009). In addition to an effect of time of day on risk, disrupted circadian rhythms also increase risk. Rotating shift work, which disrupts circadian rhythms, has also been associated with a 4% increased risk for ischemic stroke for every 5 years, with an increased risk of stroke in women with longer durations of shift work (≤15 years) (Nelson and DeVries, 2017).

Together, the literature supports the notion that a time of day variation in stroke outcome exists, and that patients who experience stroke-like symptoms during the morning, or onset of the active period, have an increased risk for poor outcome. The data also suggest that it likewise could be possible to optimize therapeutic strategies based on the biological time that the onset of stroke occurs to improve survival rates. Time of day and circadian rhythms are both important variables in ischemia, and few clinical studies have considered these variables as biological factors. In this review the existence of circadian variations in ischemia and efficacy of thrombolytic intervention for clinicians, physicians, and pharmacists is characterized with the aim that time of day be recorded and reported as a biological variable in future clinical studies.

Other aspects of temporal organization influence and alter stroke recovery. For example, exposure to artificial lighting from hospital recovery units is a major issue (Korompeli et al., 2017). Indeed, clinicians need to monitor and treat patients 24 hours a day, however, patients may be especially vulnerable to the influence of light at night due to their compromised state; frequent aspects of intensive hospital care units include frequent waking for vitals and procedures, and noise from equipment and staff (West et al., 2017). Few clinical studies to our knowledge have directly evaluated the role of dim nighttime lighting compared to dark nights on functional recovery and outcome in stroke, however, literature focused on normalizing naturalistic lighting during recovery have shown significant improvements (Wright et al., 2013).

In the context of disrupted circadian rhythms, the use of naturalistic lighting optimizes light exposure similar to solar time, allowing for resynchronization of the internal biological clock to the natural light dark cycle (West et al., 2019). Using 24 h naturalistic lighting in rehabilitation units post-stroke found that patients experienced improved sleep with decreased disturbances, improved cognitive function, and endocrine function (S. J. Wang & Chen, 2020). Other studies using artificial sunlight exposure therapy during the day, with increasing morning blue-spectral illuminance peaking in the afternoon to imitate daylight for a minimum of 14 days of exposure in 4 weeks post stroke reported improved daily function through the Bartel Index and depressive test scores (Durgan et al., 2017). Thus, normalization through re-entrainment of circadian rhythms to light dark cycles post cerebrovascular injury through the use of controlled lighting in a hospital setting is a non-invasive intervention that can potentiate improved recovery rates.

Time of Day Alterations, Disruptions to Circadian Rhythms, and Genetic Manipulations to Core Clock Genes Alters Ischemic Stroke Outcome in Murine Models

As highlighted above, stroke incidence has a distinct time of day frequency, where studies identified a morning increase in frequency and mortality compared to any other time point in clinical studies (Marler et al., 1989). In addition to these time of day variations, basic science research has identified a circadian aspect in stroke severity, infarct volume, and functional outcome in rodents (Weil et al., 2009; Beker et al., 2018; Ramsey et al., 2020). In a controlled study, variation in ischemia/reperfusion tolerance across time of day has been previously explored, showing an increase in infarct volume and fibrosis at the onset of activity (ZT12) compared to the beginning of the inactive period (ZT0) in WT mice (Durgan et al., 2010). In addition, genetic ablation of the molecular clock in cardiomyocytes abolished this diurnal variation (Durgan et al., 2011). Global cerebral ischemia from cardiac arrest during the light

(inactive) phase impaired survival and exacerbated outcome characterized by increased microglial activation, degenerating neurons, and proinflammatory cytokines in the hippocampus compared to dark (active) phase in C3H mice (Weil et al., 2009). Studies into the molecular mechanisms underlying the time of day differences post ischemia revealed that ischemic/reperfusion injury at ZT 18 had reduced infarct volume, edema, neurological deficits, apoptotic death, and improved neuronal survival compared to other time points (ZT0, ZT6, ZT12, and ZT18), coinciding with a decrease in PRAS40 and increased expression of *Bmal1, Per1, Clock, AKT, Erk-½, mTOR, S6*, and *BAD* (Beker et al. 2018).

Disruptions to circadian rhythms prior to ischemic events leads to a prothrombotic state resulting in a heightened predisposition for enhanced stroke damage and poor outcome. Chronic circadian disruption through phase advances increased infarct volume in mice that received an MCAO (Ramsey et al. 2020). Another recent study reported that chronic disruption of circadian rhythms by a 12-hour phase advance every 5 days for 7 weeks prior to MCAO increased infarct size in male and female Sprague Dawley rats. Further, male rats had increased mortality, while surviving females displayed a significant decrease in serum IGF-1 and nearly a doubling of infarct volume and sensorimotor deficits (Earnest et al., 2016).

In addition to disruption of circadian rhythms by repeated phase shifts (jet lag), foundational science research has examined the role of circadian disruption by light at night in stroke outcome. Studies evaluating the impact of ecologically relevant levels of dLAN (5 lux) have reported that stroke lesion size was significantly larger in dLAN mice compared to mice housed in dark nights after 24 hours (Weil et al., 2020). Furthermore, this study reported that mice exposed to three days of dLAN post MCAO increased TNF-α, IL-6, and IL-1β in the ipsilateral hemisphere of the brain, and displayed increased anxiety-like behavior compared to control mice

(Weil et al., 2020). Another study evaluating the effect of dLAN reports that as few as four days is sufficient to induce neuroinflammation in female mice (Walker et al. 2020) which can contribute towards neurotoxicity, increased neuronal damage within the penumbra, and delayed recovery post ischemia.

Disruptions to core circadian clock proteins, specifically *Bmal1*, has also resulted in increased reactive oxygen species, which is critical during mediation/exacerbation of damage during reperfusion in ischemia (Peek et al., 2017). Meanwhile, overexpression of *Bmal1* and *Clock* may activate HIF-1α, involved in the regulation of innate neuroprotection (Lembach et al., 2018). Additionally, there is a difference between biological outcomes when comparing males and females post ischemic injury; the infarct core and both microglial activation and astrogliosis were significantly increased in females compared to males; furthermore, this sex difference persisted in female *Bmal1* knockout mice that displayed increased core infarct size compared to wild type females (Wiebking et al., 2013). *Per1* knockout mice increased neuronal cell death post ischemia compared to wild-type control mice. Importantly, ischemic injury during time points coincident with low *Per1* expression were more susceptible to apoptosis compared to high Per1 expression (Jickling et al., 2014). Disruptions to core clock gene expression, which can occur from exposure to light at night or repeated phase shifts resulting from social jet lag can result in significant disruption in the tissue recovery process, which in the context of stroke, could result in increased ischemic damage and could be detrimental to patient outcome.

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CHAPTER 2

DIM LIGHT AT NIGHT ALTERS BRAIN-IMMUNE INTERACTIONS AND STROKE OUTCOME IN MICE

Introduction

In the context of disease states, innate immune response and the initiation of inflammation play critical roles in pathophysiological progression of neuronal injury following ischemic stroke (Iadecola et al., 2020). During acute ischemia, cerebral arteries become occluded from the formation of an embolism or thrombus, and within minutes after hypoperfusion or loss of blood flow to regions of the brain, this initiates the ischemic cascade, which is comprised of a rapid series of biochemical events that occur resulting in neuronal death at the center and core of the infarct (Kuriakose and Xiao, 2020). After focal hypoperfusion of the brain, the initial core infarct develops from loss of oxygen, that alters neuronal ATP production, resulting in depolarization after ion transport pumps fail in neurons, allowing intracellular calcium ($Ca2+$) levels to increase. triggering excitotoxic cellular damage will occur from the release of excitatory glutamate from the presence of calcium. Glutamate stimulation of AMPA receptors and $Ca2+$-permeable NMDA receptors overexcites neurons, resulting in the production of ROS, free radicals, resulting in excitotoxicity of the neurons (Orellana-Urzua et al., 2020). Phospholipases will break down cell membranes, and mitochondria will initiate apoptotic factors and caspase-dependent apoptosis cascade. The degree and duration of ischemia can determine the extent of cerebral damage, which results in a corresponding loss of neuronal function (Li and Yang et al., 2018).

After initial formation of the ischemic core infarct, there are several pathophysiological mechanisms involved in ischemic progression. However, inflammation can account for a main component of pathogenic progression (Muir et al., 2007), and interactions and bi-directional

crosstalk between the nervous and immune system is an important component in the pathophysiology of stroke. This can affect brain tissue surrounding the ischemic core, referred to as the penumbra, which is damaged from reduced blood flow, but is not unsalvageable. The penumbra represents a therapeutic target to rescue neuronal tissue post-stroke to improve functional outcome and reduce disability. Indeed, previous literature has well-demonstrated that there are multiple phases of inflammation across stroke-recovery; inflammatory events are dynamic processes (McColl et al., 2009). At the acute phase post-stroke, inflammation is initiated by microglia, which are highly dynamic and actively survey and monitor microenvironments in the brain to maintain homeostasis and to initiate inflammation. Microglia play an important role in early-stage stroke formation, and are critical in attenuating apoptosis, and contributing to functional recovery through clearing debris and tissue remodeling to stimulate anti-inflammatory cytokine growth factors (Wang et al., 2011). For example, anti- or pro-inflammatory cytokine and chemokine mediators, orchestrate infiltrating immune cells, including neutrophils, macrophages/monocytes, lymphocytes, natural killer cells, T cells, and others (Iadecola and Anrather, 2011). that will further secrete pro-inflammatory and neuroprotective mediators that increase and suppress inflammatory response.

As previously reviewed in **Chapter 1**, physiology and behavior are synchronized to light/dark cycles via direct photic input from the retina to the suprachiasmatic nucleus (SCN) of the hypothalamus, that serves to entrain (synchronize) expression of circadian clock genes to the external 24-h day. In mammals, rhythms are driven by endogenous transcriptional translational feedback loops at the cellular level composed of main core clock genes, *Clock, Bmal1, Per,* and *Cry* (Takahashi, 2017; Cox and Takahashi, 2019). The duration and intensity of light as a

zeitgeber, or external entraining cue, plays a key role in entraining molecular clock genes to time of day; therefore, prolonged exposure to light into the typically dark night has deleterious effects on physiology and behavior (Navara and Nelson, 2007; Fonken et al., 2013). Emerging evidence highlights the adverse consequences associated with nighttime lighting and how disruptions to circadian rhythms alter health outcomes and risk for disease; even low or dim levels of light at night (dLAN) disrupt molecular clock rhythms (Fonken et al., 2013). Further, dLAN disrupts immune function by impairing innate immune response (Bedrosian et al., 2011), disrupting daily rhythms of circulating immune cells (Okuliarova et al., 2021), and inducing neuroinflammation (i.e., TNF, IL-6) in as few as four nights of exposure to light in otherwise healthy mice (Walker et al., 2020; Bumgarner et al., 2020). As highlighted, immune response and inflammation is a crucial component that contributes to the exacerbation of injury while also playing a central role in promoting clearance of debris and tissue and repair. Because previous studies identified that light at night, which disrupts circadian rhythms, can influence neuroinflammation and induce changes to peripheral immune responses, this study sought to investigate the role of dLAN on stroke outcome. The mechanisms driving neuro-immune interactions with light at night in a disease state such as ischemic stroke remain unspecified. Therefore, in the present study early infarct development and changes in microglia following post-stroke exposure to dim light at night were investigated. The hypothesis tested for this study is that exposure to post-ischemic dim light at night (i.e., 5 lux) alters microglial phenotype, in turn, increasing secondary neuronal damage.

Materials and Methods

Animals

Six to twelve month-old male and female Swiss-Webster mice (Charles River Laboratories, Wilmington, MA) were individually housed in a controlled vivarium (Ventilated Light Controlled Animal Housing System, Lab Products Inc.) under a 12:12 hour light dark (LD) cycle (150 lux light; 08:00 EST, 0 lux, 0 mW/cm 2 night 20:00 EST) with *ad libitum* access to standard rodent chow (Envigo Teklad 2018 Rodent Chow) and reverse osmosis water for the duration of the study. All experiments were performed in accordance with NIH Animal Welfare guidelines and were approved by the West Virginia University Institutional Animal Care and Use Committee.

Surgical Procedures

Ischemic stroke was induced as previously described (Weil et al., 2020) using a middle cerebral artery occlusion (MCAO) during the light phase, prior to lights off (zeitgeber time; ZT 6-12). Mice were anesthetized with isoflurane vapors until unresponsive, confirmed by toe pinch, then, were placed on a homeothermic mat and body temperature was regulated and maintained at 37 ± 0.5°C. A unilateral right incision was made, exposing the right middle cerebral artery and occlusion was achieved through insertion of a 6-0 nylon filament into the internal carotid artery, past the pterygopalatine artery bifurcation. Cerebral blood flow was monitored using laser doppler flowmetry for confirmation of blood flow drop during occlusion. After 45 min of occlusion, reperfusion was initiated via removal of the occluder. Mice received 1 ml of warmed saline through subcutaneous injection, and then were monitored for recovery in clean home

cages placed on top of warming pads set to 37 °C, then were returned to the animal facility. For permanent middle cerebral artery occlusions (MCAO), mice received a right insertion of 6-0 nylon filament into the internal carotid artery, then were monitored for 15 min on laser doppler to confirm cerebral blood flow drop prior to suturing up and allowing the mice to recover.

Lighting Conditions

Following reperfusion, mice were pseudorandomly returned to standards light-dark conditions or were placed into dim light (7.32e-7mW/cm 2) produced by Ventilated Light Controlled Animal Housing System Chambers and were confirmed using a light meter (Mavolux 5032C illuminance meter (Nürnberg, Germany) from the center of an empty cage with the light sensor facing the Ceiling.

Neurobehavioral Testing

24 hours post MCAO, mice were given a neurological scale evaluation using Clark's general and focal test that were positively correlated with infarct volume at early time points (Clark et al., 1997; Wen et al., 2017). Clark's general test identifies six features, including (1) spontaneous activity (2) posture (3) eyes (4) ears (5) hair along with (6) epileptic behavior of the mice on a 0-12 defined scoring system based on severity. Clark's focal test scores seven deficits on a scoring range from 0-4 for severity of presentation, consideration (1) body symmetry (open bench top) (2) gait (open bench top) (3) climbing (gripping surface, 45 degree angle) (4) circling behavior (open bench top) (5) front limb symmetry (mouse suspended by its tail) (6) compulsory circling (front limbs on bench, rear suspended by tail) (7) whisker response (light touch from behind). Performance was analyzed by investigators blind to experimental conditions.

Infarct Size

Brains were collected and sectioned into three 2 mm thick coronal sections and were placed in 2,3,5-triphenyltetrazolium (TTC) solution to delineate brain infarct regions (Isayama et al., 1991) and were incubated at 37 °C for 15 min, flipping sections every 2 min. Slices were post-fixed in 4% buffered paraformaldehyde overnight at 4°C then were switched to a 30% sucrose solution and photographed within 72 h. Infarct size was analyzed using Fiji and was quantified as a percentage of the contralateral hemisphere to correct for edema, using the formula: [1-(ipsilateral hemisphere – infarct)/total hemisphere] x 100. Infarct volume quantification was performed by investigators blind to experimental conditions.

RNA Sequencing and Bioinformatics

Mice were intracardially perfused with ice cold 1X PBS after sodium barbiturate injection, then brains were collected. Cerebrums were separated from olfactory bulbs, cerebellum, and hindbrains and were divided into ipsilateral and contralateral hemispheres, homogenized, and filtered through a 40 μm filter and washed with PBS prior to RNA isolation with Trizol (Sigma-Aldrich) according to the manufacturer's instructions as previously described (1). RNA concentrations were quantified by Qubit fluorometer (Thermo Fisher Scientific) and quality was assessed by Bioanalyzer Nanochip. Bulk RNA sequencing was performed by Admera Health for RNA library preparation and sequencing. TruSeq Stranded with RiboZero Plus. Data analysis was performed using CLC Genomics Workbench and Ingenuity. Gene ontology was analyzed using Geneontology Unifying Biology and PantherGO Classification System v.17.0 (https://pantherdb.org/). Significance in experiments for RNA-seq was considered statistically different using Empirical Analysis of Differential Gene Expression (EDGE) test (Robinson et al.,

2010) along with a fold-change of ≥1.5.

Immunofluorescence and Analysis

Double labeled immunofluorescence for Iba1 and P2RY12 occurred on 30 µm free-floating sections and were stored at -80° C in cryoprotectant prior to use. Sections were washed in 1X PBS, replacing washes every 5 minutes for a total of 5 washes, prior to a 1 h blocking solution incubation (1X PBS, 2.5% NDS, and 0.2% Triton-X100). Sections were incubated overnight at room temperature in the primary antibody solution (1:500 rabbit anti-Iba1, Abcam Catalog #178846; 1:1000 rat anti-P2RY12, Biolegend Catalog #848002, and blocking solution). Next, sections were washed as described previously, then were placed into a secondary antibody solution (1:200 Alexa Fluor 488 donkey anti-rabbit IgG, Invitrogen Thermo FisherThermofisher Scientific Catalog #A21206; 1:1000 Alexa Fluor 594 donkey anti-rat IgG Invitrogen Thermofisher Scientific, Catalog #A21209) for a 2 h incubation at room temperature, then were washed and mounted. Slides were cover slipped with Vectashield + DAPI hardset mounting media (Vector Labs, Burlingame, CA). To quantify percent area fraction of P2RY12, 3 images of respective cortical hemispheres of the brain were imaged at 20X. Images were randomly selected from an anterior (0.97 – 1.21) portions of the cerebellum, then were loaded into Fiji, where images were separated into RGB channels (Red – P2RY12; Blue - DAPI) and microglia positive cells were manually counted. Infarct volume quantification was performed by investigators blind to experimental conditions.

CSFR1 Inhibitor (PLX5622) Administration

Plexxikon 5622 (PLX5622) (MedChemExpress, Catalog #HY-114153) at a concentration of 1200 ppm was incorporated into a standard research diet D20030403 by Research Diets Inc. that was fed 7 days prior to surgical intervention. Previous studies using PLX5622 at 1200 ppm reported selective microglia elimination of ~80% after 3 days of administration (Badimon et al., 2021) and <90% reduction within 5 days of treatment (Spangenberg et al., 2019); open standard diet (D11112201;Research Diets Inc.) was used as a control.

Flow Cytometry

After transcardial perfusion with 30 ml of cold sterile 1X PBS, brains were harvested and divided between the interhemispheric line into two hemispheres; ipsilateral and contralateral, then were rinsed with PBS prior to being mechanically digested with glass homogenizers in 6 ml of 4% Glucose Buffer at room temperature. Cell suspensions were filtered through 40μm cell strainer filters (Falcon), spun down at 1000 G for 10 min at room temperature. Immune cells were extracted from the interphase of a 70/30% Percoll gradient, then were washed and blocked with Mouse Ig (1:100, Jackson ImmunoResearch Catalog #015-000-003) for 1 h prior to staining with primary antibody-conjugated fluorophores (0.4μg CD11b - BV 650, Biolegend Catalog #101263; 0.125 μg CD45 – APC-eFluor 780, Thermo FisherThermofisher Catalog #47-0451-82; 0.25 μg Ly-6C – APC, Biolegend Catalog #128016; 0.5 μg IA8 Ly-6G – PE, Thermo FisherThermofisher 15-9668-82; 0.5 μg CD192 – BB700, BD Biosciences Catalog #747965; 0.25 μg Mannose Receptor – PE-Cy7, Biolegend Catalog #141720, 0.5 μg CD86 – APC R700, BD Biosciences Catalog #565479, 0.25 μg MHC II – BV711, Biolegend Catalog #107643, and 0.06 μg CX3CR1 - BV510, Biolegend Catalog #149025) diluted with BD Bioscience Brilliant Stain Buffer for a total of 20μl, and incubated on ice for 30 min in the dark. Cells were washed

with PBSAz then resuspended in BD Cytofix/Cytoperm buffer for 30 min in the dark on ice. Cells were then washed with BD Permwash buffer and resuspended into 0.4% paraformaldehyde for 20 min, at room temperature, in the dark, then left overnight in brilliant stain buffer. The following day, intracellular staining was performed in 20 μl of intracellular primary antibody-conjugated fluorophores (1/50 dilution IL-1β – FITC, Biorbyt Catalog #orb15831-100ug, 0.25 μg IL-6 – PE, Biolegend 504504, 0.125 μg TNF-α – BV785, Biolegend Catalog #506341; 0.4 μg IL-4 – BV421, Biolegend Catalog #504126, 0.125 μg IL-10 – PE-Dazzle 594, Biolegend Catalog #505034, 0.5 μg TGF- β1- BV421, Biolegend 141408) and were incubated for 30 min at room temperature in the dark. Cells were washed with permwash then analyzed using a four-laser 15 color instrument flow cytometer, Cytek Aurora, and results were analyzed using FCS Express 7 (De Novo software).

Serum Cytokine and Corticosterone ELISA

24 hours post MCAO mice were acclimated to the experimental room for 30 min prior to collection. Blood samples were obtained from the trunk (ZT 6-12), allowed to coagulate at room temperature, then were centrifuged at 4°C for 25 min at 2,500 x g. Serum was stored at -80°C for subsequent analysis. Serum cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, keratinocyte chemoattractant / human growth – regulated oncogene (KC/GRO), IL-10, IL-12p70, and TNF-α) were determined using MSD Mouse Proinflammatory Panel 1 (Meso Scale Discovery) in duplicates according to the manufacturer's instructions using the MSD QuickPlex SQ120). Standards and two-fold dilutions of mouse serum were applied to pre-coated wells and were incubated at room temperature for 2 h, received washes, and were incubated for an

additional 2 h with the detection antibody and was immediately read following the addition of the read buffer. Total serum corticosterone concentrations were determined using a Corticosterone Enzyme Immunoassay Kit (DetectX Arbor Assays, Ann Arbor, MI, USA) in duplicates according to the manufacturer's instructions for the 50 µL assay on a plate reader (SpectraMax iD3, Molecular Devices) (https://myassays.com/arbor-assays-corticosterone-enzyme-immunoassay-kit-high-sensitivity.assay).

Statistical Analyses

Survival was assessed through the Kaplan-Meier method using the Mantel-Cox test. Infarct sizes, neurological deficit scores, and interleukin/cytokine concentrations in serum comparing lighting conditions were analyzed using an unpaired two-way t-test. Samples that were out of range for the multiplex panel were excluded from analysis. Flow cytometry data were analyzed using a two-way ANOVA with ischemic hemisphere (ipsilateral and contralateral) and lighting condition as independent factors. Infarct data and functional deficit scores for PLX5622 studies were analyzed using a two-way ANOVA with diet and lighting condition as independent variables. A three-way ANOVA was performed with hemisphere, lighting condition, and diet as independent variables to assess P2RY12+ cells for IHC to confirm a reduction of microglia in the brain. Post-hoc comparisons were made using Fisher's LSD test. Normality was tested using the Shapiro-Wilk test. Outlier tests were performed prior to analyses, defined as a Z-score greater than 2 within a group. No more than one outlier was removed from each group. Data were analyzed separately in both sexes independently, due to running sexes in separate cohorts as a result of aging constraints. All statistical analyses were performed using GraphPad Prism software and data are represented as mean ± standard error of the mean (SEM). Statistical tests and

comparisons were considered significant if $p \leq 0.05$. Additional detailed information on statistical tests for each figure is shown below.

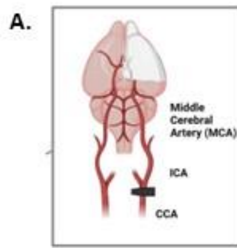
Results

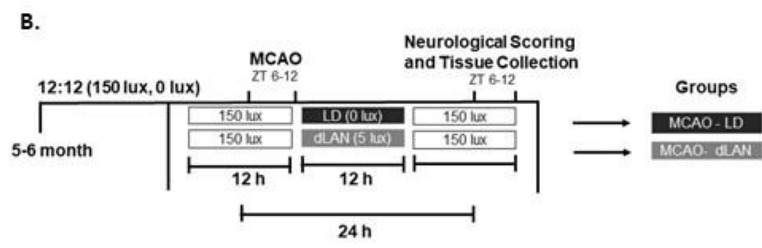
Exposure to Post-Stroke Dim Light at Night (dLAN) Increases Infarct Size and Sensorimotor Deficits in Male and Female Mice and Reduces Survival

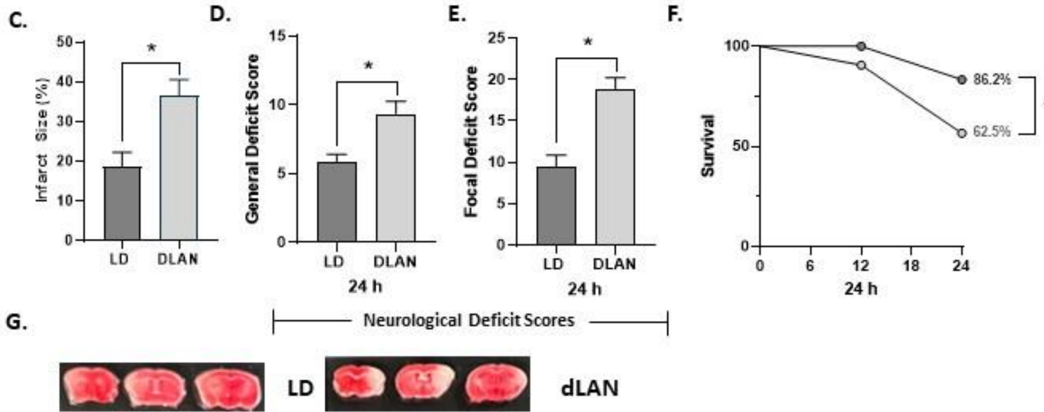
Previous studies demonstrated that in 10-week-old male mice, three nights of exposure to dLAN disrupts molecular rhythms and increases infarct size (Weil et al., 2020). Therefore, the effects of a single night of dim light exposure immediately post-MCAO was investigated to determine whether this influenced infarct size and deficit severity in adult mice.

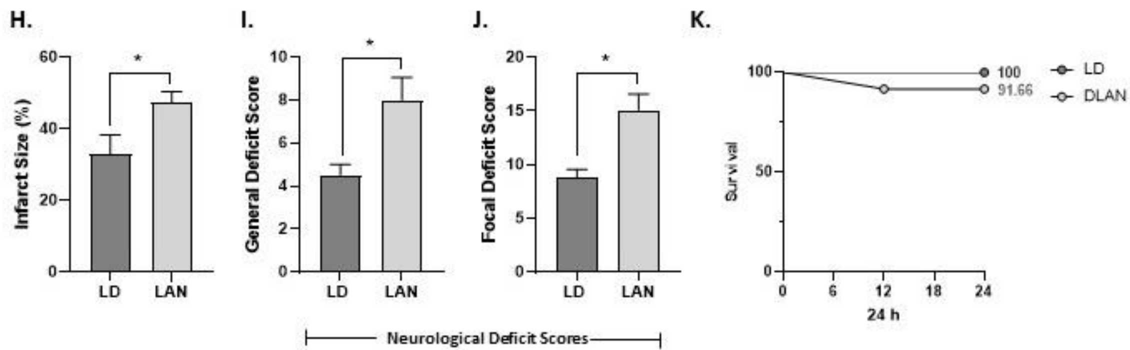
To determine how acute exposure to dLAN influences infarct evolution, six- to twelve-month-old male and female mice received a transient (45 min) right middle cerebral artery occlusion (MCAO) during the light phase (Zeitgeber time (ZT) 6-12), then were assigned to either dark night (LD) or dim light at night (dLAN) conditions. 24 h post-stroke, neurological scores were obtained to determine functional measures of stroke severity (Fig. 2.1A-B). A single night of exposure to dLAN was sufficient to significantly ($p<0.05$) increase infarct size and Clark Deficit Scores among male mice (Fig. 2.1C-E). Additionally, over the first 24 h post stroke, male mice exposed to dLAN displayed a significant reduction (23.7%) in survival compared to mice housed in dark nights (Fig. 2.1F). A representative image of infarct size visualized through TTC stain is observed in Figure 2.1G. Females exhibit a similar significant increase in infarct size and

neurological deficit scores compared to mice housed in dark night control conditions (Fig. 2.1H-J; $p<0.05$), however, in contrast to males, lighting condition did not reduce survival in female mice (Fig. 2.1K; $p<0.05$). To test whether the detrimental effects of dLAN on stroke outcome were dependent on reperfusion damage, the previous experimental timeline in male mice was repeated using permanent rather than transient middle cerebral artery occlusion and observed a similar pattern of significantly increased infarct size among mice exposed to dLAN compared to mice housed in dark nights (Fig. 2.1L-O). General and focal deficit scores were positively correlated with infarct size across both sexes and models of ischemia (Fig. 2.1P) and laser doppler flowmetry confirmed that mice subsequently assigned to LD and dLAN groups had similar reductions in relative blood flow during MCAO (Supplemental Fig. 2.1; $p<0.05$). These results demonstrate that dLAN increases infarct size and functional deficits independent of reperfusion following both transient and permanent MCAO and exacerbates stroke outcome in both males and females.


A.
Middle Cerebral Artery (MCA)
ICA
CCA


B.
MCAO
ZT 6-12
Neurological Scoring and Tissue Collection
ZT 6-12
12:12 (150 lux, 0 lux)
5-6 month
150 lux
LD (0 lux)
150 lux
150 lux
dLAN (5 lux)
150 lux
12 h
12 h
24 h
Groups
MCAO - LD
MCAO- dLAN


Male Transient MCAO
C.
Infarct Size (%)
LD
DLAN
D.
General Deficit Score
LD
DLAN
24 h
E.
Focal Deficit Score
LD
DLAN
24 h
F.
Survival
86.2%
62.5%
24 h
Neurological Deficit Scores
G.
LD
dLAN


Female Transient MCAO
H.
Infarct Size (%)
LD
LAN
I.
General Deficit Score
LD
LAN
J.
Focal Deficit Score
LD
LAN
K.
Survival
100
91.66
LD
DLAN
24 h
Neurological Deficit Scores

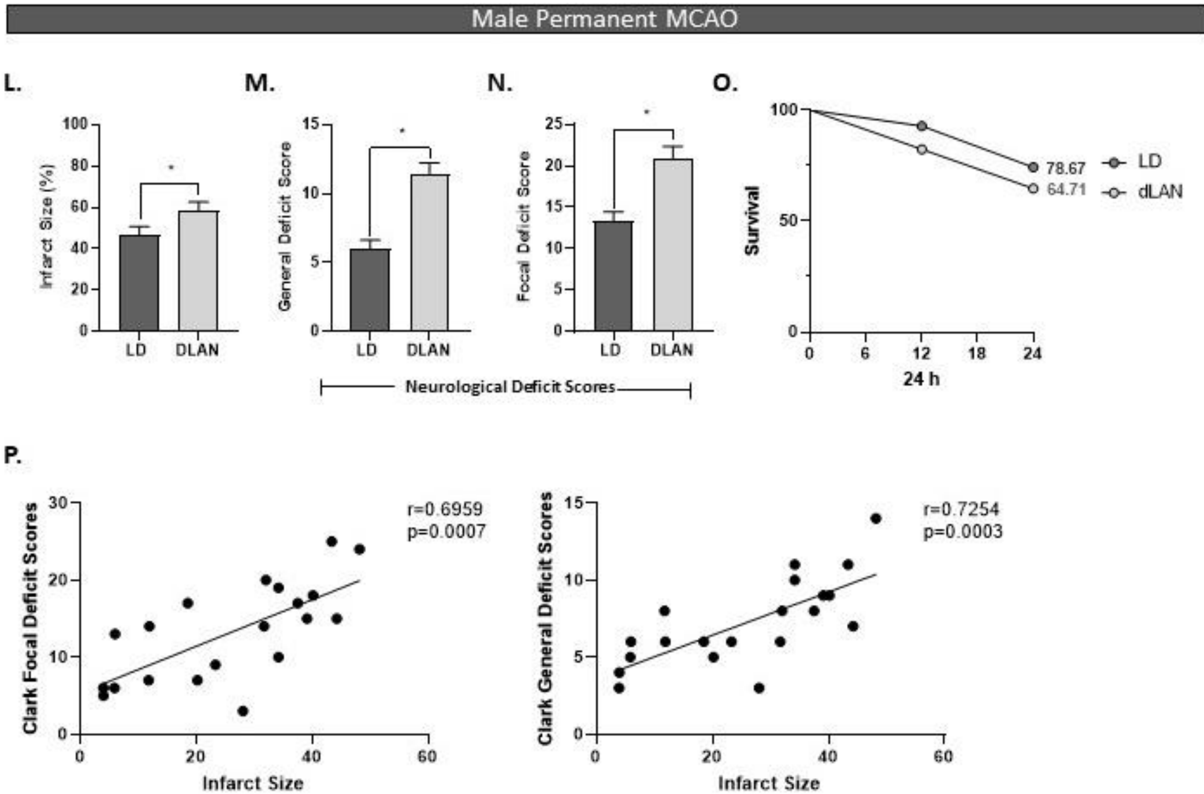


Figure 2.1. (A) Diagram of middle cerebral artery occlusion in a murine brain (B) Experimental timeline. (C) 24 hours post-transient MCAO, male mice housed in dLAN had increased infarct sizes and (D) impaired general and (E) focal deficit scores compared to mice in dark nights ($p<0.05$). The data are represented as mean ± SEM (LD n=11-12, dLAN n=7-8). (F) Mice housed in dLAN post MCAO had reduced survival (23.7%) across a 24 h period compared to dark night conditions ($p<0.05$).The data are represented as mean ± SEM (LD n=29, dLAN n=32). (G) Representative TTC Image depicting infarct size across mice housed in LD and dLAN conditions. (H) Female mice exposed to one night of dLAN, collected 24 h post-transient MCAO had increased infarct size and (I and J) sensorimotor deficits compared to dark night conditions ($p<0.05$). (K) Females between lighting conditions did not differ in survival across the first 24 h.

The data are represented as mean ± SEM (LD and dLAN n=10-11).(L) Mice exposed to dLAN after permanent occlusion had increased infarct sizes and (M and N) sensorimotor deficits compared to dark night conditions. (O) No statistical difference in survival across mice receiving permanent MCAO ($p>0.05$) (LD and dLAN n=10-11). (F) Infarct size positively correlates with focal and (I) general deficit Clark scores across sexes and lighting conditions.

Exposure to Post-Stroke dLAN Alters Pathways Associated with Innate Immune Response, Cell Response to Stress, and Apoptosis

Next, the cellular and molecular mechanisms that may be driving the detrimental effects of dLAN on stroke outcome were investigated. Bulk RNA-sequencing (RNA-Seq) analysis was performed to compare the ipsilateral (ischemic) and contralateral (control) hemispheres of male mice exposed to post-MCAO LD or dLAN. Brains were collected 12 h after MCAO to evaluate gene expression changes in the acute recovery period. Nighttime light exposure induced gene expression changes across the ipsilateral and contralateral hemispheres of the brain visualized in the volcano plot (Fig 2.2A and B). There were 51 differentially expressed genes (DEGs) (42 upregulated; 9 downregulated) in the ipsilateral (ischemic) hemisphere and 26 DEGs (24 upregulated; 2 downregulated) in the contralateral hemisphere. There were 21 overlapping DEGs between ipsilateral and contralateral hemispheres in mice exposed to dLAN compared to mice in LD conditions after stroke (Fig 2.2C). Biological processes enrichment and reactome pathway analyses were performed using the Gene Ontology database (http://geneontology.org/) and the PANTHER tool to identify biological processes associated with the differentially expressed genes after exposure to dLAN (Fig 2.2D; Supplemental Table 2). The data set of DEGs was

annotated under Reactome Pathways. The greatest differential expression occurred among genes associated with innate immune function (Mmp8, Mpo, Lyz2, Hsp90aa1, Ctsg, Mif, Camp, Ttr, Hbb-bs, S100a8, and S100a9), overlapping with neutrophil degranulation (Mmp8, Mpo, Lyz2, Hsp90aa1, Ctsg, Mif, Camp, Ttr, Hbb-bs, S100a8, and S100a9), cellular responses to stress (Cox8a, Hsp90aa1, Cox6a1, Hbb-bs, Hist1h3i, Hsph1), and regulation of neuronal apoptotic processes. Among genes involved in the apoptotic pathway; mice exposed to dLAN had increased gene expression of Gadph, S100a8, S100a9, and Nr4a1 in the ipsilateral hemisphere compared to those in dark nights. Indeed, these gene expression changes support our initial findings visualized by TTC, demonstrating that dLAN increases neuronal death post-stroke, as indicated by increased infarct size (Fig. 2.1C, G). Cell stress response occurs during acute ischemia and involves cytosolic chaperone proteins that facilitate and prevent aggregation of proteins, most notably through Hsp70 and Hsp90 (Kim et al., 2018). There was an ipsilateral decrease in gene expression associated with heat shock response, including Hsp90aa1, Hsp60, Hsp10, and Hsph110 in mice exposed to dLAN. These results suggest that post-stroke dLAN exposure downregulates expression of genes involved in cell-stress responses and associated compensatory mechanisms that prevent neuronal apoptosis. Furthermore, Per1, a circadian clock gene that is induced by exposure to light (Shigeoshu et al., 1997), was significantly upregulated across the ipsilateral and contralateral hemispheres of the brains of mice after exposure to nighttime light (12 h) post-ischemic injury.

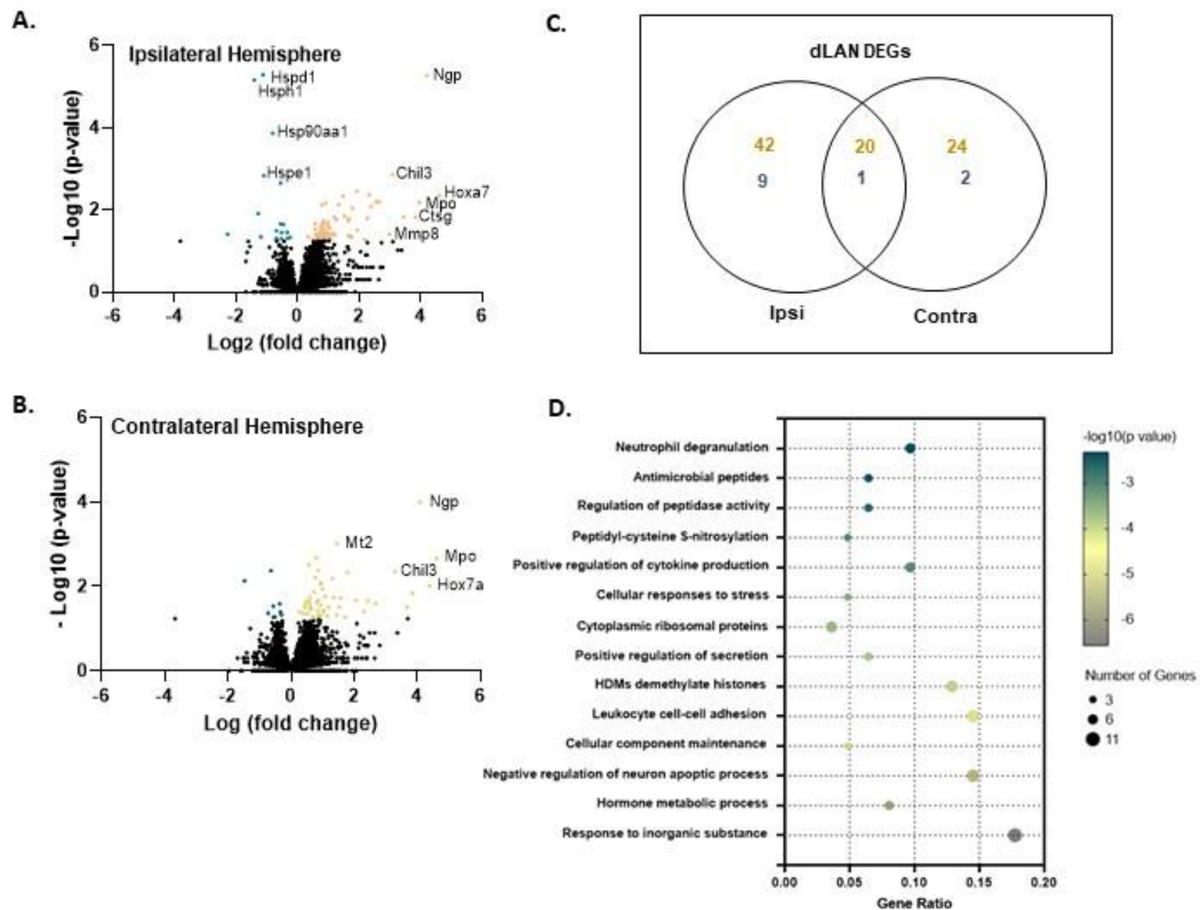
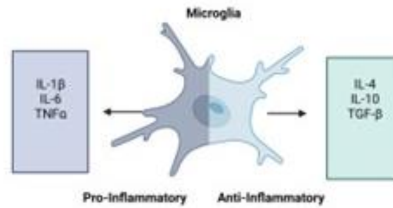


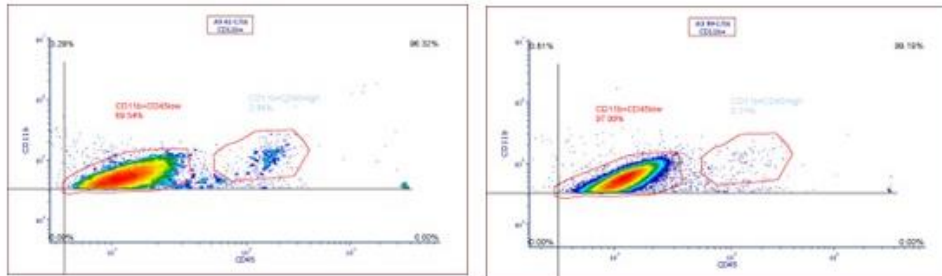
Figure 2.2. (A) Volcano Plot depicting statistical differential expression analysis. Scatterplot depicts differentially expressed genes (DEGs) in mice exposed to dLAN compared to LD conditions across the ipsilateral and (B) contralateral hemisphere. Statistical significance was determined using EDGE test ($p<0.05$) and a 1.5 or greater fold increase. Dots depicted in yellow denote statistically increased genes, blue denotes statistically decreased in dLAN mice relative to LD conditions (LD and dLAN n=4). (C) Venn diagram depicting statistically upregulated and downregulated genes in mice housed in dLAN compared to LD. (D) Bubble plot showing 14 most upregulated biological pathways based on significant DEGs in the ipsilateral hemisphere and gene ratio (LD and dLAN n=4).

dLAN Shifts Microglia to Pro-Inflammatory Phenotypes Post-Stroke

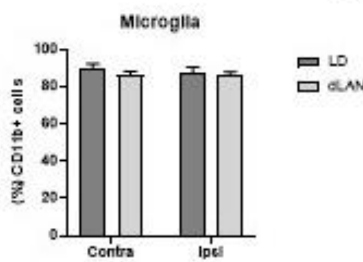
After determining that genes involved in innate immune function displayed the highest differential expression in mice exposed to dLAN, cell populations involved in inducing and recruiting immune cell changes in the brain were investigated. Microglia shift towards pro- or anti-inflammatory phenotypes after becoming activated; these phenotypes can induce and affect post-stroke inflammation. Therefore, we characterized how disrupted circadian rhythms by dLAN after stroke affected these microglial populations that modulate immune response after stroke. Flow cytometry was performed to analyze microglial phenotypes in the ipsilateral and contralateral hemispheres of brains 24 h post-stroke. Gating strategies for separating microglia from infiltrating immune cells are provided in Supplemental Figure 2.S2. The percentage of CD11b+ microglia (CD11b + CD45 low CX3CR1+) in the ipsilateral and contralateral hemispheres was not affected by dLAN (Fig 2.3B; $p<0.05$), but there was a significant increase ($p<0.05$) in the percentage of CD11b + microglia expressing major histocompatibility complex II (MHC II) and Interleukin-6 (IL-6) indicating that dLAN exposure post-stroke increases pro-inflammatory microglia (Fig. 2.3C and D). Additionally, there was an increased percentage of CD11b + microglia expressing CD206 and IL-10 ($p<0.05$; Fig 2.3E and F). There was no statistical difference in the percentage of CD11b+ cells expressing CD86, TNF-α, IL-4, or IL-1β between lighting conditions post-stroke (Fig. 2.3G-J; $p<0.05$). Finally, among CD11b + MHC II + microglia, exposure to dLAN increased the percentage of cells expressing TNF-α (Fig 2.3K) with no difference in the percentage that express IL-1b or IL-6 (Fig. 2.3L-M). Taken together, these data support the hypothesis that dLAN exposure alters the phenotype of microglia after stroke

A.

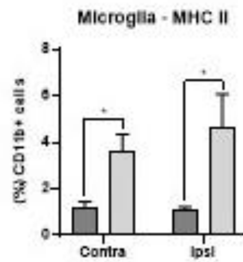




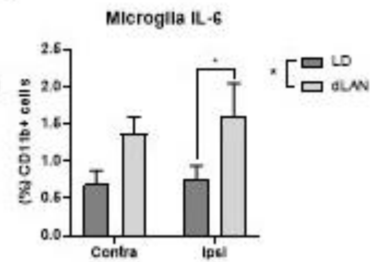
B.



C.



D.



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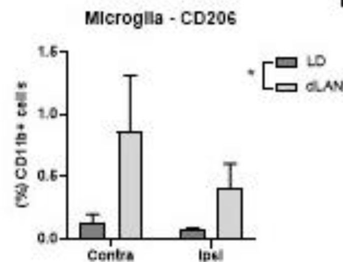

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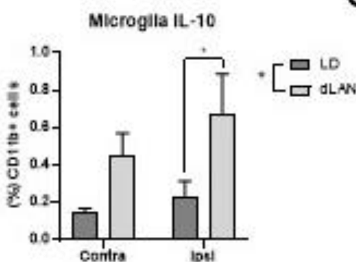

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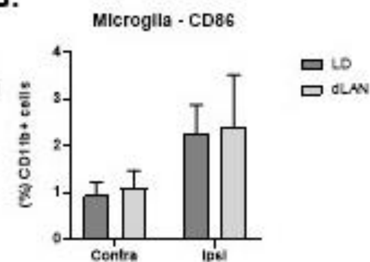

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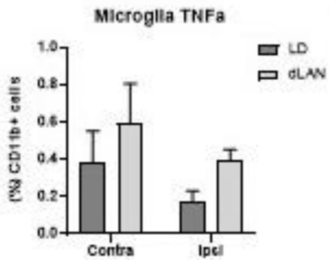

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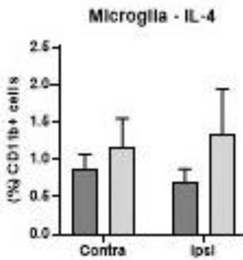

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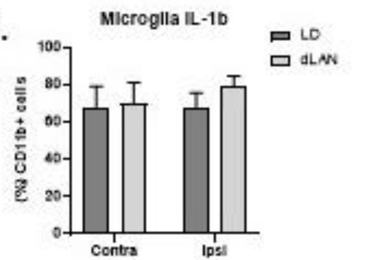

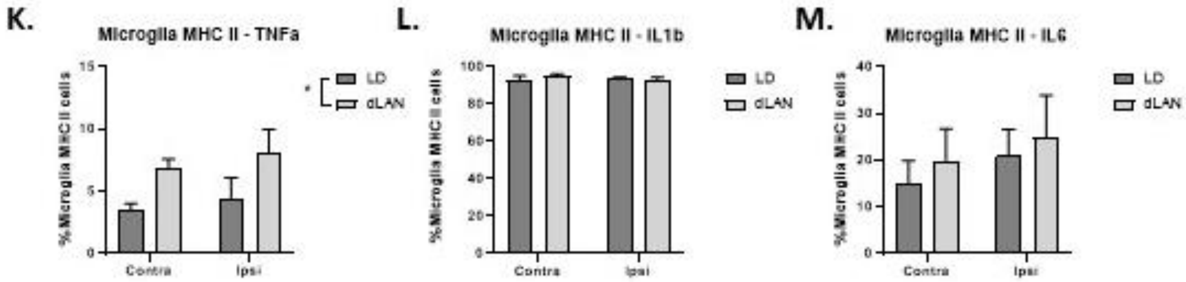

Figure 2.3. (A) Diagram and visual representation of Microglia ($CD11b^+$, $CD45^{low}$, $CX3CR1^+$) and peripheral immune cells across CD11b and CD45 axes. (B) Flow cytometry data comparing microglia ($CD11b^+$, $CD45^{low}$, $CX3CR1^+$) in mice after 24 h post stroke. There was no significant effect in the number of total microglia reported as a percentage of total CD11b+ cells in the brain of mice exposed to dLAN compared to dark night conditions ($p>0.05$); however, we observed an increase in microglia expressing $MHCII^+$ and (D) IL-6 representing a shift of microglia to a proinflammatory phenotype across the ipsilateral (IL-6) and both hemispheres (MHC II) (C and D) ($p<0.05$). (E) dLAN increased the percentage of microglia that express CD206 compared to dark night conditions ($p<0.05$; not statistically different on post-hoc analyses) (F) and an ipsilateral increase in microglia that express IL-10. No statistical difference between lighting conditions across the ipsilateral or contralateral hemisphere in the percentage of microglia that express (G) CD86, (H) TNF-α, (I) IL-4, or (J) IL-1β ($p>0.05$). (K) MHC II+ microglia from mice housed in dLAN have increased expression of TNF-α ($p<0.05$; not statistically different on post-hoc analyses) and compared to dark night controls. (L) No statistical difference in MHC II+ microglia that express IL-1β or (M) IL-6 ($p>0.05$).The data are represented as mean ± SEM (LD n=5-6 and dLAN n=4-5).

Microglia Modulate Post-Stroke Inflammatory Responses to dLAN

Our hypothesis was that microglia are responsible for the neuroinflammatory responses that contribute to increased post-stroke damage among mice exposed to dLAN. To test this hypothesis, microglia were depleted in the brain through the use of a CSFR1 inhibitor, Plexikkon 5622 (PLX5622), that selectively reduces brain microglia (Badimon et al., 2021; Spangenberg et al., 2019). PLX5622 was administered for 7 days prior to MCAO, then as described for the studies above, mice were exposed post-stroke to either one night of dark or dLAN (Fig 2.4A). 24h

post-stroke PLX5622 treatment resulted in a ~70% reduction of P2RY12 + immunoreactivity (P2RY12; Fluor 594) in the cortex of mice exposed to either dark nights or dLAN compared to control diet (Representative Image Fig 2.4B; Fig C). There were no differences in food intake between mice receiving PLX5622 incorporated diet for 7 days prior to MCAO, that were subsequently placed into dark nights or nighttime lighting conditions (Supplementary Figure 2.3; $p<0.05$). Cytokine production in serum was evaluated using the Proinflammatory Panel 1 (mouse) ELISA multiplex (Meso Scale Discovery, LLC) to determine if exposure to dLAN alters peripheral inflammation after depletion of microglia. In mice receiving the control diet (without depletion of microglia), dLAN exposure significantly increased serum TNF-α concentration compared to mice exposed to dark nights (Fig 2.4D; $p<0.05$) as expected; in contrast, among mice consuming the PLX5622 diet there was no significant difference in serum TNF-α in between the dLAN and LD groups (Fig 2.4E; $p<0.05$). There were no significant effects of diet or nighttime light exposure on other serum cytokines (Fig. 2.4F-U). Serum corticosterone was not affected by light at night exposure in mice receiving the control diet, (Fig 2.4V), however dLAN exposure increased corticosterone in mice that received PLX5622 (Fig 2.4W) suggesting

that microglia may also modulate the post-ischemia stress response. Among LD mice, depletion of microglia via PLX5622 administration caused a significant increase in infarct size relative to LD mice that received the control chow (Fig. 2.4X; $p<0.05$). These data suggest that during the early development of an infarct, microglia are beneficial to stroke recovery whereas their depletion exacerbates stroke outcome. These data are consistent with previous studies that report that PLX5622 administration increases infarct sizes compared to control diet (Jin et al., 2017; Otxoa-de Amezaga et al., 2018; Lee et al., 2021), suggesting that microglia have important neuroprotective functions after stroke, including phagocytic properties for accumulating neutrophils and removing debris after ischemic injury (Otxoa-de-Amezaga et al., 2018). In contrast, among dLAN mice, PLX5622 administration caused a significant decrease in infarct size relative to dLAN mice that received the control chow (Fig. 2.4X; $p<0.05$), suggesting that the microglial phenotype in mice exposed to dLAN are detrimental to stroke recovery. Thus, administration of PLX5622 produces similar infarcts 24h post-stroke in the LD and dLAN groups through the depletion of microglia with a neuroprotective phenotype in mice exposed to dark nights and by depletion of microglia with a neurodegenerative phenotype in mice exposed to light at night. In other words, dLAN causes a fundamental shift of microglia from a beneficial to detrimental state at this early and crucial time point after stroke, resulting in irreversible neuronal death.

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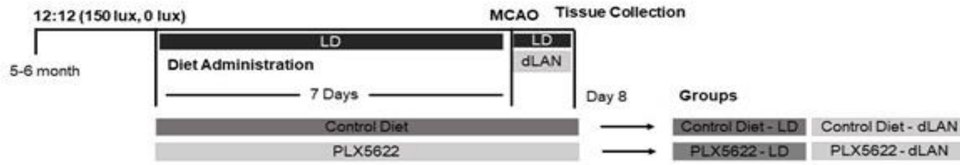

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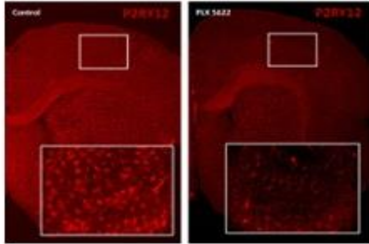

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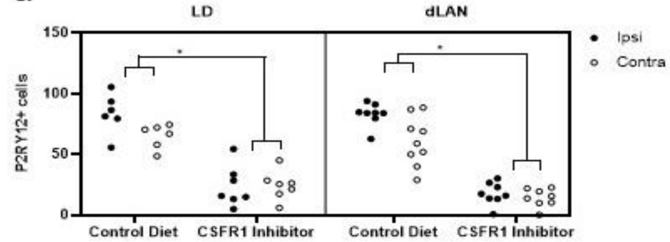

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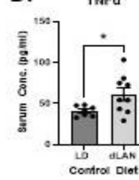

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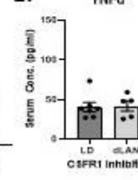

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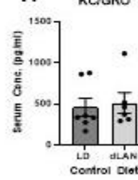

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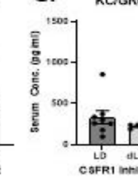

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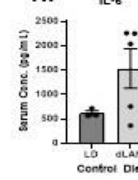

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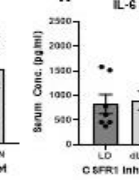

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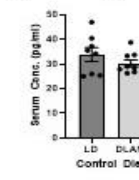

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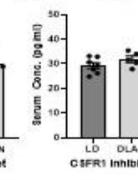

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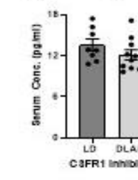

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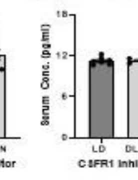

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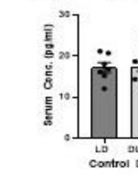

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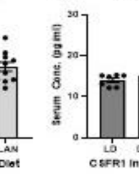

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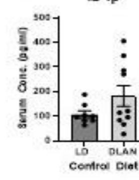

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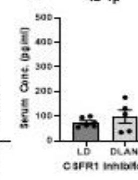

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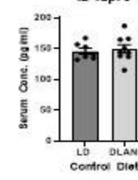

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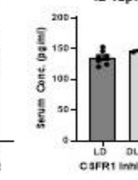

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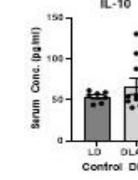

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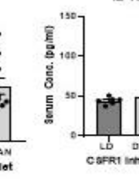

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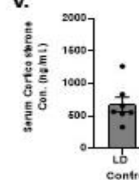

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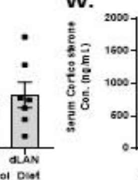

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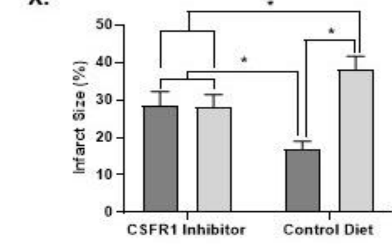


Figure 2.4. (A) Experimental Timeline for CSFR1 administration. (B) Immunofluorescence staining was performed on representative brain sections for the microglial specific marker P2RY12. (C) 7 Days of PLX5622 1200 ppm treatment led to depletion of P2RY12+ microglia in the cortex of mice post-MCAO relative to the control diet across mice housed in LD and dLAN. The data are represented as mean ± SEM (n=10/group). (D) TNF-α concentrations were increased in mice exposed to one night of dLAN under control diet conditions compared to dark nights ($p<0.05$) (E) which becomes normalized after elimination of microglia with administration of PLX5622 ($p>0.05$). The data are represented as mean ± SEM (LD n=7-8; dLAN n=4-5). (F-U) Other pro-inflammatory cytokine concentrations in serum were not statistically different ($p>0.05$), samples that did not contain enough serum or were out of range were excluded from analyses. (V) corticosterone concentrations in serum were not statistically different between lighting conditions 24 h after MCAO in control diet conditions ($p>0.05$). (W) Mice exposed to dLAN that received PLX5622 to deplete microglia had significantly increased corticosterone concentrations in serum compared to dark night controls. The data are represented as mean ± SEM (n=7/group). (X) Mice housed in dLAN had normalized infarct sizes comparable to LD conditions with the depletion of microglia using PLX5622 (CSFR1 Inhibitor). Under control diet conditions, mice exposed to dLAN had increased infarct sizes compared to dark nights ($p<0.05$) as previously described in Figure 1. Mice that received PLX5622 across light conditions had increased infarct size relative to control diet dark night conditions ($p<0.05$) and infarct sizes in mice receiving PLX5622 between light conditions were significantly decreased relative to control diet dLAN infarct sizes ($p<0.05$). The data are represented as mean ± SEM (n=9-10/group).

Discussion

Optimal circadian rhythm function is predicated on predictable and consistent exposure to alternating phases of light and dark. The widespread adoption of artificial and electrical lighting over the past century has blurred the distinction between day and night and altered both the timing and duration of light exposure across the day (Cao et al., 2022). Inopportune nighttime lighting now extends into households and naturally dark ecosystems and environments.

LAN affects up to 80% of the world population and ~99% of people in the United States and Europe (Falchi et al., 2016). This is concerning because circadian disruption has been shown to influence the pathology and progression of a wide range of chronic diseases. Less is known about its potential effects on recovery from acute illness. We chose to study the effects of dLAN on ischemic stroke recovery because it is a condition that typically requires hospitalization; stroke patients in the United States spend an average of 3.28 days in intensive care units (Lilly et al., 2011) and it is common to expose patients to dimly illuminated hospital rooms during both the day and night (Lusczek and Knauert, 2021). These lighting practices are considered necessary to properly monitor patients and to promote rest and safety, but they are disruptive to the maintenance of circadian rhythms, and our data suggest that nighttime exposure to dLAN causes excess neuronal damage following stroke. Our study provides insights into the biological mechanism underlying nighttime light exposure and post-stroke infarct development. Here, our data suggest that exposure to dLAN significantly alters microglial phenotype and in turn compromises post-stroke sensorimotor function and increases neuroinflammation and infarct size. Specifically, during the first 24 h after stroke, dLAN appears to cause a shift in microglial phenotype from promoting neuroprotection to promoting neurodegeneration. Microglial activation occurs early after ischemic injury (Ma et al., 2018) and due to their multifunctional

role in the production of pro- and anti-inflammatory cytokines, phagocytosis, and antigen presentation, growing evidence supports both a protective and detrimental role of microglia in the progression of ischemic insult and secondary damage (Lalancette, 2007; Ma et al., 2018). Our data highlight the importance of this biphasic response of microglia in the early development of an infarct and the recovery from an ischemic event. Modulating post-stroke neuroinflammation is crucial during the acute development of the ischemic infarct in order to limit stroke severity; prolonged inflammatory responses mediated by microglia and macrophages contributes to reduced functional recovery (DiSabato et al., 2017) and increased mortality in stroke patients (Hou et al., 2021). Increasing evidence supports the adverse health consequences associated with nighttime lighting, particularly for individuals with compromised physiological or disease states (Nelson and DeVries, 2007). Our data demonstrate that a single night of light exposure causes excess neuronal death in two different murine models of focal ischemic stroke. Further, dLAN reduced survival by ~23% among males across the first 24 h compared to dark night conditions. Reperfusion of the brain after ischemic stroke occurs in 3-9% of patient populations by receiving thrombolytic therapies (Adeoye et al., 2011) or up to 15% who are eligible to receive mechanical thrombectomy (El Tawil et al., 2016). However, this can cause secondary injury and increase peripheral leukocytes and proinflammatory cytokines to the site of injury (Onwuekwe and Ezeala-Adikaibe 2012). Therefore, a permanent occlusion model was used to clarify whether damage caused from disrupted circadian rhythms via exposure to dLAN is driven by reperfusion injury. Exposure to dLAN after both transient and permanent occlusion significantly increased infarct size relative to LD controls.

There is a well-supported relationship between circadian rhythms and stroke, and growing

support for the hypothesis that circadian disruption exacerbates stroke outcome (Liu et al., 2021). Previous studies identified the role of dLAN as a disruptor to circadian rhythms in 10-week-old C57BL/6 male mice and demonstrated that exposure to 3 nights of dLAN is sufficient to exacerbate stroke volume (Weil et al., 2020). In other models of injury, e.g., global ischemia, LAN exposure provoked increased hippocampal neuronal damage, and elevated proinflammatory cytokine production (Fonken et al., 2019). Other studies using genetic manipulations of clock genes including Per1 -/- mice have increased neuronal damage post-ischemic stroke compared to wildtype mice and suggest that apoptosis and autophagy is modulated by PER1 (Wiebking et al., 2013). Independent of circadian rhythm disruption, time of day is another biological variable that can alter neuronal injury after ischemic stroke. Mice receiving MCAO between ZT 18-24 compared to other time points (ZT 0, ZT 6, ZT 12, and ZT18) had improved infarct size, edema, neuronal survival, and reduced apoptotic cell death compared to other time points (Beker et al., 2018). Additionally, clinical studies reflect circadian variations in timing of stroke onset (Elliot, 1998) and poor outcome (Ryy et al., 2022). Together, these findings provide supporting evidence that altering components of circadian rhythms increases risk for neuronal death after injury. Biological sex affects brain inflammatory profiles (Felzein et al., 2001) and ischemic outcomes (Liu et al., 2009; Seiber et al., 2011), and so the effects of dLAN on stroke outcomes were examined in adult mice of both sexes. These results demonstrated that exposure to dLAN increases infarct size and sensorimotor deficits in both sexes, although mortality was increased among males, but not females, undergoing transient MCAO. This outcome is consistent with the literature; sex differences in stroke have been well-documented in rodent models and in the clinic (Branyan and Sohrabji, 2020). Adult and aged female mice have improved long-term stroke outcomes compared to adult males despite

exhibiting larger infarcts (Branyan and Sohrabji, 2020). Furthermore, males experience increased stroke-induced mortality compared to females (El-Hakim et al., 2021; Liu et al., 2009). Sex differences in autophagy (Patrizz et al., 2021), immune function (Dotson and Offner, 2017), and cell death pathways post-ischemia have been well characterized (Liu, et al., 2009). Studies evaluating other models of environmental circadian disruption, such as shift work, likewise demonstrate the detrimental impact of circadian rhythm disruption on stroke outcome (Earnest et al., 2016). Rats that underwent 12 h phase advances every 5 days for 7 weeks prior to MCAO had significantly larger infarcts and sensorimotor deficits than the control group (Earnest et al., 2016). Additionally, this study identified sex differences in pathological outcomes whereby males subjected to phase shifts had significantly elevated rates of mortality compared to females, consistent with our findings. Lastly, mice deficient in *Bmal1*, one of the core circadian clock genes, displayed a sex-dependent interaction effect between sex and genotype which the authors concluded was dependent on differences between astrocytes and microglia in peri-infarct regions (Lembach et al., 2018). Together, these studies suggest that circadian rhythm disruption is associated with poor stroke outcome.

The next step was to understand how dLAN alters stroke outcome. The majority of transcriptomic changes occur within the initial 24 h after ischemic insult and these changes coincide with neurons undergoing reperfusion, hypoxic conditions, and cell death (Li et al., 2020). To understand the influences of dLAN on this process, bulk RNA-sequencing was performed on the ipsilateral (ischemic) and contralateral (non-ischemic) hemispheres of the brain 12 h post-stroke. This allowed comparison of gene expression after a single exposure to either dark or illuminated night conditions as the infarct is forming. The most striking patterns of

differential expression were observed among genes involved in innate immune function and the cell-stress response within the ischemic hemisphere. Increasing evidence supports that the circadian clock is involved in several aspects of immune response (Keller et al., 2009; Walker et al., 2021), including phagocytic capacity (Hayashi et al., 2007; Olivia-Ramierz et al., 2014), cytokines and chemokines (Curtis et al., 2014). Previous work examining the role of dLAN as a disruptor to circadian rhythms reported dysregulated immune response after immune challenge (Fonken et al., 2012; Fonken et al., 2013). Immune challenge, however, is not necessary to demonstrate that dLAN can alter aspects of the immune system. The steady state of daily variations of circulating immune cells are altered after as little as 2 weeks of exposure to dLAN (Okuliarova et al., 2021). Our sequencing data demonstrated increased expression of genes involved in innate immune function and neutrophil degranulation, which serve as the first response after innate immune response initiation (Wang et al., 2018). In mice exposed to nighttime lighting, there was a decrease in gene expression involved in cell stress response within the ischemic hemisphere (Fig 2.3B). Heat shock proteins (HSP) are molecular chaperones that under typical conditions play a regulatory role in protein folding, intracellular protein transport to the cytosol and mitochondria (Hendrick and Hartl, 1993); they are essential for cell survival. In addition, they become activated during acute stressors, such as neuronal injury, and may modulate the inflammatory response (Lovett et al., 2014) and counteract apoptosis and cell death (Choi et al., 2014). Thus, reduced post-stroke HSP expression among the dLAN group may have contributed to increased infarct size. Future studies should also examine a potential role for TNF-α. This proinflammatory cytokine is increased in the serum of mice housed in dLAN after stroke and has previously been shown to downregulate heat shock factor 1 (HSF1; Schett et al., 2003). Together, these findings suggest that exposure to dLAN decreases gene expression

involved in cell-stress response that is vital for counteracting injury in ischemic tissue, resulting in fewer protective cellular measures and greater susceptibility to damage leading to neuronal death.

Elevated systemic inflammation is associated with an increase in stroke-related comorbidities (Esenwa et al., 2016) and because predominant changes in differentially expressed genes (DEGs) related to innate immune function were observed, we next investigated microglia as one of the glial cell populations that are responsible for initiating post-stroke inflammation and the recruitment of infiltrating peripheral immune cells. Microglia are an important component of ischemic injury repair (Ma et al., 2017; Zhang et al., 2019). Our results demonstrated that one night of exposure to dLAN alters microglial phenotypes, specifically increasing the percentage of CD11b + microglia that express MHC II, surface heterodimers involved in presenting processed antigens, and IL-6, a pro-inflammatory cytokine marker of inflammation post stroke (Zhu et al., 2022). Previous studies evaluating imbalances between microglial phenotypes after injury observed increased neuroinflammation and adverse stroke outcomes (Yong et al., 2019). Other studies investigating inhibition of microglial activation display improved infarct size (Gelosa et al., 2014), neurological deficits, and inflammatory response (Zhang et al., 2016; Shi et al., 2015), and reductions in blood-brain barrier disruption (Yenari et al., 2006; Li et al., 2014). The role of microglia in photothrombotic ischemic stroke models, demonstrated that selective deletion of microglia in CX3CR1 CreER R26 iDTR mice through tamoxifen and diphtheria toxin administration during the first three days after ischemic stroke, resulted in a decrease in infarct volume and improved motor ability performance three days post-stroke (Li et al., 2021). Further, this study demonstrated that microglial depletion reduced inducible nitric oxide synthase positive

(iNOS+) cells along with increasing anti-inflammatory cytokine factors (TGF-β1, Arg1, IL-10, IL-4, and Ym1) and decreasing pro-inflammatory factors (TNF-α, iNOS, and IL-1β; Li et al., 2021). Notably, disparities between infarct size in other studies appear to depend on the timing of microglial depletion prior or after ischemic stroke when comparing findings to studies using CSFR1 inhibitors in the context of stroke. However, no studies to date have directly compared prior and post-microglial depletion in modulating stroke outcome. There is other supporting evidence suggesting that microglial activation is necessary for modulating inflammation (Liu et al., 2014), neuronal apoptosis (Parada et al., 2013), and clearance of cell debris and infiltrating neutrophils (Neumann et al., 2008). Among our results, we observed an increased percentage of microglia that express CD206 and IL-10 in mice exposed to dLAN (Fig 2.4B) which reflect anti-inflammatory activated phenotypes (Lawrence et al., 2011). Although, this does not appear to be biologically relevant as mice displayed increased sensorimotor deficit scores reflecting those collected for infarct analysis, and immune cells were extracted at 24 h post stroke. After ischemic stroke, activated microglia polarize to an "M2-like" or anti-inflammatory phenotype which gradually switches to an "M1-like" or pro-inflammatory phenotype in peri-infarct regions (Hu et al., 2012), which may reflect why we observe increased microglia that express CD206 that have not yet shifted phenotypes at 24 h. Future analyses of microglia between 7-14 days where recovery occurs could provide insight on other mechanisms.

Serum concentrations of cytokines and interleukins post-stroke were evaluated as a measure of peripheral immune response, and TNF-α concentrations were increased in mice exposed to one night of nighttime lighting compared to mice housed in dark nights. When further characterizing pro-inflammatory microglia using flow cytometry through evaluating cytokine

expression in microglia that express MHC II+, we identified that MHC II+ microglia from mice exposed to dLAN increased expression of TNF-α compared to dark night conditions. This suggests that TNF-α secreted by pro-inflammatory microglia may play a prominent role in dLAN phenotypes and worsening acute injury. Neuroinflammation after ischemic injury can increase neurological deficit severity (Dugue, 2017); and these data are supported by and are consistent with previous studies that reported that dLAN can induce proinflammatory cytokine production, specifically TNF-α (Bedrosian et al., 2013), and neuroinflammation in otherwise healthy mice (Walker et al., 2020), as well as alter immune responses in rodents (Bedrosian et al., 2011; Fonken et al., 2013). Other studies using different models of disruption to circadian rhythms such as chronic phase advances prior to ischemic injury displayed dysregulated cytokine expression (Ramsey et al., 2020; Earnest et al., 2022). Future studies modulating TNF-α through selective inhibitors may additionally serve as a therapeutic target for reducing secondary damage progression related to circadian disruption and dLAN.

Microglial survival is dependent on CSFR1 signaling (Elmore et al., 2014); thus, we next employed a treatment targeting microglia in the brain, without directly affecting neurons in the brain (Erblich et al., 2011). Depletion of microglia in the brain reduced the infarct size in mice exposed to dLAN, suggesting that microglia are involved in the stroke microenvironment through shifting microglia to pro-inflammatory states and increasing pro-inflammatory mediators that recruit peripheral immune cells to the site of injury. Further, serum TNF- α concentrations were normalized in mice that received PLX5622 across both lighting groups. PLX5622 has previously been reported to increase stroke severity in adult rodent models of MCAO (Jin et al., 2017) and aged mice as well (Lee et al., 2021), consistent with our results. The elimination of

microglia in the context of stroke is detrimental, especially during acute injury formation, which occurs early in the recovery process and coincides with the time where neuronal death occurs. Microglia's phagocytic properties removes accumulating neutrophils at the site of infarct that contribute to secondary damage (Otxoa-de-Amezaga et al., 2018). Indeed, other myeloid cells including monocytes and macrophages express CSFR1, so we cannot rule out the possibility that use of inhibitors can affect off-target populations such as peripheral macrophages (Lei et al., 2020); however, other studies to date have noted that PLX does not affect neurological function (Elmore et al., 2014; Jin et al., 2017) or baseline inflammatory status (Luo et al., 2013).

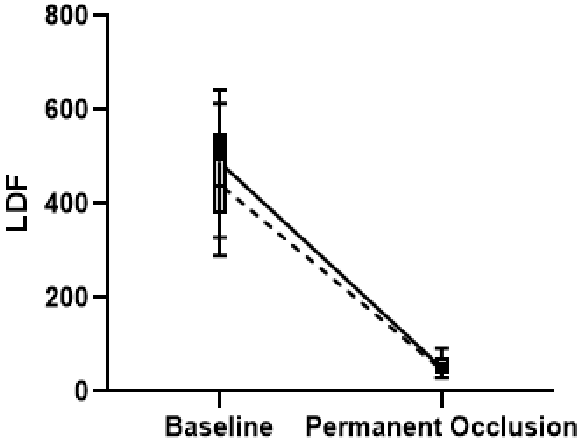
Current clinical studies linking circadian rhythms and stroke are focused on the role of disrupted sleep (West et al., 2019) or are correlative studies associating night shift work with increased stroke risk (Brown et al., 2009). Over twenty percent of people work non-traditional hours in the United States which has been linked to increased risk and higher incidence of disease, including obesity and metabolic disorders, diabetes, cardiovascular disease (Tenkanen et al., 1998), and cancer (Schernhammer et al., 2003). In addition to these risks, a study evaluating nursing shift workers revealed an increased risk of ischemic stroke and vascular disease correlating to the length of rotating shift work (Brown et al., 2009). Other studies evaluating patients exposed to shift work identified a greater risk for circulatory diseases (Tuchsen et al., 2006) and further, an increase in stroke and cardiovascular-related mortality in male shift workers (Karlsson et al., 2005). Light at night is another biological consideration that can further exacerbate injury and impair recovery that requires further and direct investigation in clinical studies. Disrupted sleep and lighting exposure is another factor that can potentially affect clinical outcomes (Mason et al., 2022).

To separate out the effects of disrupted sleep from disrupted circadian rhythms, nocturnal rodents that do not experience disrupted sleep from dim nighttime lighting were used (Borniger et al., 2013). Corticosterone values were assessed in mice receiving control diet treatment that were subsequently subjected to dark night and nighttime lighting conditions post-stroke to rule out differences in stress driving initial increases infarct size in dLAN mice, supporting previous findings in rodent models and cortisol in humans (Jung et al., 2010). Mice that were housed in dLAN and received PLX 5622 had elevated serum corticosterone concentrations compared to dark night controls. Corticosterone concentrations display robust circadian rhythms and are affected by sleep (Scher et al., 2010). Glucocorticoid receptors are expressed by several cell types in the brain including microglia and changes in the environment from stress can alter microglial morphology (Picard et al., 2021) and phagocytosis (Lehmann et al., 2016). Current studies investigating the relationship between microglia and sleep identified that depletion of microglia increases the number of wake-NREM sleep transitions which occur only during the night-time, indicating its role in stabilizing wakefulness (Liu et al., 2021). Future studies investigating circadian disruption in diurnal rodents that experience dim lighting exposure during the sleep period and microglia may delineate the mechanism driving this response.

Our results have clinical implications by identifying a biological mechanism delineating how light at night adversely affects stroke outcome in a murine model of stroke. We have identified that disruption of circadian rhythms by exposure to dLAN affects immune cascades in a rodent model of ischemic stroke by altering activated pro-inflammatory states that increase infarct size and neurological deficits, as well as reduce survival. Further, we identified that microglia, which

display robust circadian rhythms, are affected by nighttime lighting post-stroke, increasing the proportion of microglia to a pro-inflammatory state and increase serum TNF-α concentrations. Elimination of microglia post-stroke prevents the elevated infarct size in response to disrupted circadian rhythms. These results provide evidence that light at night can significantly exacerbate injury/disease outcomes and emphasize that hospital lighting settings may adversely affect patient outcomes. Future studies should consider identifying microglia as a potential therapeutic target point in chronotherapy or chronopharmacological studies. Reduced disruptions to circadian rhythms by harmonizing light conditions with the solar day in patient hospital rooms may reduce functional deficits and improve patient outcome.

Supplementary Figures and Figure Captions



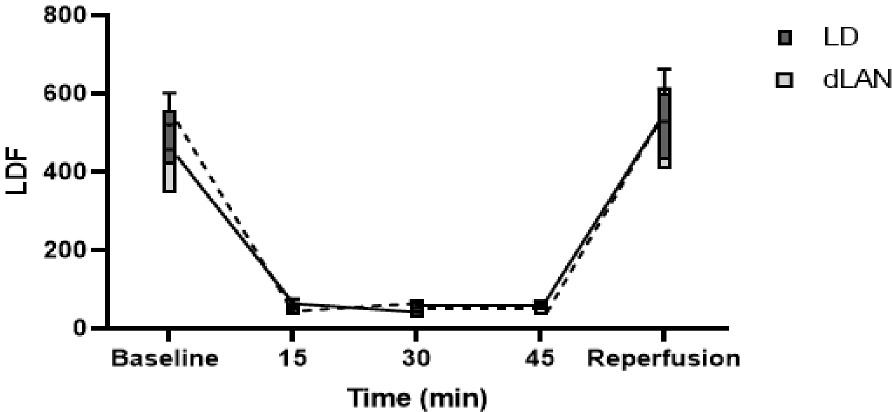


Fig. 2. S1. Laser doppler flowmetry (LDF) assessing cerebral blood flow changes across permanent or transient middle cerebral artery occlusions. (A) Laser doppler flowmetry (LDF) was performed in mice receiving transient intraluminal middle cerebral artery occlusions to measure changes in cerebral blood flow over 45 minutes. Changes in LDF after middle cerebral artery occlusion (MCAO) and visualization of reperfusion is demonstrated. A repeated measures two-way ANOVA was assessed to provide evidence that there were no differences in cerebral blood flow changes between mice under dark night conditions that were subsequently placed into dim light at night conditions (dLAN) or dark night (LD) conditions after MCAO ($p<0.05$). (B) LDF was assessed in mice receiving permanent MCAO to confirm a permanent reduction in cerebral blood flow. A repeated two-way measures ANOVA was conducted to support that there were no differences in mice prior to placement into respective post-stroke lighting conditions. *$p<0.05$.

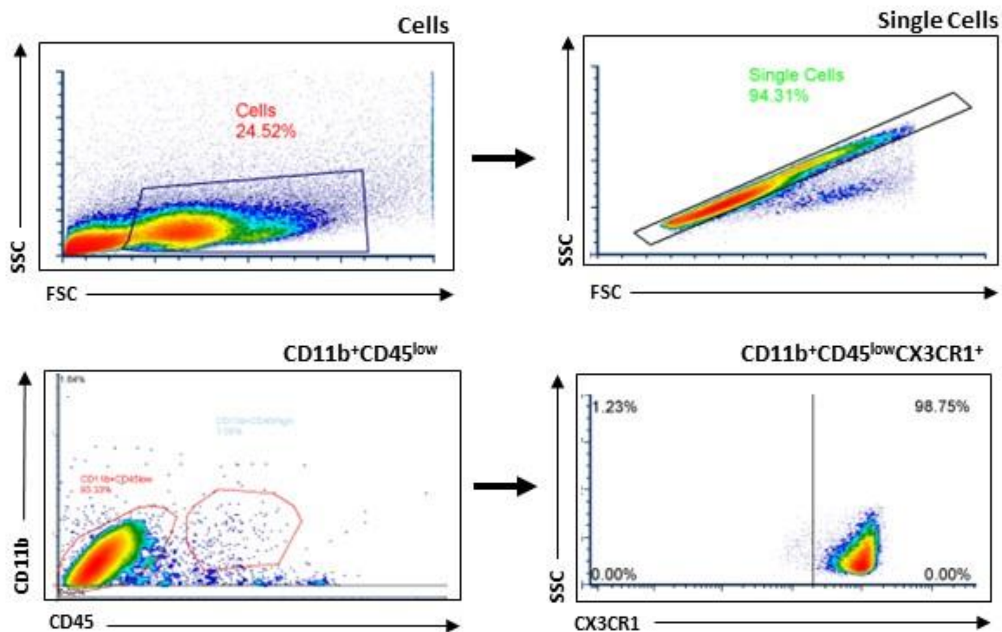
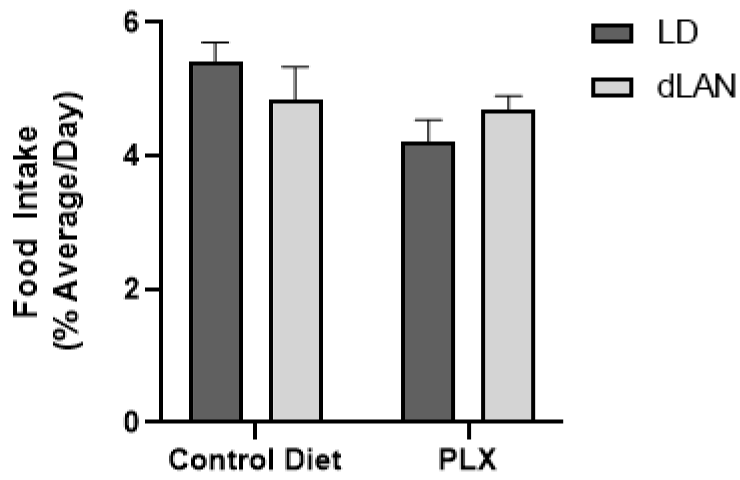


Fig. 2. S2. Representative plots indicating gating strategies for microglia. Microglia as CD11b+CD45lowCX3CR1+. Total numbers of each cell population were further quantified to evaluate pro – or anti – inflammatory profiles through markers (MHC II, CD86, CD206, and CD192) and interleukins/cytokines (IL-1β, IL-4. IL-10, IL-6, and TNF-α


Average food intake under dark night conditions prior to MCAO
Food Intake (% Average/Day)
6
4
2
0
Control Diet
PLX
LD
dLAN

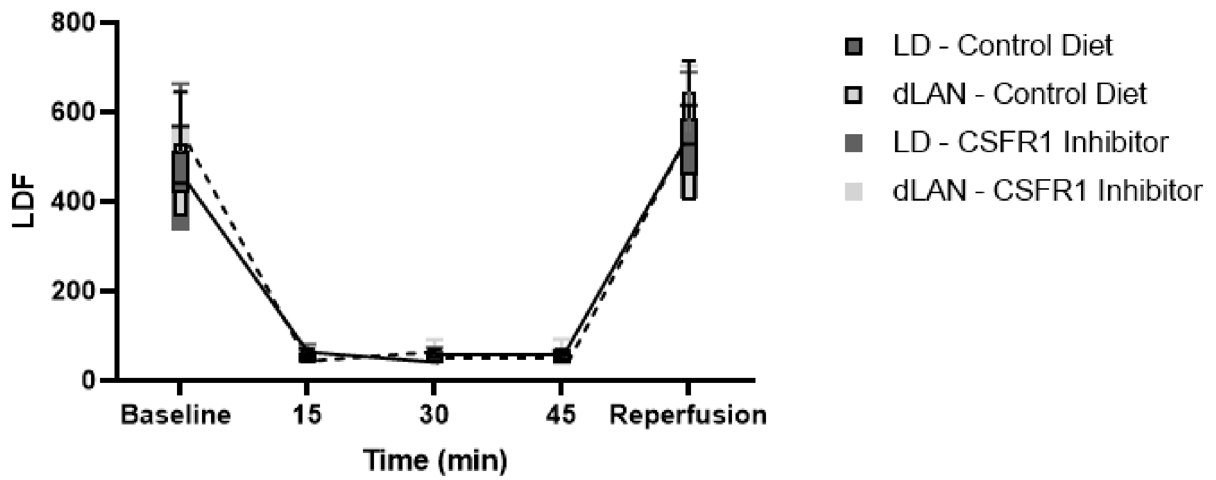

800
600
400
200
0
LDF
Baseline
15
30
45
Reperfusion
Time (min)
LD - Control Diet
dLAN - Control Diet
LD - CSFR1 Inhibitor
dLAN - CSFR1 Inhibitor

Fig. 2. S2. LDF and PLX5622 Diet intake under control dark night conditions seven days prior to MCAO do not differ between mice subsequently placed into one night of LD/dLAN. (A) Mice had *ad libitum* access to control diet (Open standard diet (D11112201; Research Diets Inc.) or PLX5622 (CSFR1 inhibitor; MedChemExpress, Catalog #HY-114153) at a concentration of 1200 ppm incorporated into a standard research diet D20030403 by Research Diets Inc. for 7 days. Mice had *ad libitum* access to diets and food intake was measured and averaged across 7 days. A two-way ANOVA assessing food intake was performed to provide evidence that there were no differences in PLX5622 diet intake between mice under dark night conditions that were subsequently placed into dim light at night conditions (dLAN) or dark night (LD) conditions after MCAO ($p<0.05$). (B) Laser doppler flowmetry (LDF) was performed during MCAO that received control diet conditions or PLX5622. A repeated measures three-way ANOVA was assessed to provide evidence that there were no differences in cerebral blood flow changes between diet conditions or mice under dark night conditions that were subsequently

placed into dim light at night conditions (dLAN) or dark night (LD) conditions after MCAO ($p<0.05$). *$p<0.05$.

Table 2. S1. Statistical Analyses for each Data Figure. Figure letters with diagrams or experimental timelines were excluded from this table.

Figure		Statistics	Treatment	P-value	Statistics	Mean
1. tMCAO Males Infarct Size General Deficit Score Focal Deficit Score	C	Unpaired two-tailed t-test	M - LD/dLAN	* p=0.0042	t=3.276 df = 18 SEM = 5.466	Mean LD = 18.74 dLAN = 36.64
	D	Unpaired two-tailed t-test	M - LD/dLAN	* p=0.0032	t=3.404 df = 18 SEM = 1.041	Mean LD = 5.833 dLAN = 9.375
	E	Unpaired two-tailed t-test	M - LD/dLAN	* p=0.0003	t=4.503 df = 18 SEM = 2.054	Mean LD = 9.500 dLAN = 18.75

<table>
<tr><td>1. Correlation Focal Deficit Infarct Analysis</td><td>F</td><td>Correlation</td><td>Infarct size vs Focal Deficit Scores</td><td>*
p=0.0007</td><td>r=0.6959
r2 = 0.4843</td><td></td></tr>
<tr><td>1. Survival – tMCAO Males</td><td>H</td><td>Mantel-Cox</td><td>M - LD/dLAN</td><td>*
p=0.0272</td><td></td><td></td></tr>
<tr><td>1. Correlation General Deficit Infarct Analysis</td><td>I</td><td>Correlation</td><td>Infarct size vs General Deficit Scores</td><td>*
p=0.0003</td><td>r=0.7254
r2=0.5263</td><td></td></tr>
<tr><td rowspan="3">1. Permanent MCAO Males

Infarct Size General Deficit Score Focal Deficit Score</td><td>J</td><td>Unpaired two-tailed t-test</td><td>M - LD/dLAN</td><td>*
p=0.0477</td><td>t=2.110
df = 20

SEM = 5.617</td><td>Mean LD = 46.77
dLAN = 58.62</td></tr>
<tr><td>K</td><td>Unpaired two-tailed t-test</td><td>M - LD/dLAN</td><td>*
p<0.0001</td><td>t=5.041
df = 20

SEM = 1.064</td><td>Mean LD = 6.000
dLAN = 11.36</td></tr>
<tr><td>L</td><td>Unpaired two-tailed t-test</td><td>M - LD/dLAN</td><td>*
p=0.0005</td><td>t=4.151
df=20

SEM = 1.818</td><td>Mean LD = 13.36
dLAN = 20.91</td></tr>
</table>

1. Survival - Permanent MCAO Males	M	Mantel-Cox	M - LD/dLAN	ns p=0.4177		
1. tMCAO Females Infarct Size General Deficit Score Focal Deficit Score	N	Unpaired two-tailed t-test	F - LD/dLAN	* p=0.0313	t=2.316 df=20 SEM = 6.229	Mean LD = 32.95 dLAN = 47.38
	O	Unpaired two-tailed t-test	F - LD/dLAN	* p=0.0059	t=3.067 df=21 SEM = 1.141	Mean LD = 4.5 dLAN = 8.000
	P	Unpaired two-tailed t-test	F - LD/dLAN	* p=0.0010	t=3.802 df=21 SEM = 1.668	Mean LD = 8.750 dLAN = 15.09

1. Survival - tMCAO Females	Q	Mantel-Cox	F - LD/dLAN	ns p=0.3173		
3. (% CD11b+ cells) Microglia	B	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.6307 Hemisphere (Row), ns p=0.5193 Lighting Condition (Column) p=0.2780	F(1, 18) = 0.2392 F(1, 18) = 0.4321 F(1, 18) = 1.251	
3. (% CD11b+ cells) Microglia – MHC II	C	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.4719 Hemisphere (Row), ns p=0.5400 * Lighting Condition (Column) p=0.0020	F(1, 16) = 0.5430 F(1, 16) = 0.3922 F(1, 16) = 13.62	
		Post-Hoc – Fisher's LSD	Contra LD vs Contra dLAN Contra LD vs Ipsi LD Ipsi LD vs Ipsi dLAN Contra dLAN vs Ipsi dLAN	* p=0.0531 ns p=0.9386 * p=0.0065 ns p=0.3494		

3. (% CD11b+ cells) Microglia – CD86	D	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.9780 Hemisphere (Row), ns p=0.0586 Lighting Condition (Column), ns p=0.8146	F(1, 18) = 0.0007802 F(1, 18) = 0.4.077 F(1, 18) = 0.05665	
3. (% CD11b+ cells) Microglia – CD206	E	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.4538 Hemisphere (Row), ns p=0.3217 * Lighting Condition (Column), p=0.0470	F(1, 16) = 0.5896 F(1, 16) = 1.046 F(1, 16) = 4.630	
		Post-Hoc – Fisher's LSD	Contra LD vs Contra dLAN Contra LD vs Ipsi LD Ipsi LD vs Ipsi dLAN Contra dLAN vs Ipsi dLAN	ns p=0.0556 ns p=0.8593 * p=0.3424 ns p=0.2236		

3. (% CD11b+ cells) Microglia – IL-1β	F	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.6394 Hemisphere (Row), ns p=0.6337 Lighting Condition (Column), ns p=0.5158	F(1, 18) = 0.2271 F(1, 18) = 0.2350 F(1, 18) = 0.4394	
3. (% CD11b+ cells) Microglia – IL-6	G	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.7442 Hemisphere (Row), ns p=0.5704 *Lighting Condition (Column), p=0.0086	F(1, 18) = 0.2271 F(1, 18) = 0.2350 F(1, 18) = 0.4394	
		Post-Hoc – Fisher's LSD	Contra LD vs Contra dLAN Contra LD vs Ipsi LD Ipsi LD vs Ipsi dLAN Contra dLAN vs Ipsi dLAN	ns p=0.0805 ns p=0.8569 * p=0.0322 ns p=0.5459		

3. (% CD11b+ cells) Microglia – TNF-α	H	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.9654 Hemisphere (Row), ns p=0.1833 Lighting Condition (Column), ns p=0.1613	F(1, 17) = 0.001933 F(1, 17) = 0.1.924 F(1, 17) = 2.145	
3. (% CD11b+ cells) Microglia – IL-4	I	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.6143 Hemisphere (Row), ns p=0.9623 Lighting Condition (Column), ns p=0.1960	F(1, 18) = 0.2630 F(1, 18) = 0.002299 F(1, 18) = 1.803	
3. (% CD11b+ cells) Microglia – IL-10	J	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.5822 Hemisphere (Row), ns p=0.2345 * Lighting Condition (Column), p=0.0077	F(1, 17) = 0.3146 F(1, 17) = 1.519 F(1, 17) = 9.132	

		Post-Hoc – Fisher's LSD	Contra LD vs Contra dLAN Contra LD vs Ipsi LD Ipsi LD vs Ipsi dLAN Contra dLAN vs Ipsi dLAN	ns p=0.1067 ns p=0.6335 * p=0.0191 ns p=0.2313		
3. % Microglia MHC II – IL-1β	K	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.3798 Hemisphere (Row), ns p=0.7543 Lighting Condition (Column), ns p=0.5192	F(1, 18) = 0.8105 F(1, 18) = 0.1010 F(1, 18) = 0.4323	
3. % Microglia MHC II – IL-6	L	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.9635 Hemisphere (Row), ns p=0.4205 Lighting Condition (Column), ns p=0.5316	F(1, 18) = 0.002149 F(1, 18) = 0.6796 F(1, 18) = 0.4069	

3. % Microglia MHC II – TNF-α	M	2-way ANOVA	**Lighting Condition, Hemisphere** LD Contra LD Ipsi dLAN Contra dLAN Ipsi	Interaction, ns p=0.9165 Hemisphere (Row), ns p=0.4855 *Lighting Condition (Column), p=0.0264	F(1, 17) = 0.01133 F(1, 17) = 0.5085 F(1, 17) = 5.911	
		Post-Hoc – Fisher's LSD	Contra LD vs Contra dLAN Contra LD vs Ipsi LD Ipsi LD vs Ipsi dLAN Contra dLAN vs Ipsi dLAN	ns p=0.1260 ns p=0.6665 ns p=0.0841 ns p=0.0841		
Figure		**Statistics**	**Treatment**	**ANOVA Source of Variation**	**F**	**SE of diff**
	B	2-way ANOVA	**Diet, Lighting Condition** Control Diet - LD Control Diet – dLAN CSFR1 – LD CSFR1 – dLAN	*Interaction p=0.0032 Diet (Row), ns p=0.8643 *Lighting Condition (Column) p=0.0044	F(1, 36) = 9.982 F(1, 36) = 0.02962 F(1, 36) = 9.215	

4. Infarct Size		Post-Hoc – Fisher's LSD	CSFR1 inhibitor LD vs CSFR1 Inhibitor dLAN	ns p=0.9267		4.600
			CSFR1 inhibitor LD vs Control Diet LD	* p=0.0282		5.013
			CSFR1 inhibitor LD vs Control Diet dLAN	* p=0.0437		4.714
			CSFR1 inhibitor dLAN vs Control Diet LD	* p=0.0342		5.013
			CSFR1 inhibitor dLAN vs Control Diet dLAN	* p=0.0358		4.714
			Control Diet LD vs Control Diet dLAN	* p=0.0002		5.117
Figure		**Statistics**	**Treatment**	**ANOVA Source of Variation**	**F**	**SE of diff**

4. P2RY12+ cells Cortex 20X (IHC)	D	Three-way ANOVA (2 x 2 x 3)	**Diet, Lighting Condition, Hemisphere** Control Diet – LD Ipsi Control Diet – dLAN Ipsi CSFR1 – LD Ipsi CSFR1 – dLAN Ipsi Control Diet – LD Contra Control Diet – dLAN Contra CSFR1 – LD Contra CSFR1 – dLAN Contra	* Diet $p<0.0001$ Lighting Condition $p=0.1419$ * Hemisphere $p=0.0032$ Diet x Lighting Condition, ns $p=0.4454$ *Diet x Hemisphere, $p=0.0092$ Lighting Condition x Hemisphere, ns $p=0.5749$ Diet x Lighting Condition x Hemisphere, ns $p=0.9941$	$F(1,51) = 225.3$ $F(1,51) = 2.226$ $F(1,51) = 9.551$ $F(1,51) = 0.5914$ $F(1,51) = 7.323$ $F(1,51) = 0.3187$ $F(1,51) = 5.536e-005$	

		Post-Hoc – Tukey	Control Diet LD Ipsi vs CSFR1 Inhibitor LD Ipsi Control Diet LD Contra vs CSFR1 Inhibitor LD Contra Control Diet dLAN Ipsi vs CSFR1 Inhibitor dLAN Ipsi Control Diet dLAN Contra vs CSFR1 Inhibitor dLAN Contra	* p<0.0001 * p<0.0001 * p<0.0001 * p<0.0001		7.493 7.493 6.734 6.544
4. TNF-α – Control Diet (serum conc. pg/ml)	E	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	* p=0.0421	t=2.237 df = 14 SEM = 9.145	Mean LD = 41.23 dLAN = 61.69
4. TNF-α – CSFR1 inhibitor (serum conc. pg/ml)	F	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	p=0.8809	t=0.1537, df = 10 SEM = 8.827	Mean LD = 41.12 dLAN = 42.48
4. KC/GRO – Control Diet (serum conc. pg/ml)	G	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	p=0.7546	t=0.3205, df = 11 SEM = 165.1	Mean LD = 457.8 dLAN = 510.7
4. KC/GRO – CSFR1 inhibitor (serum conc. pg/ml)	H	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	p=0.3037	t=1.079 df = 11 SEM = 104.4	Mean LD = 331.6 dLAN = 218.9

4. IL-6 – Control Diet (serum conc. pg/ml)	I	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	p=0.1421	t=1.690 df = 6 SEM = 541.1	Mean LD = 618.5 dLAN = 1533
4. IL-6 – CSFR1 inhibitor (serum conc. pg/ml)	J	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	p=0.8028	t=0.2564 df = 10 SEM = 280.3	Mean LD = 826.6 dLAN = 898.5
4. IL-5 – Control Diet (serum conc. pg/ml)	K	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	p=0.2621	t=1.165 df = 15 SEM = 3.011	Mean LD = 33.91 dLAN = 30.40
4. IL-5 – CSFR1 inhibitor (serum conc. pg/ml)	L	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	p=0.1830	t=1.431 df=10	Mean LD = 29.32 dLAN = 31.94
4. IL-4 – Control Diet (serum conc. pg/ml)	M	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	p=0.2207	t=1.275 df=16	Mean LD = 13.59 dLAN = 12.14
4. IL-4 – CSFR1 inhibitor (serum conc. pg/ml)	N	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	p=0.8609	t=0.1798 df=10 SEM = 0.2768	Mean LD = 11.27 dLAN = 11.32
4. IL-2 – Control Diet (serum conc. pg/ml)	O	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	p=0.8959	t=0.1329 df = 16 SEM = 1.679	Mean LD = 17.14 dLAN = 17.36

4. IL-2 – CSFR1 inhibitor (serum conc. pg/ml)	P	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	p=0.4185	t=0.8438 df=10 SEM = 1.433	Mean LD = 14.00 dLAN = 15.21
4. IL-1β – Control Diet (serum conc. pg/ml)	Q	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	p=0.1486	t=1.518 df = 16 SEM = 48.84	Mean LD = 107.5 dLAN = 181.6
4. IL-1β – CSFR1 inhibitor (serum conc. pg/ml)	R	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	p=0.3943	t=0.8946 df = 9 SEM = 26.08	Mean LD = 84.49 dLAN = 97.81
4. IL-12p70 – Control Diet (serum conc. pg/ml)	S	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	p=0.6822	t=0.4181 df = 14 SEM = 9.018	Mean LD = 145.8 dLAN = 149.6
4. IL-12p70 – CSFR1 inhibitor (serum conc. pg/ml)	T	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	p=0.0888	t=1.908 df = 9 SEM = 5.991	Mean LD = 135.2 dLAN = 146.6
4. IL-10 – Control Diet (serum conc. pg/ml)	U	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	p=0.3014	t=1.070 df= 15 SEM = 11.95	Mean LD = 54.12 dLAN = 66.91
4. IL-10 – CSFR1 inhibitor (serum conc. pg/ml)	V	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	p=0.1394	t=0.1394 df = 8 SEM = 3.117	Mean LD = 44.22 dLAN = 49.34

4. Corticosterone -Control Diet (serum conc. ng/ml)	W	Unpaired two-tailed t-test	Control Diet LD vs Control Diet dLAN	p=0.5260	t=0.6531 df = 12 SEM = 225.8	Mean LD = 677.7 dLAN = 825.2
4. Corticosterone -CSFR1 inhibitor (serum conc. ng/ml)	X	Unpaired two-tailed t-test	CSFR1 LD v.s CSFR1 dLAN	* p=0.0239	t=2.584 df = 12 SEM = 179.9	Mean LD = 515.1 dLAN = 980.0
Figure		**Statistics**	**Treatment**	**ANOVA**	**F**	**SE of diff**
Supplementary Figure Food Intake		2-way ANOVA	**Diet, Lighting Condition** Control Diet - LD Control Diet – dLAN CSFR1 – LD CSFR1 – dLAN	Interaction, ns p=0.1440 Diet (Row), ns p=0.0622 Lighting Condition (Column), ns p=0.9073	F(1, 39) = 2.223 F(1, 39) = 3.686 F(1, 39) = 0.01373	

Table 2. S2. Statistical Analyses for RNA-seq dataset comparing each respective hemisphere of mice housed in dark night conditions compared to dLAN. Comparisons were made using EDGE test, and gene list order in bold includes genes statistically different across the ipsilateral and contralateral hemisphere of the brain. After, gene lists are corresponding to fold change values,

*$p < 0.05$.

	LD v.s. DLAN Ipsilateral Hemisphere				LD v.s. DLAN Contralateral Hemisphere		
	Gene	**Fold**	**EDGE test: p-value**		**Gene**	**Fold**	**EDGE test: p-value**
1	**Hoxa7**	24.46007	0.0044516	1	**Hoxa7**	20.66386227	0.009705083
2	**Ngp**	18.44967139	5.31848E-06	2	**Ngp**	16.96796666	0.000100304
3	**Mpo**	15.58319893	0.006520215	3	**Mpo**	24.28506598	0.00216702
4	**Ctsg**	14.29323989	0.014701204	4	**Ctsg**	14.33912243	0.014701204
5	**S100a8**	5.236707232	0.008386413	5	**S100a8**	6.357494731	0.024853242
6	**Gm11808**	3.808364325	0.003469685	6	**Gm11808**	3.450021983	0.004636444
7	**Hist1h4c**	3.19294099	0.016081672	7	**Hist1h4c**	2.856732848	0.030617776
8	**Lyz2**	3.137259276	0.042770668	8	**Lyz2**	4.17714763	0.0211909
9	**Hist1h2al**	3.126658033	0.018609606	9	**Hist1h2al**	2.959674646	0.018808217
10	**Cox8a**	2.519767914	0.007404721	10	**Cox8a**	2.046343491	0.036619501
11	**Hist1h4d**	2.402060391	0.017322091	11	**Hist1h4d**	2.47460263	0.006430863
12	**Rpl36**	2.246185288	0.037989115	12	**Rpl36**	2.269653891	0.030739166
13	**Egr3**	1.993646093	0.036826076	13	**Egr3**	2.540365323	0.018460204

14	**Per1**	1.986550131	0.020153248	14	**Per1**	1.905393	0.050217637
15	**Agt**	1.868592875	0.033815685	15	**Agt**	1.830331111	0.021413828
16	**Rps29**	1.859403729	0.039934885	16	**Rps29**	1.909973645	0.027682063
17	**Fbxl16**	1.758379098	0.028255236				

18	**Leng8**	1.737326355	0.007584023	17	**Leng8**	1.647839407	0.01378485
19	**Ftl1**	1.705208273	0.024377794	18	**Ftl1**	1.917908647	0.008591702
20	**Gapdh**	1.634897529	0.040416472	19	Gadph	1.614866167	0.051326469
21	**RP23145I16.3**	-2.29529182	0.045585514	20	**RP23145I16.3**	-2.76609845	0.007402867
22	Camp	10.94239679	0.014701204	21	Hist1h2ai	12.72807823	0.031255993
23	Chil3	8.495481337	0.00136765	22	Chil3	9.820769398	0.004441783
24	Mmp8	7.931707879	0.039271416	23	Sap18b	5.389348562	0.022061759
25	S100a9	6.30344672	0.006445977	24	Hist1h3h	4.987259799	0.039073287
26	Hbb-bs	5.866853867	0.006445977	25	Lcn2	3.281523583	0.053661658
27	Hist1h3i	5.019099837	0.004339001	26	Mt2	2.727707235	0.000972292
28	Rpl37rt	4.830203289	0.015805897	27	Hist2h3b	2.727461689	0.047734728
29	F13a1	4.512502388	0.052499842	28	Nptx2	2.222651059	0.050103672
30	Alas2	3.848254523	0.03118859	29	Hist1h1e	2.095524586	0.017048631
31	Gm9843	3.435578728	0.00920737	30	Rps28	2.030377798	0.040901423
32	Egr4	3.393474639	0.046365989	31	Gm6311	1.98183849	0.011420319

33	Fosb	3.272013039	0.042283745	32	Rplp1	1.928136869	0.008512961
34	Ttr	2.876616388	0.014238217	33	Rps21	1.885038367	0.051773776
35	Rpl10-ps3	2.78415524	0.004572889	34	Rps27	1.848253396	0.046756684
36	Avp	2.313898851	0.040055081	35	Tceb2	1.817058285	0.05193937
37	Nr4a1	2.293693398	0.015040495	36	Fau	1.809691314	0.004236096
38	Junb	2.191031349	0.039546383	37	Rpl24	1.792247851	0.037033515
39	Higd2a	2.089896039	0.049888744	38	B2m	1.75280296	0.024005762
40	Abhd17a	2.067151315	0.027959327	39	Rps15	1.735218422	0.002077437

41	Ssbp4	2.019889552	0.051850456	40	Rps24	1.703161508	0.006220188
42	Rnf208	2.003567981	0.041549623	41	Rpl18a	1.564012024	0.03540595
43	Pcsk1n	2.002337432	0.024978219	42	Git1	1.527310019	0.028945915
44	C1qtnf4	1.945809199	0.025212458	43	Apoe	1.526803999	0.033852624
45	Pde1b	1.885821104	0.006667421	44	Arl6ip1	-1.55728361	0.004221655
46	B3gat3	1.853532612	0.039921004	45	Rps24	1.703161508	0.006220188
47	Itpka	1.832492711	0.04773942	46	Wsb1	-1.64987031	0.043120881
48	Psd	1.793688209	0.018858329				
49	Mif	1.785803432	0.039410103				
50	Cnih2	1.782410221	0.037688231				
51	Cxx1a	1.781603374	0.021392248				
52	Cbarp	1.780490368	0.035289246				
53	Ypel3	1.753645667	0.045646217				
54	Rps12-ps3	1.741739788	0.02973805				
55	Lmtk3	1.733836162	0.023252956				

56	Mast3	1.704750045	0.02763181				
57	Phyhip	1.69738786	0.041548507				
58	Cox6a1	1.647717817	0.03451319				
59	Rplp1	1.644428531	0.044582388				
60	Dlgap3	0.040149601	1.618409408				
61	Dgkz	0.049381594	1.588151674				
62	Nnat	0.041006565	1.548621078				
63	Arf5	0.041805668	1.547717484				
64	Syngap1	1.506289433	0.039618937				
65	Ociad2	-1.6153706	0.032251781				
66	Azin1	-1.61964967	0.050333779				
67	Hsp90aa1	-1.75401990	0.000135953				
68	Hspe1	-2.14036721	0.001463549				
69	Hspd1	-2.18919826	5.07115E-06				
70	Spp1	-2.42265469	0.012292632				

71	Hsph1	-2.65995358	6.90828E-06				
72	Gm6467	-4.83521060	0.039073287				

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CHAPTER 3

ALTERNATIVE SPECTRAL LIGHTING MINIMIZES ISCHEMIC DAMAGE ASSOCIATED WITH NIGHTTIME LIGHTING CONDITIONS

Introduction

Circadian entertainment relies on *zeitgebers* including light as the most potent entraining cue for synchronizing endogenous internal rhythms to the natural solar day and environment. During the past century, exposure to artificial environmental lighting has become a ubiquitous part of modern lifestyles, resulting in changes to light cycles. Lighting sources more recently, have shifted to artificial light emitting diode (LED) light. This has provided significant advantages over halogen bulbs, including greater energy efficiency, reduced heat generation, and significantly longer lifetimes and efficiencies compared to previous models developed in the 19th and 20th century. However, LED lighting sources utilize a combination of broad spectrum yellow phosphor and greater intensity of blue wavelengths (488 nm) to create the appearance of whiter lighting in industrialized societies (Navara and Nelson, 2007). Light's effect on physiology is dependent on the duration, timing, spectral component of light, and intensity. Notably, responsivity of the circadian system to lighting is not equal across all wavelengths. Intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina are non-imaging forming photoreceptors that contain melanopsin (OPN4), a photopigment that is maximally sensitive to short-wavelengths (blue; 480 nm) visible light. Photic information derived from cones and rods will communicate and project to the SCN through the retinohypothalamic tract (Beaulé et al., 2003) and additionally relay signals to the olivary pretectal nucleus (OPN) to control pupillary light reflex (Hattar et al., 2002). Indeed, these shorter wavelengths are present across natural sunlight, and contain prominent peaks in artificial broad spectrum white light or light emitting diodes (LED) lighting to create the appearance of “whiter” visual lighting. Exposure to blue wavelength intensities in the beginning of the day are important for alertness, cognitive

performance, and suppressing melatonin production across mammals, however, exposure to light into the evening from room lighting (Gooley et al., 2011), LED-backlit computer screens (Caochen et al., 2011), or eBooks (Chang et al., 2015) can suppress and delay melatonin onset in humans. Further, 90% of adults surveyed in America report using a light-emitting device within an hour before bedtime (Gradisar et al., 2013). This is a mounting concern, given that recent studies have identified an increased risk for negative health consequences with exposure to light at night (Stevens and Zhu, 2015; Bedrosian et al., 2016; Fonken et al., 2019).

Several aspects of the circadian system are conserved between rodents and humans. During nighttime lighting exposure, both rodents and humans are most responsive to blue wavelength lighting (460 nm) (Brainard et al., 1982; Ruger et al., 2013) and longer wavelengths such as red light, minimally influence the circadian system because low intensities of red light do not activate melanopsin-containing retinal ganglion cells which project to the SCN (Brainard et al., 2008; Figuerio and Rea, 2010). However, chronic or prolonged exposure to artificial lighting conditions to low-intensities of blue wavelength lighting can disrupt circadian periods (Wahl et al., 2019). Even short ten minute pulses of 5 lux lighting intensity of blue wavelength light are sufficient to activate cFOS+ cells in the SCN in mice maintained in dark conditions for 3 days (Walker et al., unpublished data) .

In **Chapter 2**, exposure to dim levels (5 lux) of artificial white LED nighttime lighting significantly increases poor health outcomes in a murine model of ischemic stroke. Additionally, this chapter identified that there are mounting changes in neuroinflammatory response after exposure to dLAN. Studies investigating the role of dim blue LAN in otherwise healthy mice

reported increased neuroinflammation and activated hippocampal microglia after four weeks of exposure compared to dark night conditions. Further, mRNA expression of TNF-α, and IL-6 were upregulated in microglia in the hippocampus (Liu et al., 2022) that was correlated to changes in hippocampal structure resulting in spatial working memory impairments. Several previous studies have reported neuroinflammation associated with exposure to light at night in healthy rodents, and this occurs across white LED lighting conditions with predominantly blue wavelengths (Walker et al., 2020; Bumgarner et al., 2020, Tam et al., 2021; Liu et al., 2022). Further, white LAN that contains strong blue spectral components exacerbates pro-inflammatory cytokine production in disease states including global ischemia (Fonken et al., 2013) and ischemic stroke (Weil et al., 2020) that contributes to functional injury. Chronic activation of microglia and inflammation can contribute to neurodegeneration and neurotoxicity (Saitgareeva et al., 2020). Therefore, reducing neuroinflammatory response and neuronal damage is an essential goal of cerebrovascular research. Because environmental lighting conditions can exacerbate injury, I sought to utilize different wavelength lighting to which photosensitive retinal ganglion cells are not sensitive to. Therefore, the hypothesis for this experiment is that eliminating blue wavelength lighting ameliorates the negative consequences associated with dim light at night post-stroke. To test this hypothesis, the role of light exposure conditions after ischemic stroke was examined. Five lux of dim white LED light was compared to alternative spectral lighting, including matched intensities of dim white LED light with filtered out blue wavelengths (5 lux), dim-red wavelength light (5 lux), and dark night conditions as a control.

Methods

Animals

5-6 month old male Swiss-Webster (CFW) mice were obtained from Charles River Laboratories, Wilmington, MA. Upon arrival, mice were single-housed in polypropylene ventilated cages with autoclaved paper nesting material and were habituated in animal holding rooms (Ventilated Light Controlled Animal Housing System, Lab Products Inc.) for 1 week, then were moved to the satellite facility to a controlled vivarium 12:12 hour light dark (LD) cycle (~150 lux day, 0 lux night) with *ad libitum* access to standard rodent chow (Envigo Teklad 2018 Rodent Chow) and reverse osmosis water.

Surgical Procedures

Ischemic stroke was induced using a middle cerebral artery occlusion (MCAO) during the light phase (zeitgeber time ZT 1-5). Mice were anesthetized with isoflurane vapors until unresponsive, confirmed by toe pinch, then, were placed on a homeothermic mat and body temperature was regulated and maintained at 37 ± 0.5°C. A unilateral right incision was made, exposing the right middle cerebral artery and occlusion was achieved through insertion of a 6-0 nylon filament into the internal carotid artery, past the pterygopalatine artery bifurcation. Cerebral blood flow was monitored using laser doppler flowmetry for confirmation of blood flow drop during occlusion. After 30 minutes of occlusion, the mice were re-anesthetized and initiated reperfusion via removal of the occluder. Mice received 1 ml of warmed saline through subcutaneous injection, were monitored for recovery in clean home cages placed on top of warming pads set to 37 °C, then were returned to the animal facility.

Lighting Treatments post MCAO

Following reperfusion, mice were pseudo-randomly returned to normal dark night conditions or were placed in the corresponding lighting treatments; dim light (dLAN) (5 lux), dim white light with a blue-blocking filter (Bf-dLAN adjusted to 5 lux; Zircon UV Blue Blocker, Lee Filters, Burbank, CA USA), and red light (rLAN 5 lux). Lighting was provided by LUMA5 LED light strips (Hitlights Inc; 1.5W/ft, 5000K "cool white", 1200 lumens, Hitlights Inc; 1.5 W/ft, 5000k "red", 1200 lumens) placed equidistant in the back of each cage facing up. Light measurements were determined using a light meter (Mavolux 5032C illuminance meter (Nürnberg, Germany) from the center of an empty cage with the light sensor facing the ceiling.

Tissue Collection

24 hours post MCAO, mice were given a neuroscore evaluation using Clark's test, then brain tissue was collected and sectioned into 2 mm thick coronal sections and were placed in 2,3,5-triphenyltetrazolium (TTC) solution, responsible for staining live mitochondria, and was incubated at 37 °C for 15 minutes, flipping sections every two minutes. Slices were post-fixed in 10% buffered formalin and photographed within 72 h. Infarct size was analyzed using ImageJ software by a blind experimenter, and was determined as a percentage of the contralateral hemisphere [(total ipsilateral hemisphere - infarct)/total contralateral hemisphere x 100]. In a second cohort, mice were injected with euthasol, then once unresponsive to toe-pinch, was perfused intracardially with PBS, then brains were removed and stored in RNAlater for 24 h at 4 degrees C then stored at -80 until dissection. Blood was obtained via submandibular bleed and samples were allowed to coagulate at room temperature, then were centrifuged at 4 °C for 25

minutes at 2,500 x g. Serum was collected and stored in a 1.5 mL microcentrifuge tube at -80 °C for subsequent analysis.

RNA Extractions, cDNA, and qt-PCR

The cerebral cortex was dissected and isolated from the frontal lobe and dermal punches isolating the caudate nucleus were collected from whole brains stored in RNAlater. RNA extractions were performed as previously described using Trizol Reagent according to manufacturer instructions (Ambion, Waltham, MA). RNA quality and quantity was assessed by using a spectrophotometer (Thermofisher Nanodrop One), then, cDNA was synthesized using SuperScript IV VILO reverse transcriptase for qt-PCR (Invitrogen) according to the manufacturer's protocol. 40μg in 4 μl of 1:10 diluted cDNA was combined with 16μl of a master-mix solution (Tacman Fast Advanced Master Mix (Life Technologies), and using corresponding probes: TNFα, IL-6, and IL-1β (Life Technologies), inventoried probe (Applied Biosystems LIfe Technologies). Samples were run in duplicate and polymerase chain reaction (PCR) cycling conditions were ran using the 2-step real-time PCR cycling conditions: 95 °C for 20 s, followed by 95 °C 3 s cycles 40 times, and a 30 s 60 °C. Gene expression was quantified using the relative standard curve (Pfaffl) method (Pfaffl, 2001).

Serum Cytokines and Corticosterone

The V-PLEX Proinflammatory Panel 1 measuring IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, KC/GRO, IL-10, IL-12p70, and TNF-α was used according to the manual using serum. Total serum corticosterone concentrations were determined using a Corticosterone Enzyme Immunoassay Kit (DetectX Arbor Assays, Ann Arbor, MI, USA) in duplicates according to the

manufacturer's instructions for the 50 µL assay on a plate reader (SpectraMax iD3, Molecular Devices).

Statistical Analyses

Infarct size, Functional Deficit scores (Clark's General and Focal Deficit scores), cytokine concentrations in serum, and qtPCR were compared using a one-way ANOVA between lighting condition groups . Correlations were compared using a simple linear regression including infarct size and neurological scores across all lighting conditions. Multiple comparisons were conducted using Fisher's LSD test. Data were tested for normality using the Shapiro-Wilks test. Grubbs test was performed prior to analysis to identify outliers, a maximum of one outlier was removed, defined as having a >2 Z-score within-treatment group. Statistical tests were analyzed using GraphPad Prism 9.0 software. Data are presented as the mean ± standard error of the mean (SEM); differences were considered statistically significant if $p \leq 0.05$.

Results

Exposure to Alternative Wavelengths Ameliorates the Effect of LAN on Infarct Size and Neurological Functional Deficits Post-Stroke

There was a main effect of lighting condition on infarct size in mice collected 24 h after MCAO ($F(3, 43) = 2.950$; $p=0.0432$). Mice exposed to a single night of dim white LED light at night (dLAN) after MCAO had significantly increased infarct size compared to dark night conditions ($p<0.05$) and alternative wavelength lighting (rLAN $p=0.0389$; Bf-dLAN $p=0.0074$). Filtering

out blue wavelength lighting in white LED lighting ameliorated the increased infarct size observed from exposure to dLAN, resulting in comparable infarct sizes between Bf-dLAN to dark nights (p=0.4757) and 5 lux of rLAN (p=0.5005). General Deficit Scores (F (3, 66) = 16.74; p<0.0001) and Focal Deficit Scores (F (3, 66) = 0.2336; p<0.0001) were significantly affected by lighting conditions. Mice exposed to dLAN displayed increased general deficit scores compared to dark night conditions (p<0.0001), Bf-dLAN (p<0.0001), and rLAN (p<0.0001). Additionally, dLAN mice displayed increased functional deficit scores compared to dark night conditions (p<0.0001), Bf-dLAN (p<0.0001), and rLAN (p<0.0001). There were no differences between general or functional deficit scores between alternative lighting spectras or dark night conditions (p>0.05). Infarct size was positively correlated with Clark General (r^2 = 0.3784; p<0.0001) and Focal Deficit scores (r^2 = 0.3168; p<0.0001).

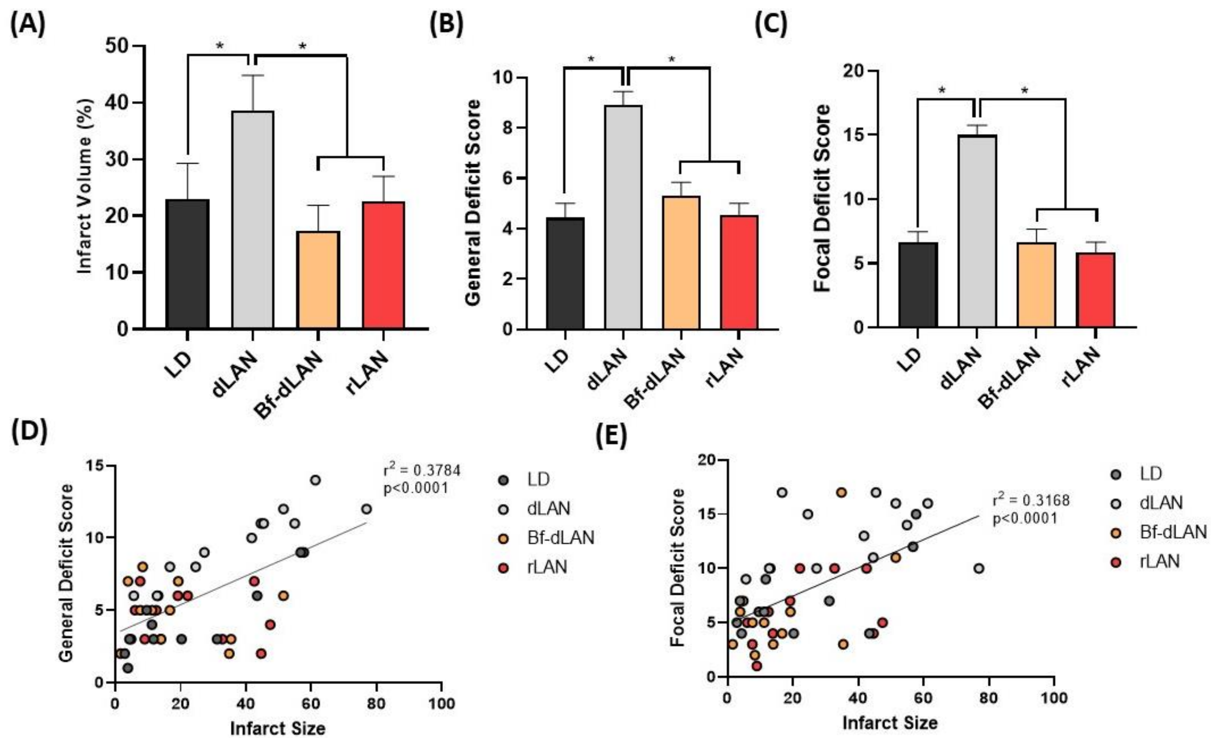

Figure 3.1. Alternative Spectral Lighting Normalizes Infarct Size and Neurological Deficit Scores Comparable to Dark Night Conditions. (a) Exposure to dLAN significantly increases infarct size relative to dark night conditions, blue-filtered dLAN (bf-dLAN), and red light at night (rLAN) ($p<0.05$). No significant differences between dark night conditions and alternative spectral lighting ($p>0.05$). (b and c) General and focal deficit scores display similar trends to infarct size where dLAN is significantly increased relative to other lighting conditions ($p>0.05$). (d) General and (e) focal deficit scores are positively correlated to infarct size. The data are represented as mean ± SEM (n=10-16/group).

Exposure to dLAN Increases TNF-α Expression in the Brain and Serum Concentrations Post-Stroke That Becomes Normalized with Alternative Spectral Lighting

Neuroinflammatory response modulates neuronal damage after ischemia (Iadecola and Anrather, 2011); therefore, regions of the brain that are affected by ischemic injury including the cortex, caudate nucleus, and hippocampus, were collected 24 h after stroke to assess relative expression and pro-inflammatory response. There was a significant effect of light condition on relative TNF-α gene expression in the caudate nucleus ($F_{(3,16)} = 3.460$; $p=0.0414$) and cortex ($p=0.0138$) One night of exposure to dLAN significantly increased relative TNF-α cytokine expression compared to LD ($p=0.05$), Bf-dLAN ($p=0.0151$), and rLAN (0.0216) in the caudate nucleus, and the cortex compared to alternative spectral lighting (Bf-dLAN $p=0.0035$, rLAN $p=0.0184$). There were no significant effects of lighting condition on relative gene expression of Il-1β or IL-6, other pro-inflammatory cytokines across regions in the brain ($p>0.05$). Peripheral immune response was evaluated through a proinflammatory cytokine multiplex panel, where we

observed a main effect of TNF-α concentrations (F (3, 50); 4.367; p=0.0287). DLAN increased serum TNF-α compared to LD (p=0.0085), Bf-dLAN (p=0.0160), and rLAN (p=0.0160). There were no significant differences in other cytokine concentrations observed across lighting conditions (p>0.05).

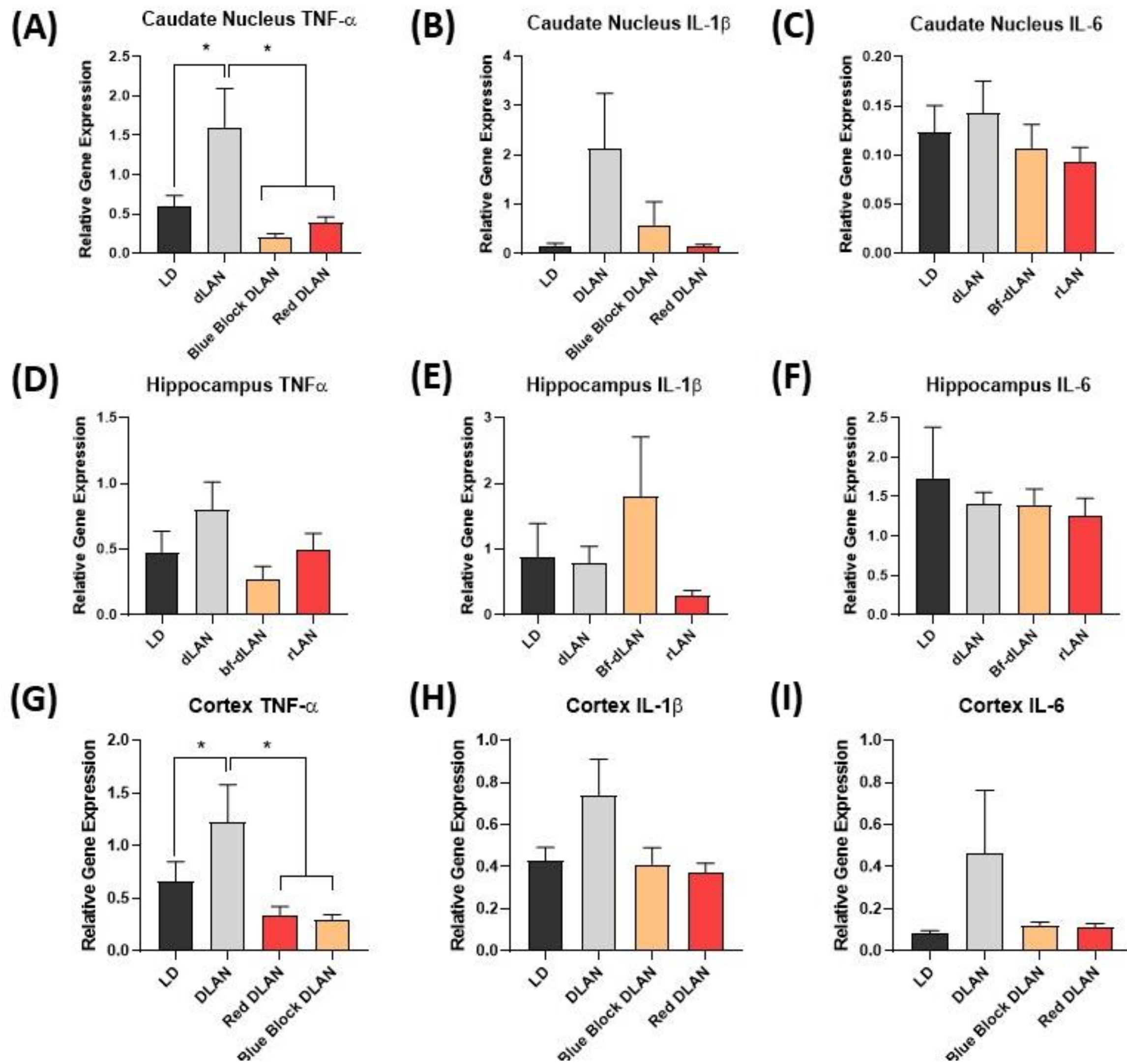

Figure 3.2. Filtering Out Blue-Wavelength Lighting Attenuates Neuroinflammatory Response. (a) Exposure to dLAN post-stroke significantly increases relative TNF-α expression

in the caudate nucleus and (g) cortex compared to dark night conditions, bf-MCAO, and rLAN ($p<0.05$). (b-c, e-f, g-i) no other significant differences between lighting conditions and relative gene expression for IL-1β or IL-6 ($p>0.05$). The data are represented as mean ± SEM (n=10-16/group).

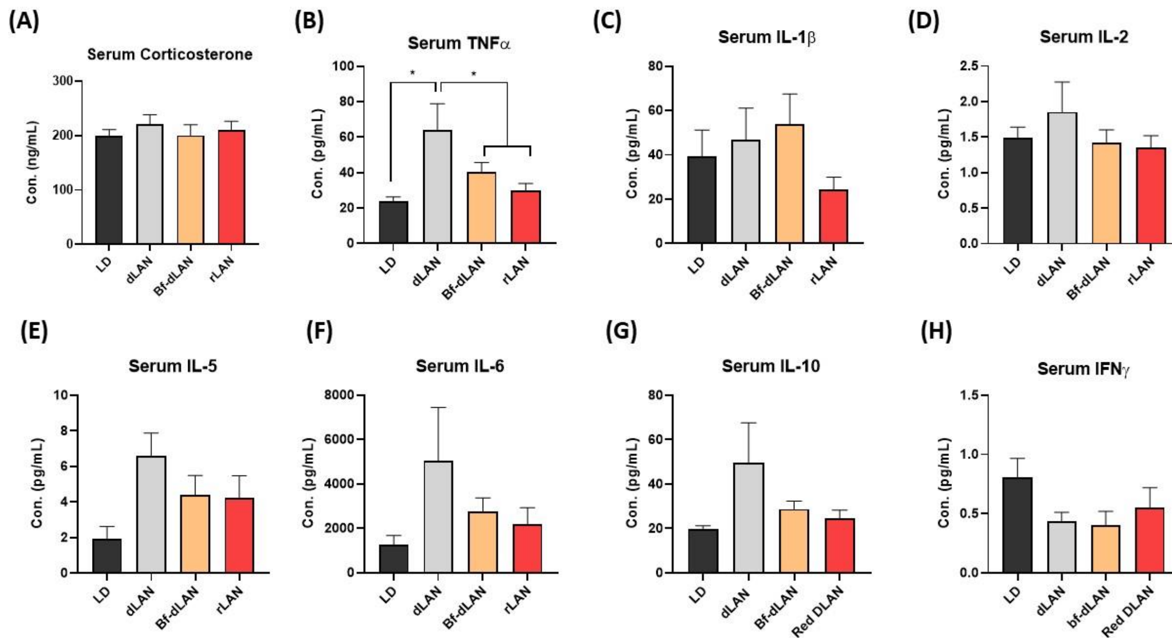

Figure 3.3. dLAN Increases Serum TNF-α after Ischemic Stroke. (a) There were no statistical differences between serum corticosterone concentrations between lighting conditions ($p>0.05$) (b) Exposure to dLAN post-stroke significantly increases serum TNF-α concentrations compared to dark night conditions, bf-MCAO, and rLAN ($p<0.05$). (c-h) no changes to cytokine concentrations in serum between lighting conditions ($p>0.05$). The data are represented as mean ± SEM (n=10-16/group).

Lighting Exposure After Ischemic Stroke Does Not Alter Corticosterone Concentrations

Corticosterone values were assessed in mice after exposure to nighttime lighting to rule out the role of stress driving increased infarct sizes. By using a One-Way ANOVA I determined that there were no significant differences in corticosterone values in serum between dark night and lighting condition treatments ($p>0.05$).

Discussion

The results from this study demonstrate that exposure to alternative spectral lighting conditions for one night after cerebral ischemia minimizes neuronal damage that is comparable to dark night conditions. These data are consistent with results observed in **Chapter 2** and in previous studies in 10 week-old mice (Weil et al., 2020), where exposure to dim white LED light during the night phase exacerbates injury and inflammatory response. This provides supporting evidence suggesting that the intensities and spectra within an environment and recovery conditions can modulate disease progression. Notably, wavelengths that are most disruptive within dim intensity lighting are short wavelengths (480 nm) in white LED light. Eliminating only this spectra within broad spectrum white LED light is sufficient to not exacerbate pathways necessary to contribute to exacerbated neuroinflammatory response that increases permanent neuronal damage.

First, previous literature provides supporting evidence that exposure to short wavelengths, perceived as blue light, restricted to exposure during the light phase can affect circadian physiology under normal light-dark conditions. Indeed, blue components of lighting can have important effects on health at appropriate intensities and timing (Czeisler, 2013), however, with the extensive use of LED light, this can result in exposure to significantly greater amounts of

short-wavelength light throughout the day and into the evening that can affect health. Studies examining daytime exposure to blue light in diurnal rats significantly alters cardiovascular physiology, including systolic blood pressure (BP) in normotensive Wistar-Kyoto rats, and heart rate in hypertensive SHR rats. Additionally, there was increased electrolyte excretion in normotensive rats compared to rats housed under white day light, suggesting that monochromatic blue light exposure during the light period can alter other aspects of physiology (Bryk et al., 2022). Exposure to white LED light that has increased intensities of blue-wavelength lighting during the day, increased brain neurodegeneration, locomotor activity, and reduced lifespan across *Drosophila* (Nash et al., 2019). Independent of disease state, other studies investigating the role of suppressing blue light within white dLAN demonstrated similar supporting findings that minimally affects circadian physiology. Reduced blue wavelengths in dLAN improves metabolic impairment compared to white LAN exposure across experimental and clinical settings (Nagai et al., 2019). Exposure to dim blue LAN for four weeks in otherwise healthy mice found increased neuroinflammation, and activated hippocampal microglia compared to dark night conditions. Further, mRNA expression of TNF-α, and IL-6 were upregulated in microglia in the hippocampus (Liu et al., 2022). Chronic activated microglia can prolong inflammation and inflammatory response, which can increase neurodegeneration and neurotoxicity (Saitgareeva et al., 2020), and here we suggest that the blue-wavelength components within white LED light is necessary to dysregulate pathways that likely affect microglia to induce exacerbated neuroinflammatory responses that contributes to secondary damage after ischemic injury

Not only does the spectral composition of light affect aspects of circadian rhythm entrainment and disruption, but light intensity also affects circadian physiology (Wahl et al., 2019). Therefore, it's important to identify that lower intensity lighting in this study plays an important factor in these findings. IpRGCs receive intraretinal synaptic input from image-forming photoreceptor circuits, including rods and cones (Sollars and Pickard, 2011) that can modulate photic information relayed to the SCN and other regions in the brain. Photoreceptors that are light intensity dependent including rods will primarily relay input from scotopic conditions ~7 log photons $cm^{-2}s^{-1}$ and cones in photopic conditions of ~11 log photons $cm^{-2}s^{-1}$ (Wahl et al., 2019). Therefore, in addition to altering wavelengths to result in amelioration of increased infarct sizes resulting from exposure to white LED dLAN, intensities of alternative lighting sources that are not sufficient to relay information from rods and cones are also necessary. One caveat to using red wavelength lighting is that mice lack long-wavelength (red) cones, which result in more limited detection of red lighting; mice M-cones are maximally sensitive to 510 nm outside of the 636 nm red light wavelength (Hattar et al., 2003). However, our blue-filtered dim white light treatment group was maintained at 5 lux intensity lighting as a control that had comparable infarct sizes compared to mice that were exposed to red light and dark nights post-stroke that would include alternative wavelengths that do not consist of only red wavelength light. Literature reports that exposure to dim intensity red wavelength lighting does not affect aspects of circadian physiology compared to full white broad spectrum light (Figueiro and Rea, 2010), and this resulted in applicable outcomes in rodents after global ischemia. TNF-α, IL-1β, and IL-6 gene expression were normalized to dark night conditions with exposure to dim red wavelength lighting (Fonken et al., 2013) and in ischemic stroke (Weil et al., 2020).

In conclusion, this study provides further evidence characterizing the adverse consequences of nighttime light. Blue wavelengths within white LED light are responsible for increasing pro-inflammatory cytokine production in the brain and serum, that likely contributes to increased infarct size observed in this experiment and as also demonstrated in Chapter 2. Eliminating blue-wavelength lighting within white LED light, or the use of 5 lux of red LAN, normalizes infarct size comparable to dark night conditions. These results have significant implications if translatable to humans, because critically ill patients require significant intensive care, and hospital settings often expose patients to dim to constant exposure to lighting. Although other factors in hospital settings can still result in disruptions during the dark phase, e.g., frequent waking for procedures disrupting sleep-wake cycles and noise, this study was interested in investigating the role of disruption to rhythms only through nighttime lighting exposure and found that this alone, was sufficient to increase infarct size and functional deficits. Few studies to date have investigated the use of red lighting or blue-wavelength filtering in the clinical setting but one study reports that wearing blue-light shield eyewear, aspects of sleep architecture including sleep latency and efficacy are improved (Ayaki et al., 2016).

CHAPTER 4

CHRONIC EXPOSURE TO DIM LIGHT AT NIGHT DISRUPTS CELL-MEDIATED IMMUNE RESPONSE AND DECREASES LONGEVITY IN AGED FEMALE MICE

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[1] Liu, et al. *Chronobiol. Int. (In press)*

Note: Part of this chapter includes text and figures obtained from publications highlighted above.

Introduction

Organisms have evolved endogenous rhythms that reflect recurring natural solar days comprising bright days and dark nights. Circadian rhythms have a period of ~24 hours and are synchronized to precisely 24 hours by the light cues from the external environment to optimize physiological and behavioral function, as well as survival. Circadian rhythms are generated at the cellular level by a transcriptional-translational feedback loop composed of core clock genes including Clock, Bmal1, Per, and Cry. External factors, or zeitgebers (time givers), act as cues to entrain the circadian system to match the solar day; the most common zeitgeber among vertebrates is light. In mammals, light input is transmitted from intrinsically photosensitive ganglion cells (ipRGCs) in the retina to the central biological clock, relaying environmental photic information to the suprachiasmatic nucleus (SCN) of the hypothalamus, which is responsible for synchronizing the central and peripheral biological clocks to the exogenous environment (Honma, 2018). With the rapid adoption of artificial lighting, individual organisms and ecosystems are exposed to increasing levels of lighting that can divorce internal temporal rhythms from natural solar days (Cinzano et al., 2001). Exposure to aberrant lighting during the night disrupts circadian rhythms of core clock gene expression resulting in altered behavior and physiology. Increasing evidence suggests that low or dim levels of illuminance levels of light at night (LAN, e.g., 5 lux) that mimic the amount of light pollution in urban environments can affect several physiological parameters (Navara and Nelson, 2007; Nelson and DeVries, 2017); night-time light exposure has been associated with health consequences including disruptions to metabolism (Fonken et al., 2013) and immune function (Bedrosian et al., 2011; Cissé et al., 2017), as well as an increased risk for cancer (Walker et al., 2020). The presence, intensity, and extent of exposure to

artificial light at night has significantly increased over the past century (Cinzano et al., 2001); nighttime artificial lighting increased by 2.2% per year between 2012 to 2016 alone (Kyba et al., 2017). Furthermore, light at night affects organisms at the individual, population, and ecosystem level, indicating its growing impact and importance (Navara and Nelson, 2007; Nelson and DeVries, 2017).

Aging is characterized through the functional decline in physiological functions and is an unmodifiable risk factor for numerous chronic diseases. Several aspects of health change as individuals age, including metabolism (van Beek et al., 2016), cognition (Duncan, 2020), and response to immune challenges (Montecino-Rodriguez et al., 2013). Endogenous and exogenous environmental factors have been suggested to drive progression of the aging process, and cellular senescence, a cell-intrinsic stress response, plays a central role in aging (López-Otín et al., 2013).

Within aged populations, the molecular clock and circadian system also undergo several changes including blunted amplitude and loss of robustness, leading to susceptibility to disrupted circadian rhythms that in turn can contribute to the development of neurodegenerative diseases (Abbott and Videnovic, 2016; Kondratova and Kondratov, 2012). Earlier studies examining disrupted circadian rhythms using a chronic jet lag model (chronic phase advances) reported increased mortality in mice (Davidson et al., 2006). Chronic exposure to dLAN also decreases survival in female Drosophila (McLay et al., 2017), suggesting a sex difference in survival in response to perturbed circadian rhythms.

Disrupted circadian rhythms also alter aspects of the immune system including the regulation of proinflammatory cytokines, which in turn, can provoke uncontrolled complement activation resulting in immune dysfunction and disease, as well as shortened life expectancy (Brodsky, 2015; Inokawa et al., 2020; Jasim et al., 2019; Oster et al., 2017). For example, acute exposure to ecologically relevant levels of dim light at night (dLAN; 5 lux) suppresses immune function in hamsters (Bedrosian et al., 2011). Additionally, exposure to dLAN impairs circulating monocytes and T-cells and alters Cd68 and Ccl2 expression in peripheral tissue in rats (Okuliarova et al., 2021). The immune system has also been implicated in cellular senescence, where impaired immune surveillance accelerates aging, accumulation of senescent cells, and chronic inflammation (Ovadya et al., 2018), however, the relationship between disruptions to circadian rhythms and its effects on immune function and aging remains unspecified. Thus, we hypothesized that disrupted circadian rhythms by exposure to chronic exposure to dim light at night impairs immune response and survival in aged mice.

Methods

Sixteen-month-old C57BL/6 unmated male and female mice were obtained from the aged mouse colony maintained by the US National Institute of Aging. Upon arrival, mice were group-housed with same-sex cohorts in the vivarium and allowed to acclimate to light-dark (LD) conditions (14h light 150 lux, lights on at 0400 h; 10 h dark 0 lux, lights off at 1800 h) to maintain a long-day phenotype (Tavolaro et al., 2015) for 4 months prior to experimental manipulation. At 20 months (2 months), mice were randomly assigned to treatment groups and either transferred to chronic dLAN (14h light 150 lux; 10 h dark 5 lux) at night or remained in LD conditions for 24

weeks (LD males n=14, dLAN males n=15, LD and dLAN females n=15). Mice were single housed in polypropylene cages (30x18x14 cm) to avoid the formation of social dominance hierarchies that could induce differences in stress responsivity and activity among cage mates (Horii et al., 2017; Robbers et al., 2021), and to avoid the possibility of reconstituting a group of mice mid-study due to death or aggression. Mice were maintained on a static rack with bi-weekly cage changes by husbandry staff and provided *ad libitum* access to standard rodent chow (Envigo Teklad 2018) and reverse osmosis filtered water.

Light at night (dLAN) was produced by LUMA5 LED light strips (Hitlights Inc; 1.5W/ft, 5000K “cool white”, 1200 lumens) placed equidistant in front of each cage. Light measurements were determined using a light meter (Mavolux 5032C illuminance meter (Nürnberg, Germany) from the center of an empty cage with the light sensor facing the ceiling. Due to a technical issue, seven female mice in the dim light at night treatment group were briefly exposed to ~15 lux during the dark period for 6 days during week 7, however, their data were not significantly different from other females and were included in the analyses (Supplemental Figure 4.1). The female cohort of mice were two months older than the males at initiation of the study due to timing constraints for behavioral testing. Body mass was measured prior to lighting treatment, then measured at 10, 20, and 24 weeks of exposure to the respective lighting conditions. Mice were monitored and survival was recorded daily. One LD male, and one female in each of the LD and dLAN conditions were removed from survival analyses due to self-injury. One male in dLAN was removed from DTH analysis due to not having sufficient pinna.

Delayed-Type Hypersensitivity Test (DTH)

To assess cell-mediated immune response, mice were sensitized to a chemical antigenic challenge as previously described. Briefly, under light anesthesia 25 µl of 2-4 dinitro-1-fluorobenzene (DNFB; Sigma, St. Louis, MO; 0.5% volume in a 4:1 acetone/olive oil vehicle) was applied to a 2 x 3 cm shaved region of the dorsum for 2 consecutive days starting at week 22. Prior to manipulation, mice acclimated to the experimental room for 30 min and the thickness of the right and left pinna was measured using a constant- loaded thickness gauge (Mitutoyo #7309, Kawasaki, Kamagawa, Japan) at the same time point each day (zeitgeber time; ZT 8-10, 1200 h to 1400 h). Mice were lightly anesthetized with isoflurane vapors during DNFB challenge and pinnae thickness measurements. Seven days post sensitization, mice were re-challenged with 20 µl DNFB (0.2% volume DNFB in vehicle) to the external right pinna, the left pinna received the vehicle solution. Pinnae thickness was measured for the following 6 days. All experiments were approved by West Virginia University Institutional Animal Care and Use Committee and animals were maintained in accordance with NIH Animal Welfare guidelines.

Statistical Analyses

All data were analyzed independently for each sex. Survival was assessed through the Kaplan-Meier method. Survival curves comparing lighting conditions were analyzed using the Mantel-Cox test. Body mass was assessed as a percent change from baseline using a repeated measures two-way ANOVA analysis with lighting condition and time as independent variables. Mice that died during the experimental timeline and study were removed from body mass analysis. A repeated measures two-way ANOVA was used to analyze the delayed-type sensitivity test with lighting condition and time post sensitization as variables. Adrenal and spleen masses were

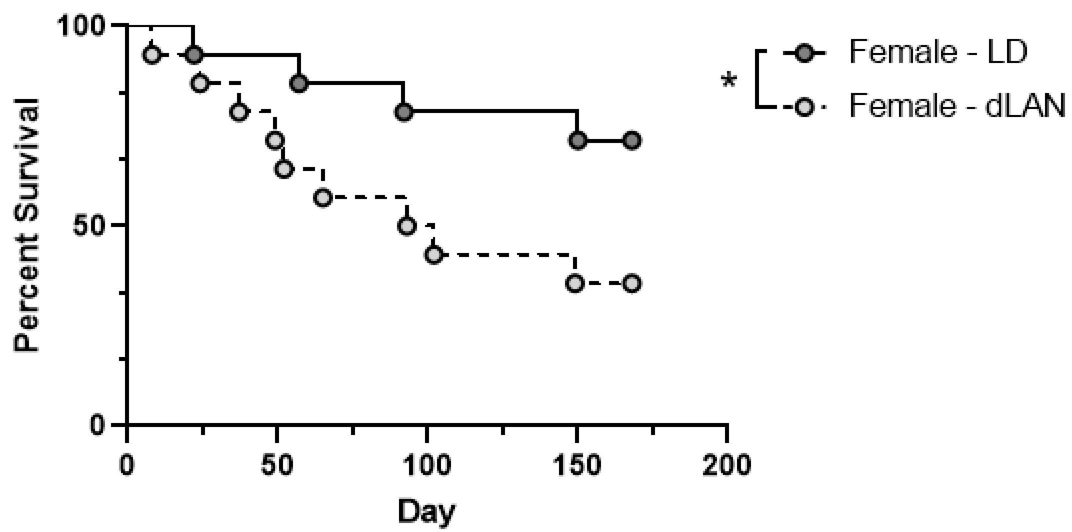
reported as a percentage of body mass and were analyzed comparing lighting conditions using an unpaired 2-tailed t test. Post-hoc analyses were performed using Fisher's LSD test. An outlier test was performed prior to analysis and a maximum of one outlier per group was removed a priori; an outlier was defined as having a within-group Z score >2. Data were tested for normality using Shapiro-Wilks test. Statistical analyses were performed using GraphPad Prism 9.0 software, and data are presented as the mean ± standard error of the mean (SEM). Mean differences were considered statistically significant if $p \leq 0.05$.

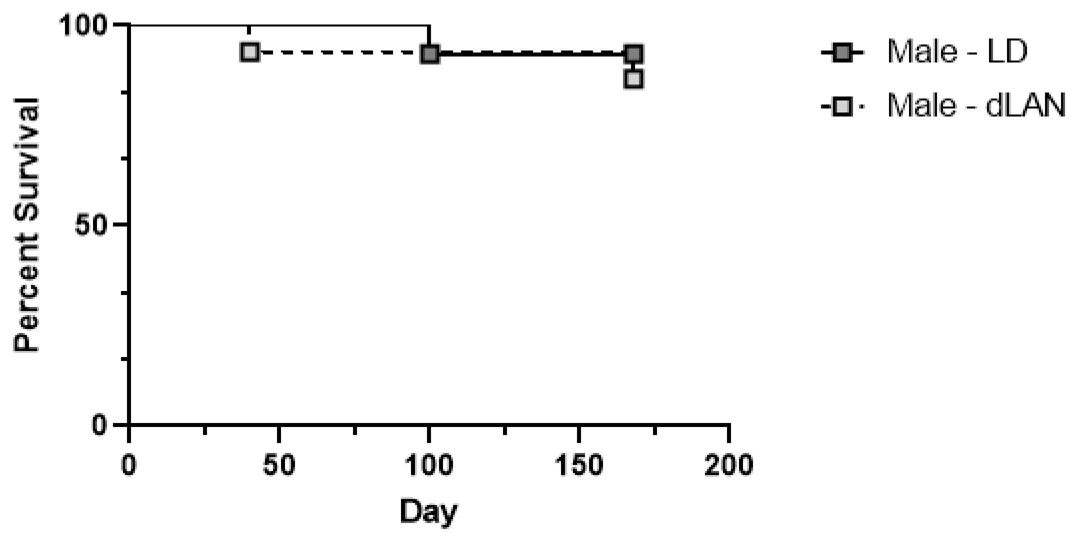
Results

Dim Light at Night Reduces Lifespan in Female Aged Mice

After 24 weeks of chronic exposure to light at night, female mice exposed to dim light at night displayed reduced lifespan compared to females housed in dark nights assessed through the Kaplan-Meier Mantel-Cox test ($p<0.05$) (Fig 4.1.A). There were no significant differences in survival between males in either lighting condition ($p>0.05$) (Fig 4.1.A).

1. **(a)**





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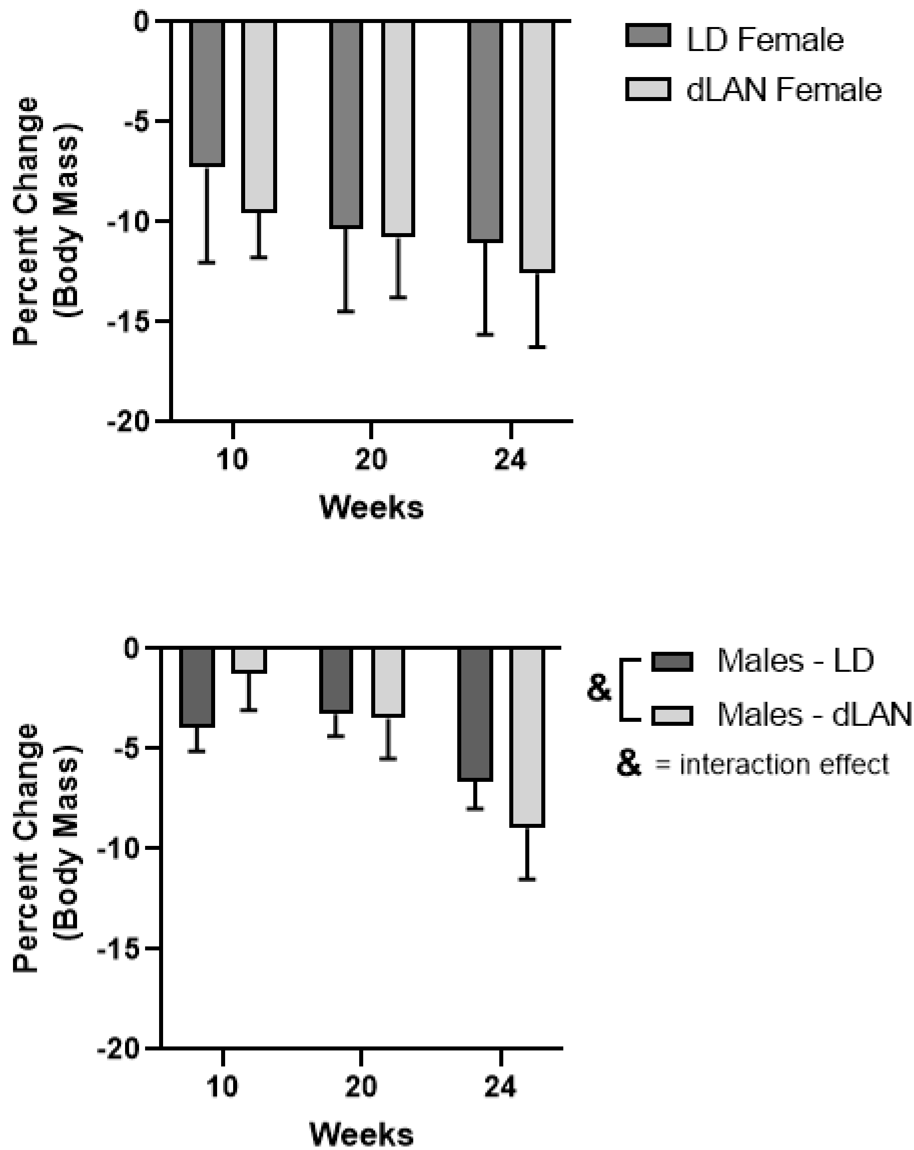


Figure 4.1. Survival and body mass after 24 weeks of exposure to dLAN. (a) dLAN decreased lifespan in females compared to LD counterparts ($p=0.05$), (LD female and dLAN female $n=14$). No observed effect of lighting condition on survival in males ($p>0.05$), (LD male $n=13$, dLAN male $n=15$). (b) There was a significant interaction of lighting condition on body mass in males ($p<0.05$). Males exposed to dLAN initially lost a smaller percentage of their body

mass at week 10, then lost a significantly larger percentage of their body mass at 24 weeks compared to LD males. There were no differences in body mass when comparing lighting conditions in females ($p>0.05$). Mean ± S.E.M.; *$p\leq0.05$ (&; denotes an interaction effect).

Dim Light at Night Altered Weight Loss in dLAN Males

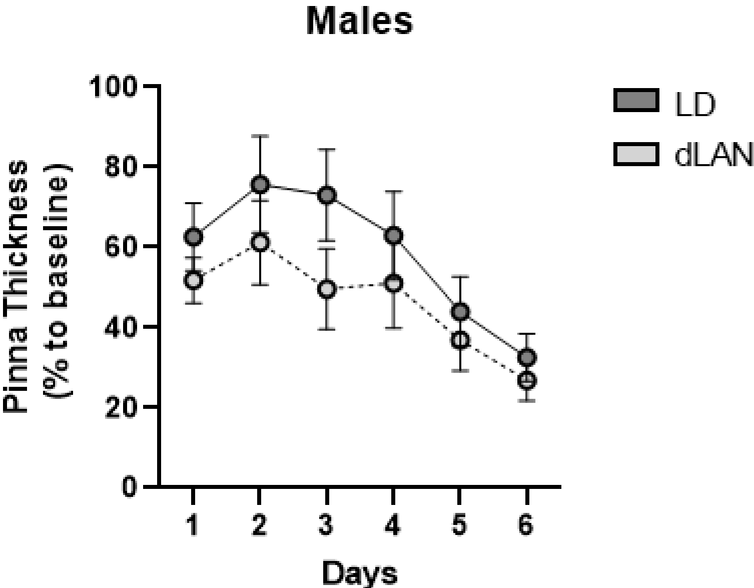
Our analysis revealed a main effect of time ($F_{2,48} = 22.92$; $p<0.05$) on body mass in males and an interaction effect between lighting and time on male body mass reported as a percentage compared to baseline ($F_{2,48} = 4.629$; $p<0.05$). When comparing male body mass at 10, 20, and 24 weeks, dLAN males lost proportionally less body mass compared to males exposed to dark nights at 10 weeks, while males housed under dLAN lost significantly more body mass at 24 weeks compared to 10 weeks, however, post-hoc comparisons were not statistically different when comparing lighting conditions across each time point (Male LD n=13, dLAN n=13). There was no effect of lighting condition on body mass in females ($p>0.05$), however, mice that died during the experimental timeline across both sexes were excluded from body mass analysis due to statistical requirements.

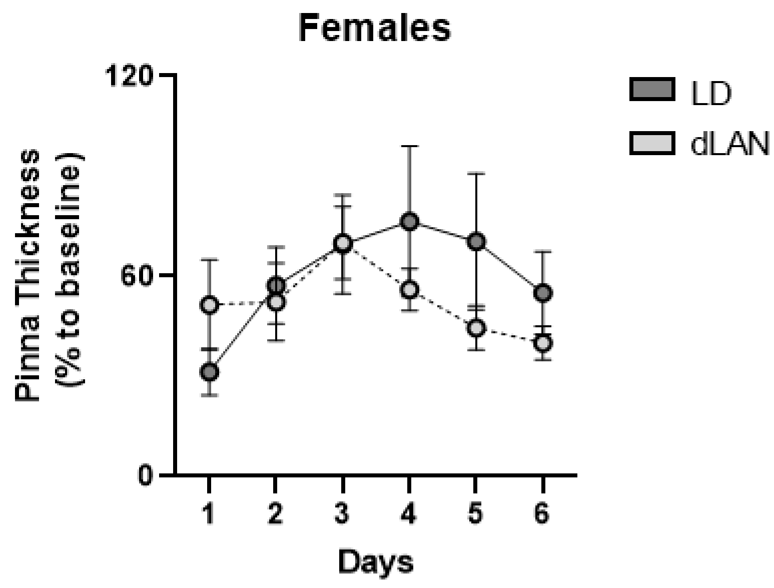
Dim Light at Night Dysregulates Cell-Mediated Immune Response in Female Mice

Re-exposure to DNFB induced swelling in the challenged pinna compared to baseline for both treatment groups ($p<0.05$; data not shown). There was an interaction effect of lighting condition and time in females (Fig. 4.2A; $F_{5,65} = 2.39$, $p<0.05$); dLAN females initially had comparable swelling to LD female mice but displayed decreased right pinna swelling beginning after day 3 post sensitization; post-hoc comparisons were not statistically different when comparing lighting

conditions across each day ($p>0.05$). No significant difference between lighting conditions was observed among males ($F_{1,24} = 1.16$, $p>0.05$).

2. (a)





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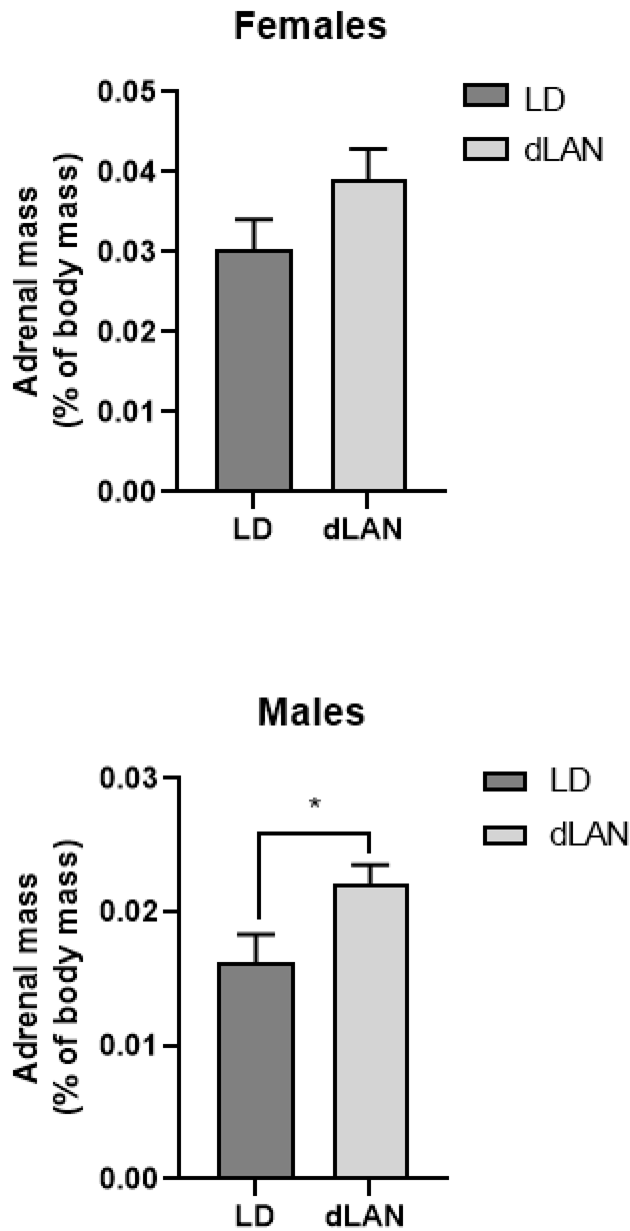

Figure 4.2. DLAN differentially affected inflammatory response in females and altered adrenal masses in males, compared to dark night sex counterparts (a) dLAN significantly impaired female inflammatory response during the later days of the delayed-type hypersensitivity response, depicted by decreased cell-mediated pinna swelling reported as a

percent change from baseline ($p<0.05$). There was no significant difference between LD and dLAN males ($p>0.05$). (b) After correction for differences in body mass, males exposed to chronic dLAN displayed increased adrenal mass as a percentage of body mass compared to LD males ($p<0.05$) and no difference in females in both lighting treatments ($p>0.05$) (LD females n=10, dLAN females n=6, LD males n=11, dLAN males n=13). Mean ± S.E.M.; *$p\leq0.05$. (# denotes a main effect of sex, @ denotes a main effect of lighting condition, and & denotes an interaction effect).

Dim Light at Night Increases Adrenal Mass in Aged Male Mice

Males housed in dLAN and after immune challenge had significantly greater adrenal masses reported as a percentage of body weight compared to dark night conditions (Fig. 4.2B; $p<0.05$, unpaired, 2-tailed t test), whereas there were no differences in adrenal masses among females (Fig. 4.2B; $p>0.05$, unpaired, 2-tailed t test). There were no effects of lighting condition on spleen masses among males (Fig. 4.2C; $p>0.05$, unpaired, 2-tailed t test) or females (Fig. 4.2C; $p>0.05$, unpaired, 2-tailed t test).

Discussion

Here, we report the presence of a sex difference in the effects of chronic exposure to dim light at night on lifespan in aged mice. These findings are consistent with previous reports in other species, such as *Drosophila*, where females housed in nighttime lighting had reduced survival, and is the first report, to our knowledge, that chronic exposure to dim light at night late in lifespan affects survival in otherwise healthy, aged mice. Our results suggest that dLAN accelerates aging in a sex specific manner, denoted through a shortened lifespan compared to LD

females. Additionally, females exposed to dLAN displayed dysregulated T-cell mediated hypersensitivity and inflammation through DTH reaction, as a measure of cell-mediated immune response. These results are consistent with previous reports that 4 weeks of dim light at night is sufficient to suppress immune responses in young Siberian hamsters (Bedrosian et al., 2011). Other studies evaluating models of disruption of circadian rhythms or genetic clock gene manipulations have also identified that even in the absence of immune challenge, disruption of circadian rhythms can produce pro-inflammatory cytokines and increase immune activation (Bedrosian et al., 2013) and does so in as few as 3 nights of exposure to dLAN in otherwise healthy mice (Walker et al., 2020). We observed no differences in cell-mediated immune challenge or lifespan in males; however, male mice housed in chronic dLAN conditions displayed dysregulated body mass and increased adrenal mass compared to mice housed in dark nights, suggesting that dLAN may differentially affect pathways and mechanisms between sexes in aged mice. Exposure to dLAN has been previously reported to disrupt molecular rhythms and core clock genes during disruption of circadian rhythms (Fonken et al, 2013). Indeed, with the widespread adoption of ALAN, the delineation between light days and dark nights is blurred, in turn resulting in disruption of clock gene function. Low levels of nighttime lighting, (i.e., 5 lux), are consistent with the exposure of populations residing in light polluted urban and suburban settings (Gaston et al. 2012). Furthermore, (5 lux) ALAN is ecologically relevant due to its behavioral and biological effects (Nelson and Navara, 2007; Gaston et al. 2013). Although we did not directly measure clock gene expression, several studies have demonstrated that there are intact circadian rhythms in this strain (Panagiotou & Deboer, 2020) and that constant levels of “daytime lighting” dysregulates clock gene expression (Hong et al., 2020). Furthermore,

constant lighting in younger, 2-month-old CBA mice disrupted estrous function, increased spontaneous tumors, and reduced lifespan (Anisimov et al, 2004).

The relationship between circadian rhythms and aging is complex; the bi-directional relationship can affect aspects of biological function including aging. Introductions of mutations in core clock genes *bmal1* and *period* in Drosophila and mice accelerates age-related impairments including tissue decline, cognitive impairments, and shortened lifespan (Kondratov et al, 2006; Krishnan et al, 2009). Previous studies have also suggested the existence of a sex difference in circadian rhythm misalignment. For example, sex differences in hypothalamic-pituitary-adrenal (HPA) function have been observed in which stress-response activation is altered in women compared to men (Kudielka & Kirschbaum, 2005). There are also notable sex differences of circadian modulation of the HPA axis attributed to differences in gonadal steroid hormones; estrogens generally enhance, whereas androgens inhibit, HPA function (Yan et al., 2016). Further, females also display significantly more disturbances in energy homeostasis, primarily via increased energy expenditure and lipid oxidation rates, compared to temporally misaligned males (Qian et al., 2019). These results are consistent with previous studies of other forms of chronic circadian rhythm disruption, including a repeated jet-lag model, in which aged mice appear susceptible to shortened lifespans compared to younger mice (Davidson et al., 2006). Whereas dLAN may seem fairly innocuous compared to constant lighting or a chronic jet lag model, dLAN disrupts molecular biological rhythms in multiple species (Bedrosian et al., 2013; Fonken et al., 2013). It is possible that the effects we report here on cell-mediated immune response and longevity in female mice are caused by circadian misalignment dysregulating physiological function.

In our study, mice were unmated and remained gonadally intact throughout the duration of their lifespan; these factors have previously been demonstrated to affect lifespan and longevity (Russell, 1996). In female mice, reproductive changes and decline are detectable by 13-14 months of age (Nelson et al., 1995), and accelerates such that 80% of female mice are acyclic or exhibit irregular cycles at 17 months of age, and 100% by 25 months (Frick et al., 2000). Thus, because the mice in our study were assigned to experimental lighting groups at approximately 20 months of age, it is likely that the majority experienced reproductive decline prior to the experimental lighting exposure. We did not gonadectomize aged mice as we addressed the experimental question of how exposure to chronic lighting conditions would affect otherwise typically aging mice. Whether gonadectomy would alter the outcome of our study, implicating a role for gonadal steroids across the lifespan, is an interesting question that should be answered in future studies.

Age and survivor effects are another consideration for interpreting this study. We enrolled mice at 20+2 months of age, at which point 75-90% of the population is still typically alive (NIA colony statistics). Whether dLAN has similar effects on survival in younger and older mice will need to be determined in the future studies. For the present study, males and females were obtained from the same National Institute of Aging (NIA) cohort, but the mean ages of females and males at exposure to dLAN were different by 2 months because we needed to adjust the experimental start timelines due to time constraints for behavioral/immune testing. Thus, data from females and males in this study were analyzed separately. However, it is important to emphasize that the greatest effects of dLAN on survival in females occurred in the first 80 days of exposure to dLAN, when the female mice were ~22-24.5 months of age. Male mice were

approximately this age between week 8 and 12 of dLAN exposure and yet there was no similar effect of dLAN on survival among males during this period, however, a greater sample size of males may be necessary to identify differences between survival (Yuan et al. 2009) and future studies will need to be conducted.

Aging is associated with changes in body mass composition, and body mass loss late in life in aged populations is correlated with adverse health consequences in rodents and humans (Ray et al., 2010; Nagy and Pappas, 2019). In the current study, a significant interaction between time and lighting condition was observed among males; during the first 10 weeks of exposure, dLAN males initially lost less mass proportionally than LD males, but by 20 weeks of exposure this pattern reversed and dLAN males lost more mass proportionally than LD males. In contrast, there were no significant differences in body mass among females at any of the time points. However, these analyses included only mice that survived to the end of the experiment, and because fewer dLAN females survived than LD females, it is possible that the differential survival skewed the body mass data if the mice that died had the greatest body mass loss.

Females housed in dLAN also exhibited changes to cell-mediated immune response that could contribute to accelerated aging and reduced lifespan that we did not observe in males. We observed increased adrenal mass in males housed in chronic dLAN compared to dark night conditions, which suggests that chronic dLAN may dysregulate stress response in males, but not females. Chronic stress is often accompanied by increased adrenal masses (Ulrich-Lai et al., 2006). Previous studies evaluating other models of disrupted circadian rhythms, including light pulses during the night (Ishida et al., 2005) or jet lag paradigms/constant illumination, report

elevated glucocorticoid concentrations (Dunnet al., 1972; Sakellaris et al., 1975). No studies to date have reported altered stress responses in aged males evoked by exposure to dim light at night; however, elderly mice may have increased risk and susceptibility to the consequences of disrupted circadian rhythms due to aging. Future studies should further characterize increased adrenal masses and stress responses to delineate whether this has functional significance. Together, these data support the proposition that chronic dLAN differentially affects aspects of physiology in aged populations.

Optimal immune response is dependent on a functioning circadian system (Okuliarova et al., 2021). Thus, we chose to use the DTH test, which is a measure of cell-mediated immunity that can be used as an indirect measure of immune response mediated by memory T-cells, antigen presenting cells, effector T cells, cytokine production, and inflammation at the exposure site. DTH is also used as an assay for increased risk of mortality and poor health in aged human populations (Wayne et al., 1990), and natural aging has been associated with reduced B and T cell production, and reduced lymphocyte function (Montecino-Rodriguez et al., 2013). Previous studies indicate that disrupted circadian rhythms alters circulating immune cells. For example, 5 weeks of dLAN reduces white blood cell populations, including monocytes and T-cells (Okuliarova et al., 2021). Furthermore, chronic disruption of circadian rhythms accelerated aging in young 10-week-old mice, likely via low-grade inflammation (Inokawa et al., 2020). The altered DTH response we observed after exposure to chronic dLAN suggests that altered immune function may also be a contributing factor to accelerated aging in this study.

In summary, our results suggest that chronic exposure to dim light at night dysregulates cell-mediated hypersensitivity reactions as a measure of cell-mediated immune response and accelerates aging, demonstrated through reduced lifespan in female mice. Further, we report that males housed in dLAN display dysregulated body mass after chronic exposure to dim light at night and increased adrenal mass which is often associated with elevated stress exposure and response. This may be a concern given that aged human populations have a particularly high incidence of chronic exposure to artificial light at night (Scheuermaier et al., 2010), in part due to the increased care requirements of elderly populations. Dysregulation of immune response can result in increased hospital visits, further exposing patients to night-time artificial lighting from recovery units (Durrington et al., 2017). Future studies should be aimed towards identifying underlying mechanisms for sex differences in response to circadian misalignment and examine how restoration of naturalistic lighting in environments to normalize circadian rhythms could improve immune function and prolong longevity.

Supplemental Results

Female mice in dLAN that were inadvertently exposed to 15 lux of dim lighting at 7 weeks for 6 days were not significantly different from females that remained in typical dLAN (5 lux) conditions (p=0.1870) (Fig. S4.1A). Notably, the sample size in this Kaplan-Meier curve differs from Figure 4.1, because this curve does not include mice that died prior to brief lighting exposure (n=3). Treatment groups had 2 and 3 respective deaths throughout the remainder of the study which is not statistically different (Fig. S4.1B).

Supplemental Figures and Captions

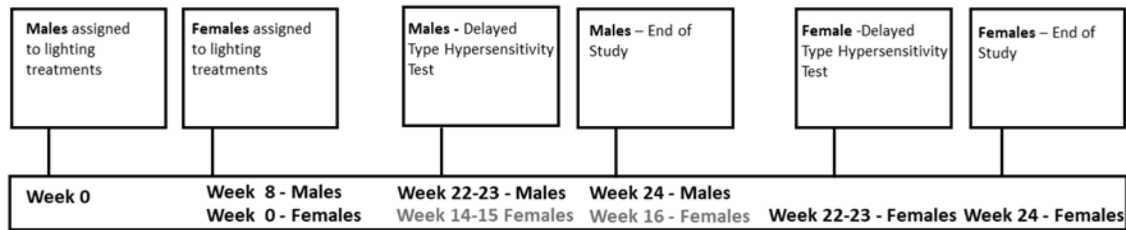

Table S41. Experimental Timeline.

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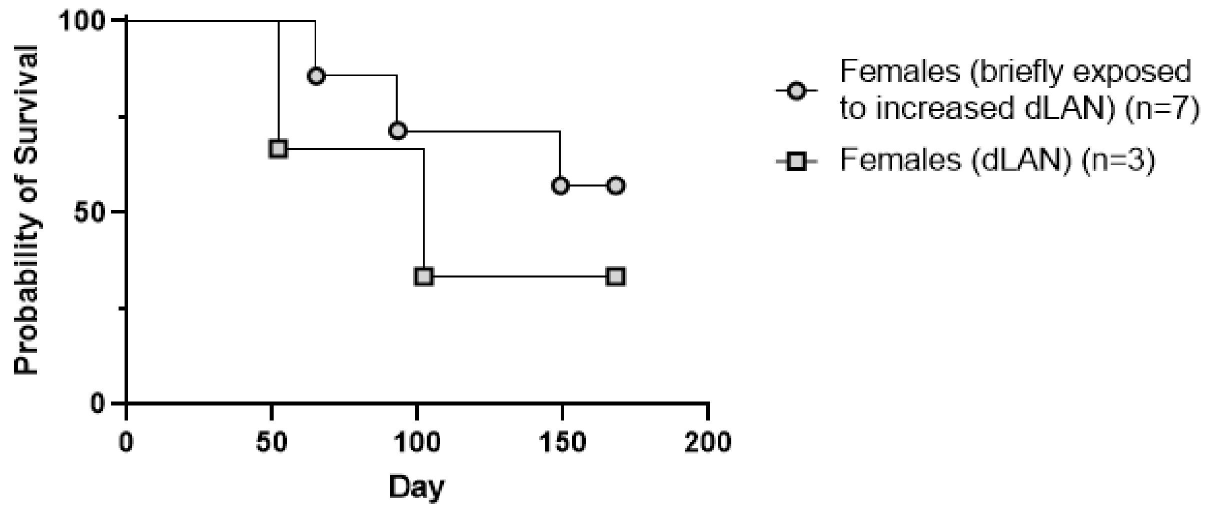

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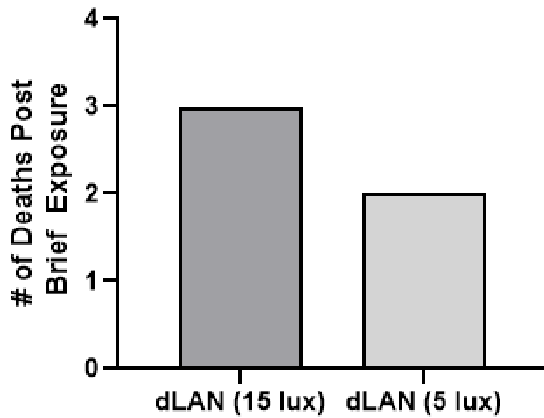

Supplementary Figure S41. Brief exposure to increased lux did not reduce lifespan in dLAN females. (a) Females that were briefly exposed to ~15 lux for 6 days during week 7 out of 24 weeks of experimental manipulation due to a technical error were not statistically different compared to the remaining cohort (dLAN (15 lux) females n=7; dLAN (5 lux) females n=4) (p=0.1870).

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CHAPTER 5

CHRONIC PHASE ADVANCES REDUCES SPATIAL RECOGNITION MEMORY AND INCREASES COGNITIVE DEMENTIA-LIKE IMPAIRMENTS IN AGED MICE

Introduction

Cognitive impairment is a condition that broadly encompasses the stages between typical cognitive decline associated with aging that are of high risk to develop dementia (Dubois et al., 2007) and approximately 12% of individuals will go on to develop Alzheimer's Disease each year (Cardinali et al., 2010). This clinical condition consists of impairments in executive function, spatial memory, and attention, which leads to impairments in general cognition (Webster et al., 2014). Working memory, one of the important components of the cognitive system that is affected by aging and dementia, involves holding and storing information that can be divided into short and long-term memory (Miyake and Shah, 1999). Previous research provides supporting evidence that short-term memory is an essential component to advanced cognitive processes, including learning and problem solving. Prefrontal cortex activation has been demonstrated to be involved in working memory, and damage from neurodegeneration or lesions in this region can affect working memory (Stern, et al., 2001). The underlying pathophysiology contributing to the development of cognitive impairments requires further investigation. Several associated symptoms have been identified in aged populations prior to diagnoses which serve as biological targets and considerations.

The circadian system directly influences cognition, memory, and aging. Light and photic information modulates cognition, memory processing, and hippocampal activation (Hassan et al., 2021) and ipRGC projecting photic information can mediate spatial working memory and learning (Fernandez et al., 2018). Previous studies have identified that disruptions to circadian

rhythms from exposure to dim white light in adult rodents can impair hippocampal-dependent learning and memory (Taufique et al., 2018; Mangyal et al., 2020). Other models that disrupt circadian rhythms involve alterations to light dark cycles, which is a common form of disruption in human populations resulting from shift work or travel. Disruption or misalignment of the internal circadian clock from the external environment can occur from jet lag (i.e. trans-meridian travel across time zones), shift work (non-standard, night shift work, and irregular work hours deviating from "9" to "5" workdays) or more commonly, social jet lag which is a more prevalent form of circadian disruption across adolescent and adult populations (Crowley et al., 2007). This can dysregulate homeostasis and adaptations to the environment resulting in an increased risk for poor health outcomes (Kyriacou et al., 2010). The circadian system can contribute to the aging process, and previous studies have associated disruptions or manipulations of the circadian clock is involved in cognitive function decline and increased risk for impairments, neurodegenerative, and neuropsychiatric diseases (Kondratova and Kondratov, 2012; Musiek et al., 2015). Changes to *Bmal1* rhythmicity have been observed in the early stages of AD patients (Cronin et al., 2017). In studies using triple transgenic murine models of AD, expression of clock genes are disrupted in the SCN and hypothalamic regions involved in circadian regulation, including the hippocampus, frontal cortex, and the brainstem in the early stages of pathology development accompanying cognitive impairments (Francesco et al., 2017). Further, in individuals with mild cognitive impairment (MCI), neuropsychiatric symptoms including anxiety and depression are common (Lyketsos et al., 2011). Neurodegenerative disorders affect over fifteen percent of the world's population, and cognitive impairments remain the seventh leading cause of death around the globe (Maiese, 2021). With the increase of the number of adults over 65 anticipated to double

by 2050 (Lowerison et al., 2022), cognitive impairments and dementia will overburden society, highlighting the prevalence and need for further investigation.

In **Chapter 4**, I identified that exposure to circadian rhythm disruption in aged mice adversely affected physiological parameters, including changes to immune function and corresponding shortened lifespan in female mice, and indicated that chronic exposure to dLAN can accelerate aging. Despite increased number of studies that associate circadian disruption and health risks, the underlying cellular mechanisms resulting in cognitive changes remains poorly characterized. Here, the hypothesis tested is that disruptions to circadian rhythms from phase advances changes vasculature in the brain and increases cognitive impairment in aged mice. In addition, circadian rhythm disruption may increase risk for cognitive aging, decline, and risk for health and specifically, vascular diseases. To test this hypothesis, 20-month old male and female mice were subjected to 8 weeks of 6 h phase advances every 7 days then behavior and cognitive function was assessed.

Materials and Methods

C57BL/6 aged male and female mice were obtained from the US National Institute of Aging colony and were housed until 20 months of age prior to experimental group assignment. After acclimation, mice were assigned to control light dark cycles (12 h light, 12 h dark 08:00 h EST; 12 h dark, 0 lux 20:00 h EST) or were subjected to chronic 6h phase advances each week. A table illustrating the experimental design is listed below (Table 1). Mice were single-housed and fed *ad libitum* access to standard rodent chow (2018 Teklad) and reverse osmosis water. Body

mass and food intake was measured each week. At night 6 prior to the phase advance each week, food intake during the light phase and dark phase were measured. Mice were checked daily during the light phase and survival was recorded. On week 8, behavior paradigms were conducted to assess spatial working memory using the spontaneous alternation Y-maze task, novel object recognition task, open field for locomotor activity measures, and to assess anxiety-like behavior, and amygdala dependent cognitive performance through toned and contextual fear conditioning. Behavioral testing was conducted during the light phase (ZT 1-8, 09:00 h to 16:00 h) starting at 09:00 h each session. The duration of behavioral testing was dependent on the task. Behavior was assessed during the light phase due to lighting exposure during the toned-fear conditioning test to avoid disrupting light-dark cycles. Tissue collection and perfusions were performed as described below between ZT 5-8 (13:00 to 16:00 h) during the same time interval behavioral testing occurred, and was collected during that time between 24-72 h after completion of behavioral testing due to perfusion timing constraints for collection. An experimental table can be found depicting the lighting condition manipulations, experimental design, and behavior testing order (Table 1). All studies were approved by the West Virginia University Institutional Animal Care and Use Committee, and animals were maintained in accordance with the NIH Animal Welfare guidelines.

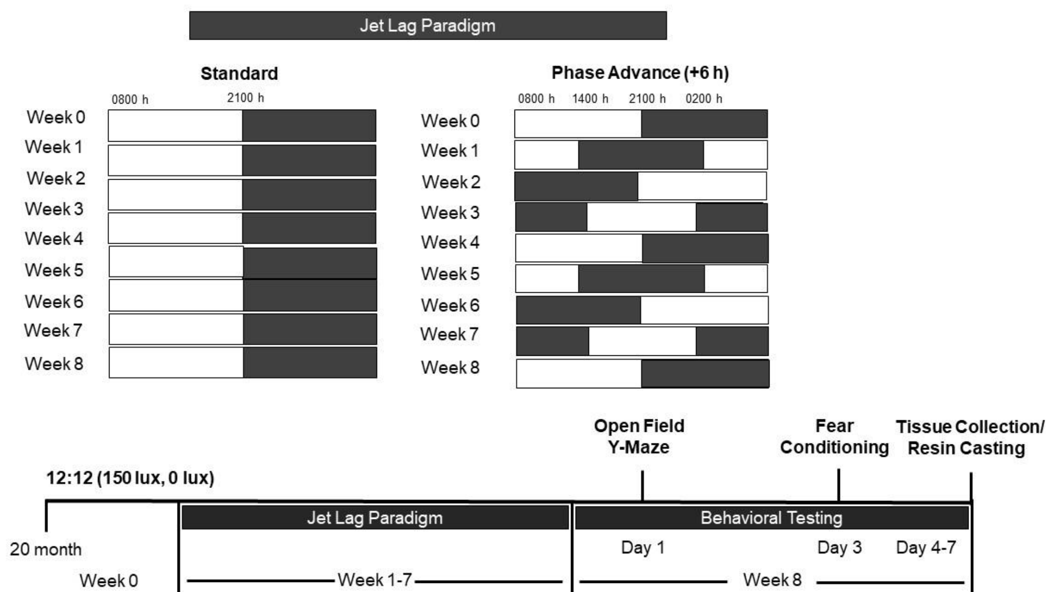

Table 1. Experimental Design for Chronic Phase Advance (Jet Lag) Paradigm.

Spontaneous Alternation Task

To assess spatial working memory as a measure of cognitive performance, spontaneous alternation behavior was measured using a standard Y-Maze apparatus (35 cm at each arm, converged at an equal angle). Mice were placed into one arm facing the same direction and allowed to freely move through the maze for 180 s and locomotor activity was recorded and tracked using Ethovision XT. Arm entries were defined as all four limbs crossing into the arm and alternations were calculated using: [(alternation/maximum possible number of alternations) x 100]. Maximum possible alternations were calculated as the total arm entries -2.

Open Field

Locomotor and anxiety-like behaviors were assessed using a 36 cm x 36 cm polypropylene open field chamber boxes (Open Field Photobeam Activity System, San Diego Instruments Inc., San Diego, CA USA) equipped with infrared sensors mounted to detect horizontal and vertical movement for 10 minutes as previously described (Walker et al., 2021). Boxes were contained in cabinets to remove outside noise or light. Total locomotor activity was determined by the total number of beam breaks. Central tendency and number of rears were calculated within the first 5 minute bin.

Auditory Fear Conditioning

Toned-fear conditioning associative learning was assessed using the Near-IR MedAssociates Video Fear Conditioning System (MedAssociates, Inc., St. Albans, VT USA) consisting of a light and sound controlled Plexiglas chamber with a metal grid. Freezing behavior was recorded using Video Freeze software (Med Associates) and was reported as a percentage of freezing during each binned interval. For the acquisition session for tone-conditioning, mice habituated to the environment 30 minutes prior to behavioral testing. Mice were placed into the test chambers and were illuminated with white light for 2 minutes with a 68 dB white noise, then, were exposed to a series of 8 conditioned stimuli including a 6 s tone (3500 Hz, 80 dB done, CS) followed by a 2 s paired 0.6 mA foot shock on the wire grid (unconditioned stimulus, US) with a 30 second interval between conditioned stimuli. Mice remained in the chamber for 60 s after the final CS/US pairing then were returned to their home cages. 24 h after acquisition, contextual fear retention was assessed and freezing behavior was recorded for 180 s in the unmodified chambers. Four hours following the contextual fear retention, mice were re-tested in a modified chamber to alter contextual cues by changing testing room and apparatus settings to avoid

context-dependent cues or freezing. Testing room settings were changed to a dimly lit room with a dull fan noise and the chamber system was modified in shape and texture with the addition of a smooth plastic semi-circular shape with extinguished lights and a gauze pad with a drop of vanilla present to present as a novel CS environment. Mice were then re-tested for retention of the CS/US pairing following the same experimental procedure described for the acquisition trial above without receiving the foot shock. Freezing behavior was reported as percent freezing per component interval.

Resin Perfusions and μCT Scanning

To assess vasculature, resin casting perfusions were performed across three days after behavioral testing on Week 8 (ZT 5-8; 13:00 to 16:00 h) during the same time interval behavioral testing occurred. Mice received a lethal dose of sodium pentobarbital. After confirming anesthetization, mice then were transcardially perfused at 4 mL/min with 15 ml of 25U/mL saline heparin solution followed with 15 mL of 4% paraformaldehyde. Radiopaque Resin for vascular corrosion casts was performed using PU4ii-RO (VasQTec). Resin and hardener were mixed directly prior to perfusion according to manufacturer guidelines and allowed to harden for 5 days in 4° C. Skulls were removed and decalcified using 5% formic acid (BDH4554; VWR International) for 5 h. The brains were then dissected and casts were isolated from remaining tissue with 7.5% KOH (BDH7622; VWR International) for two 12 h washes. Resin casts were washed with Milli-Q water, then were lyophilized prior to mounting.

Corrosion cerebrovascular casts were imaged using the SkyScan 1272 (Bruker), following 50 kV/200 μA without filter and 360°, 0.17° interval rotation. There were 900 ms exposures with 4

frame averages/step producing a 2.7µm isotropic voxel resolution. Images were reconstructed at 15% ring artifact reduction at 3, smoothing at 0, 0.02-0.40 dynamic range images with compensation across individual samples using NRecon (Bruker). Volumes were resliced coronally, then were loaded into CTAn (Bruker) and the Hippocampus and Isocortex were manually segmented between bregma -1.6 to -2.6 and were interpolated at 0.1 mm distances.

Statistical Analyses

Mean behavioral data were compared with a Two-Way ANOVA and post-hoc comparisons were formed using Fisher's LSD. Segmented vasculature hippocampal and isocortex volumes were analyzed using VesselVio (Bumgarner and Nelson, 2022) with 10µm filters for isolated segments and 5µm filters for endpoint segmenting. Vasculature datasets were collapsed between sexes due to the limited number of casts collected. Unpaired two-way t-tests were performed between vasculature datasets across lighting conditions. Data were tested for normality using the Shapiro-Wilk tests, those that did not meet assumptions for normality were subjected to nonparametric tests (Mann Whitney Test). Outlier detection was conducted using Grubb's Outlier Test; defined as a z-score >2. A maximum of 1 outlier per treatment group was removed from analysis. Differences were considered statistically significant if $p \leq 0.05$. All analyses were conducted using Graphpad Prism 9.

Results

Circadian Disruption by Exposure to Chronic Phase Advances Reduces Spatial Recognition Memory in Females

Spatial learning and memory impairment was assessed using spontaneous alternation task in Y-maze after 8 weeks of chronic phase advances. There was an interaction effect between lighting condition and sex ($F(1, 49) = 7.048$; $p=0.0107$) (Figure 5.1). Females housed in chronic jet lag displayed significantly reduced spontaneous alternation on post-hoc tests compared to females housed in dark night conditions ($p=0.005$), decreasing by 11.66%. There were no statistical differences between light conditions in males on post-hoc analyses ($p=0.0932$).

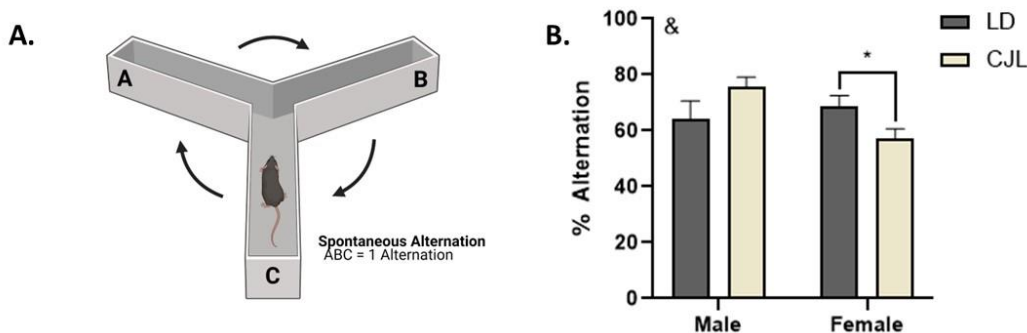

Figure 5.1. Chronic Phase Advances Reduces Spatial Recognition Memory in Females (a) Diagram of Y-Maze for Spontaneous Alternation Task (b) Females exposed to chronic phase advances display reduced spontaneous alternation as a behavioral measure of spatial recognition memory compared to dark night controls ($p<0.05$). Data are represented as mean ± SEM (LD males n=12, LD females n=15; CJL males n=10, CJL females n=16). & denotes interaction effect, * $p<0.05$.

Females Exposed to Chronic Phase Advances Display Reduced Acquisition During Toned-Fear Associative Learning Task

An associative learning task (fear conditioning) was performed as another assessment of cognitive function in aged mice. There was a main effect of time ($F(7, 210) = 28.77$; $p<0.0001$) and light condition ($F(1, 30) = 5.125$; $p=0.0310$) during the toned-fear acquisition task; females exposed to chronic phase advances display significantly reduced freezing compared to females housed in dark night conditions (post-hoc: Tone 6 $p=0.0056$, Tone 8 $p=0.0071$) (Fig. 5.2). In males, there was a main effect of time ($F(7, 133) = 10.43$; $p<0.0001$) demonstrating that males acquired the tone, but there was no effect of light condition ($p=0.9580$). Both males and females display increased freezing behavior post-tone compared to pre-tone habituation across both light conditions (males $F(1,18) = 178.2$, $p<0.0001$) (females $F(1,62) = 123.9$; $p<0.0001$). Percent freezing did not differ between light conditions of either sex ($p>0.05$).

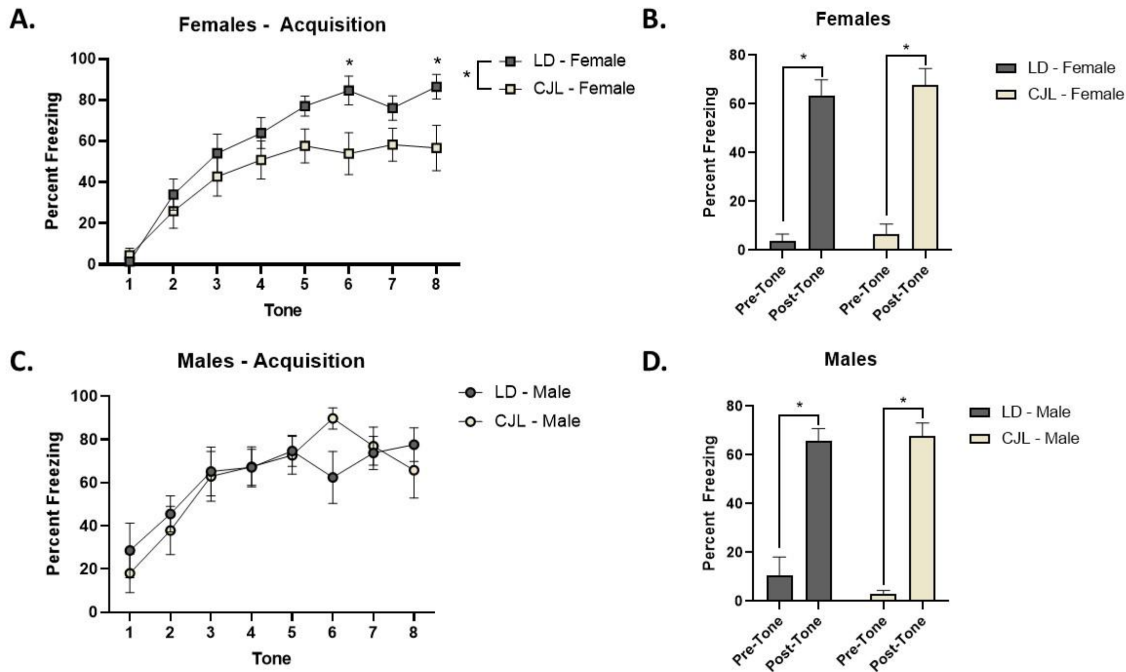

Figure 5.2. Chronic Phase Advances Reduces Spatial Recognition Memory in Females (a) Females exposed to chronic phase advances display reduced freezing behavior across tone presentations during toned-fear conditioning acquisition compared to dark nights ($p<0.05$) (b) During the acquisition phase, both CJL and LD females significantly increased freezing behavior (%) post-tone presentation compared to pre-tone habituation indicating females acquired the learning task ($p<0.05$) (c) No differences between dark night control and males housed under chronic phase advances during the acquisition toned-fear task ($p>0.05$). (d) CJL and LD males significantly increased freezing behavior post-tone compared to pre-tone ($p<0.05$) Data are represented as mean ± SEM (LD females n=15; CJL females n=16) * $p<0.05$.

Males and Females Retain Contextual Dependent Cues Across Lighting Conditions and Males Display Reduced Freezing During Retention Retrieval Tasks

24 h after acquisition, mice were placed into fear conditioning chambers to assess contextual retention by freezing behavior. Males and females across both lighting conditions displayed increased freezing compared to pre-trial percentages, indicating that mice across both sexes and lighting conditions retain context-dependent cues ($p<0.05$). Freezing behavior did not differ during retention tone trials in females between lighting conditions ($p>0.05$) In males, there was a main effect of lighting conditions on the repeated measures two-way ANOVA ($F(1, 144) = 9.356$; $p=0.0027$) where males exposed to chronic phase advances reduced freezing compared to males housed in dark night conditions (post-hoc; Tone 5 $p=0.0394$, Tone 7 $p=0.0390$).

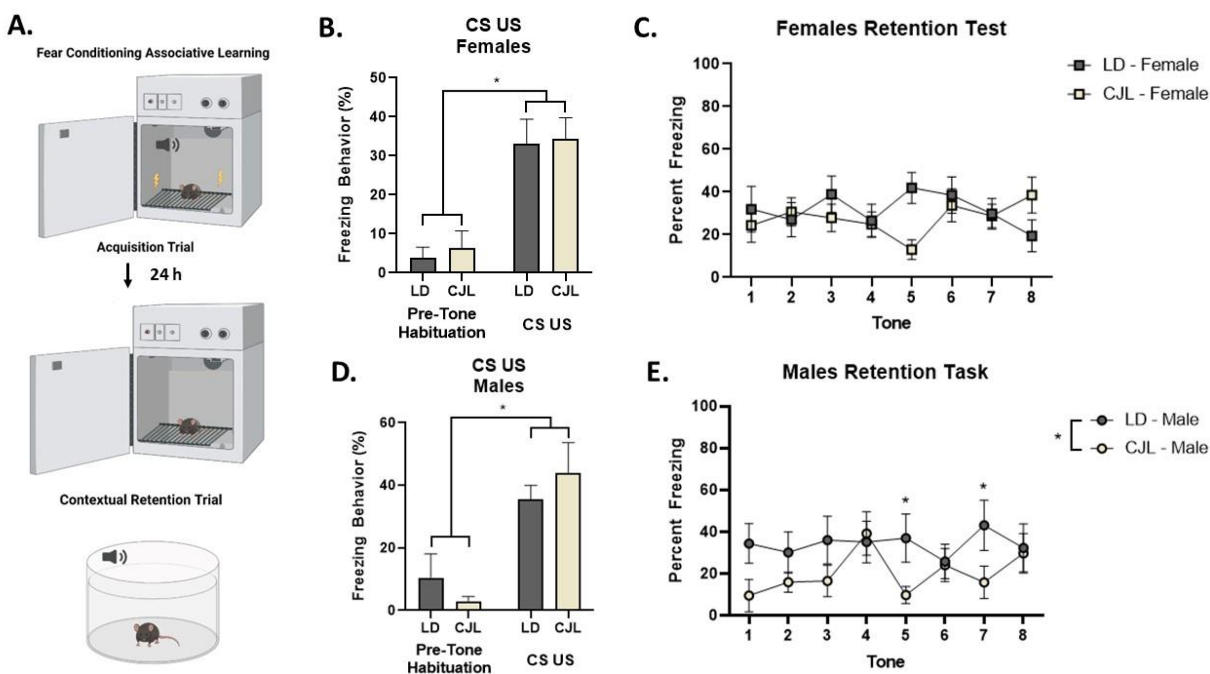

Figure 5.3. Males and Females Retain Contextual Dependent Fear Across Lighting Conditions but Males Exposed to Chronic Phase Advances Display Significantly Reduced

Freezing Behavior During Tone Retention Trials (a) Diagram of Toned-Fear Acquisition Task (b and d) Freezing behavior during contextual dependent task does not differ in male or female mice between lighting conditions ($p>0.05$) but display increased freezing behavior compared to pre-trials suggest that mice retain fear to context dependent cues ($p<0.05$). (c) No significant differences in the percentage of freezing behavior between lighting conditions in females (d) males exposed to chronic phase advances display significantly reduced freezing behavior compared to dark night conditions 24 h after acquisition during retention trials ($p<0.05$). Data are represented as mean ± SEM (LD males n=12, LD females n=15; CJL males n=10, CJL females n=16). & denotes interaction effect, * $p<0.05$.

Exposure to Chronic Phase Advances Increases Anxiety-like Behaviors in Male Mice

To determine whether chronic phase advances resulted in additional behavioral changes, locomotor activity and anxiety-like behavior was assessed using the open-field test. There was a main effect of light conditions on rearing across the first five minutes of open-field ($F(1, 27) = 6.187$; $p=0.0165$). Males exposed to chronic phase advances had significantly elevated number of rears relative to mice housed in dark night conditions on post-hoc analyses ($p=0.0198$), and there were no observable differences between females across light conditions ($p>0.05$). Central tendency and total locomotor activity (determined by the number of beam breaks) was not significantly different across sex or light condition ($p>0.05$).

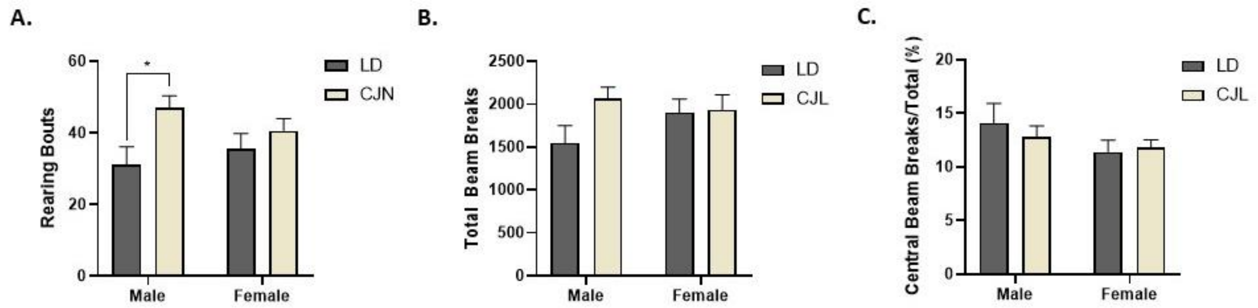

Figure 5.4. Exposure to Chronic Phase Advances Increases Anxiety-like Behavior in Male Mice. (a) Male mice exposed to chronic phase advances increase the number of rearing bouts during the first five minutes of open field activity as an indicator of increased anxiety-like behavior compared to males housed in dark conditions ($p<0.05$). (b and c) there were no significant differences in central tendency or total beam breaks representative of locomotor activity between light condition or sex. Data are represented as mean ± SEM (LD males n=12, LD females n=15; CJL males n=10, CJL females n=16).

Chronic Phase Advances Alters Vascular Tortuosity in the Isocortex

To determine whether increased cognitive impairments in aged mice exposed to chronic phase advances were the result of changes to vasculature in the brain, network volume, vascular density, and surface area of cerebrovascular casts were assessed in the isocortex and hippocampus. There were no statistical differences between lighting conditions ($p>0.05$) in either brain regions, however, we next determined mean characteristics of individual vessel segments within each region to determine whether components of vessels comprising vasculature were changed. Exposure to chronic phase advances increased mean tortuosity in the isocortex (F (3, 7)

= 3.376; p=0.0434) compared to aged mice housed under dark night conditions. No other aspects of segment characteristics were statistically different between lighting conditions (p>0.05).

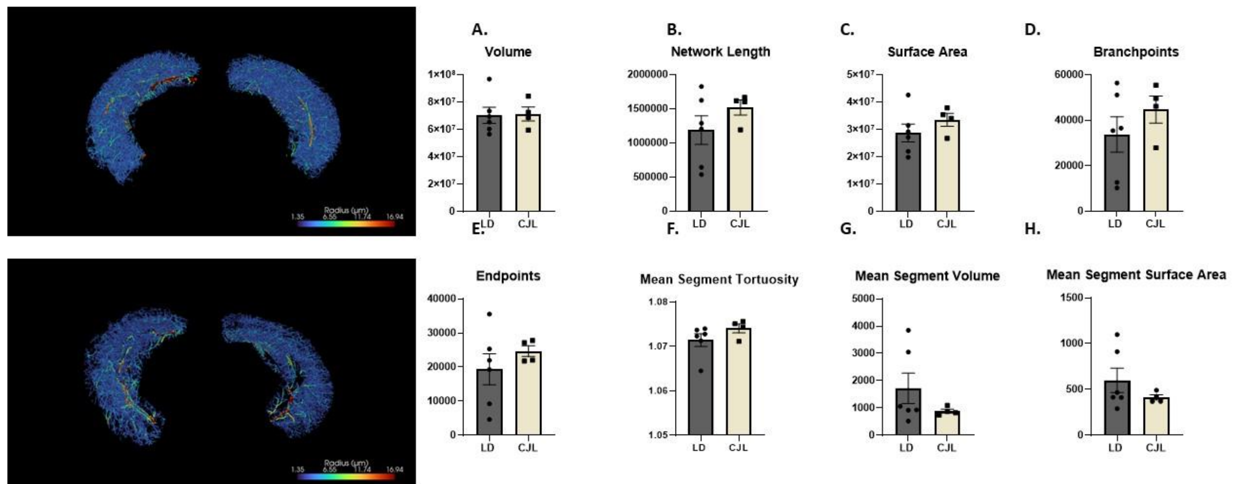

Figure 5.6. Vasculature in the Hippocampus Does Not Change After Chronic Phase Advances Diagram visualizing vasculature in the hippocampus using VesselVio (a-h) Aged mice maintained under dark night conditions or exposed to chronic phase advances do not display significant differences in vasculature or segment characteristics (p>0.05). Data are represented as Mean ± SEM (LD n= 6, CJL n=4) * p<0.05.

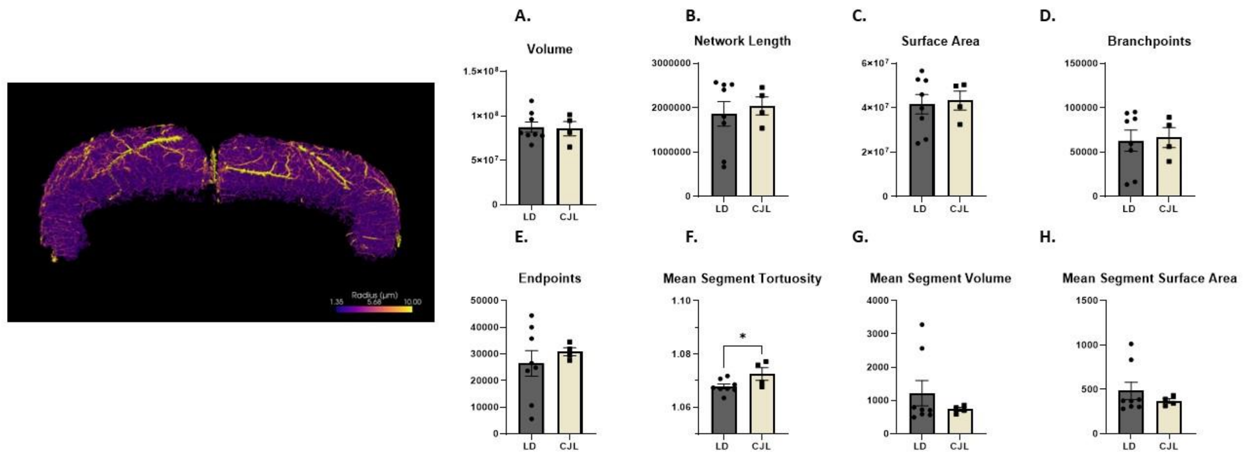

Figure 5.6. Chronic Phase Advances Increases Tortuosity of Vessels in the Isocortex. Diagram visualizing vasculature in the isocortex using VesselVio (a-f) Aged mice maintained under dark night conditions or exposed to chronic phase advances does not alter vascular volume, surface area, branch points or network length (p>0.05), however, (f) chronic phase advances does increase vascular tortuosity compared to dark night controls (p<0.05). (g-h) no significant differences in mean segment volume or surface area (p>0.05). Data are represented as mean ± SEM (LD n= 6, CJL n=4) * p<0.05.

Discussion

Here, I first identified that chronic phase advances increased spatial working memory and toned fear associative learning tasks in aged mice. Impairments in learning and memory after exposure to circadian rhythm disruption have been well-characterized to have a detrimental effect on this aspect of cognitive function. Both acute and chronic phase advance and recovery conditions (3 h day phase advances for 6 consecutive days, followed by a 10 day recovery period), but resulted

in different effects on memory and learning impairments. Acquisition and retention on spatial learning tasks including water maze were impaired in chronically exposed mice, whereas, there were no observable differences in fear conditioning (Craig and McDonald, 2008). In another study, adult female hamsters exposed to phase advances had significantly impaired learning and memory that corresponds with reductions in neurogenesis and proliferation in the hippocampus. Further, these deficits persisted after returning to normal light dark cycles suggesting potential for long term cognitive consequences (Gibson et al., 2010). Further investigations into the mechanism underlying phase advances affecting cognition, and revealed that reductions in neurogenesis are dependent on the direction and duration of phase shifts (Kott et al., 2012). Indeed, long-term memory and circadian rhythmicity become altered and impaired with age (Kondratova and Kondarov, 2012). Therefore, studies identifying mechanisms delineating age-related changes and circadian rhythms demonstrate that hippocampal expression of *Per1* is necessary for long-term memory formation. In aged mice, *Per1,* long-term memory, and synaptic plasticity is affected by histone deacetylase (HDAC 3) repression within the hippocampus, rather than from impairments of circadian rhythms, changes to sleep, or other effects from changes in other brain regions (Kwapis et al., 2018).

This study provides evidence that disruptions to circadian rhythms differentially affects aspects of cognitive function and impairments between sexes in aged mice. These results suggest that exposure to chronic phase advances affects both hippocampal dependent spatial working memory in females and the acquisition of auditory toned-fear associative learning tasks. Contextual and auditory toned fear utilizes several brain regions, including the anterior cingulate cortex (Frankland et al., 2004), dorsal hippocampus (Chowdhury et al., 2005), and medial

prefrontal cortex (Quinn et al., 2008) that involves associative memory of an unconditioned stimulus (US, e.g., foot shock) with a conditioned stimulus (CS e.g., tone). For this conditioning task, the fear response, which is characterized by non-movement of the mouse or freezing during either tone presentation or in the presence of the contextual environment, denotes an acquisition of a learned association between the US and the cue (Fanselow, 1984). The neurocircuitry behind these tasks have partial overlap in brain regions involved in these responses. Both the hippocampus and amygdala, as well as involvement from cortical regions such as the medial prefrontal cortex and anterior cingulate cortex, are directly involved in context dependent retention (Wiltgen and Silva, 2007; Phillips and LeDoux, 1992), whereas the amygdala predominantly is involved during tone acquisition. Spontaneous alternation and spatial working memory are hippocampal dependent tasks (Sarnyai et al., 2000). From these results, females exposed to chronic phase advances may experience impairments in both the hippocampus, amygdala, and cortical regions involved in fear conditioning, whereas males exposed to chronic jet lag likely only experience impairments in the amygdala. To our knowledge, this is one of the first studies to report a sex difference in these aspects of cognitive function, learning, and memory after exposure to chronic jet lag in aged mice. These differences could be due to differences in physiological progression of cognitive aging or advanced neurodegeneration in females compared to males. In **Chapter 4**, my data support this supposition; that is, a sex difference which persists in aged mice after circadian rhythm disruption from exposure to dLAN. Females exhibited significantly accelerated aging and shortened lifespan after 24 weeks of exposure to chronic dLAN and dysregulated immune response compared to males. Further, previous reports have identified sex differences in circadian regulation of physiology and rhythmicity (Santhi et al., 2016), and these provide converging evidence that cognition and

performance impairments is greater in females after circadian disruption compared to males (Santhi et al., 2016). These differences also exist at the cellular level, where sex hormone (androgen) receptors exist within the SCN that can modulate circadian regulation and downstream photic signaling (Mong et al., 2011). Sex differences in the hypothalamic-pituitary-gonadal axis (HPG) and hypothalamic adrenal-pituitary axis (HPA) and sleep-arousal systems additionally exist (Bailey and Silver, 2014). However, future studies further investigating cellular contributions from neurodegeneration may be necessary.

Coinciding with the recent increased prevalence of dementia in the aging population, literature has well-characterized the relationship between neurodegeneration, vascular dysfunction, and cognitive decline (Raz et al., 2015). In this study, we observed that exposure to circadian disruption from chronic phase advances increases vascular tortuosity in aged populations compared to control age-matched mice housed in dark nights. Tortuosity of vasculature results from hemodynamic changes to blood flow resulting in weakening of arterial walls or twisting of vasculature that increases across age (Amemiya et al., 2011) but can be indications of systemic disease (Hughes et al., 2006) and is correlated to vascular pathologies in human studies (Li et al., 2011). Other preclinical models characterizing aging, found a significant reduction in blood velocity and blood volume in the cerebral cortex, along with increases in vascular tortuosity using super-resolution ULM imaging (Lowerison et al., 2022). Indeed, tortuosity can elevate blood flow and pressure (Hughes et al., 2006) which can weaken arterial walls and reduce axial tension over time (Han et al., 2012) increasing risk of vascular disease and injury. Further, hemodynamic changes to blood flow from tortuous coronary vessels have lower wall shear stress and residence time (Rikhtegar et al., 2012) which has been associated with aneurysm

development (Meng et al., 2014). Clinical studies evaluating patients with intracerebral artery (ICA) aneurysms, found increased ICA tortuosity (Labeyrie et al., 2017). Other vascular diseases that have been correlated with increased tortuosity of the ICA include patients after subarachnoid hemorrhage (Lowerison et al., 2022). One limitation of this experiment is that we could not discern sexes within resin casts due to limitations in the number of animals we were able to resin cast, however, future studies further investigating this, and determining whether changes changes in tortuosity coincide with alterations to cerebral blood flow would be of future interest.

Within aged populations, vascular cognitive impairments, or vascular contributions to cognitive impairment (VCID) is another disease that refers to a range of cognitive deficits and impairments that are broadly associated with cerebrovascular disease and is recently recognized as the second most common form of dementia (Dichgans and Leys, 2017). Notably, VCID encompasses a range of conditions from mild forms of impairment to dementia, and can result from any form of vascular brain injury that can result in changes to structural or functional connectivity which include neurodegeneration to clinical stroke diagnoses and risk of vascular dementia doubles every 5.3 years of age after 65 (Lobo et al., 2000). In aged patients with dementia, changes to cerebrovasculature are a common pathological finding, which includes vascular remodeling of macro and microvasculature that increases risk for blood vessel integrity disruption. Further, remodeling can result in hypoperfusion that is associated with structural changes, functional damage, and neuronal injury (Honjo et al., 2012). Capillary wall deterioration, basement membrane thickening, accumulation of erythrocytes, and pericyte degeneration can occur in dementia patients which can increase blood brain barrier permeability (Gorelick et al., 2011). Other models that disrupt circadian rhythms including exposure to dim light at night (dLAN)

alters components of vasculature (Chellappa et al., 2019) including reductions in angiogenesis and vascular permeability factors, VEGF (Aubrecht et al., 2013; Walker et al., 2020). Other aspects of vasculature disrupted by alterations in light cycles to 10:10 h, resulted in reduced hypertrophy of vascular muscle cells and thinning of vascular aortic walls in a murine model of pressure overload cardiac hypertrophy (Martino et al., 2007). In clinical studies, female flight attendants exposed to chronic jet lag from travel have increased learning and memory deficits that correspond with a reduction in volume and atrophy of the temporal lobe compared to controls (Cho et al., 2001).

Altogether, this study provides evidence supporting a convergence between aging, changes to structural components of vasculature, and cognitive impairments increasing risk for cognitive dementias in aged mice exposed to circadian rhythm disruption from chronic phase advances. Chronic phase advance females exhibit increased spatial working memory deficits during a spontaneous alternation task and display significantly reduced freezing behavior during the acquisition trials of a toned-fear associative learning task. Conversely, males exposed to chronic phase advances display reduced retention of toned-fear associative learning tasks 24 h after acquisition and exhibit increased anxiety-like behaviors during an open field task compared to mice housed in dark nights. These cognitive deficits coincide with increased vascular tortuosity in the isocortex, which is associated with increased risk of vascular diseases and changes to cerebral blood flow. In conclusion, these results demonstrate that exposure to chronic phase advances results in sex-dependent differences, by increasing cognitive aging and impairments through altering spatial recognition memory, and toned fear associative learning in aged mice.

CHAPTER 6

CONCLUSIONS

Conclusions

Exposure to light at night (LAN) and the prevalence of shift work and nighttime lighting results in changes to circadian molecular clocks, desynchronizing internal physiology from the external environment. LAN has deleterious effects on the circadian system and is associated with adverse health outcomes. The goal of this dissertation was to determine the physiological consequences and influences of exposure to disrupted circadian rhythms in injury progression after ischemia, and in physiological and cognitive aging, testing the central hypothesis that exposure to light at night changes immune physiology and parameters that adversely affects injury progression in a murine model of stroke, physiology, and cognitive aging. In this dissertation, I demonstrate that exposure to dim white light at night (5 lux), has significant consequences to physiology, and in particular, immune function, in aged populations, and affecting disease states, including a murine model of stroke.

Summary

In **Chapter 2** I demonstrate that dLAN following cerebral ischemia alters ischemic pathophysiology, in turn increasing neuronal damage, functional disability, and mortality in a murine model of stroke. Transient and permanent models of ischemic injury were investigated to delineate whether the effect of nighttime lighting was limited to perfusion injury, and after one night of dLAN increased infarct sizes and functional deficits independent reperfusion and infarct size was exacerbated in both males and females. Relative to dark night conditions, dLAN

increased concentrations of TNF-α in serum and bulk-RNA sequencing identified transcriptome changes to genes involved in immune response, therefore, cell populations that are important in initiating inflammatory response and activating/recruiting immune cell populations to the brain were characterized. The percentage of microglia shifts to a pro-inflammatory phenotype (MHC II+ and IL-6) after exposure to dLAN causing a fundamental shift in microglia to a detrimental state at an early and crucial time point after stroke, resulting in irreversible neuronal death. Depletion of microglia in the CNS prior to stroke, normalizes the effect of lighting condition on infarct size and serum TNF-α. This chapter identified a biological mechanism where microglia as a resident immune cell in the brain, important in initiating inflammatory response after neuronal injury, shift to a pro-inflammatory phenotype after dLAN that consequently alters neuro-immune interactions and CNS pathophysiology, increasing permanent neuronal damage and neurological deficits.

Next, after demonstrating that nighttime lighting increased neuronal injury after ischemic stroke, in **Chapter 3** I examined the role of alternative spectra lighting that differentially activates intrinsically photosensitive-retinal ganglion cells to identify whether eliminating harmful components within white LED lighting during the evening, e.g., blue wavelengths, could ameliorate the effect of nighttime lighting on ischemic outcome. Exposure to a single night of blue-filtered dLAN and red LAN normalized infarct sizes and sensorimotor deficits comparable to dark night conditions. DLAN increased infarct size and increased relative gene expression of *TNF-α* in the cortex and caudate nucleus compared to dark night conditions and alternative spectra. Pro-inflammatory cytokines in serum were measured to assess peripheral response, and TNF-α concentrations in serum were significantly elevated in mice housed in dLAN compared to

other lighting treatment conditions. These results indicate that shifting the wavelengths of lighting exposure during nighttime to those that ipRCG's are not sensitive to, can negate the effects of dim white LED light at night by normalizing pro-inflammatory expression in regions of the brain undergoing neuronal repair mechanisms and in serum.

In **Chapter 4** I characterized the role of chronic exposure to dim white LED light in aged murine populations to investigate how physiology and immune function changes and contributes to aging. 20 month old C57Bl/6 mice were exposed to dark night conditions or 5 lux of dLAN for 24 weeks. During chronic dLAN exposure, delayed-type hypersensitivity tests reveal that females exposed to a chemical antigenic challenge displayed dysregulated cell-mediated inflammatory immune response and had significantly shorter lifespans compared to females housed in dark night conditions, suggesting that dLAN accelerates aging in a sex-specific manner. Males displayed dysregulated body mass across 24 weeks and increased adrenal mass as a percentage of body mass after immune challenge, but dLAN did not alter cell-mediated immune response or lifespan in males. This study indicated that dLAN differently affected physiology across sexes; females may be more susceptible to physiological changes and dysregulated immune response that can contribute to longevity.

Because I identified that exposure to dLAN altered physiology, immune response, and survival in aged murine populations, in **Chapter 5** I examined another model of disrupted circadian rhythms to investigate whether chronic phase advances additionally contribute to other aspects of aging including cognitive function that could result in increased cognitive impairments. This experiment proposed that in addition to circadian disruption affecting ischemic outcome, that this

may increase susceptibility to cognitive aging and deficits leading to impairment and dementia. Spatial working memory was assessed in 20 month old aged mice after exposure to either dark night conditions or 6 hour phase advances for 8 consecutive weeks; females displayed significantly impaired spatial recognition memory in a spontaneous alternation task compared to females housed in dark night conditions. Additionally, females exposed to phase advances displayed reduced acquisition during an associative fear-toned learning task, whereas, males exhibited reduced freezing during retention retrieval tasks 24 h after acquisition testing. Anxiety-like behaviors were increased in males exposed to phase advances identified by increased rearing over the first five minutes during open field, indicating that circadian disruption through phase advances increased neuropsychiatric-mood related disorders that often coincide with aged populations with cognitive impairments and dementia (Ma, 2020). Cerebrovasculature was assessed through corrosion casts to evaluate vascular characteristics and aspects of vessels at the individual segment level to identify whether vascular changes could contribute to cognitive impairments in this subset of aged mice. There were no components of vascular density or volume that were altered with phase advances. However, chronic jet lag increased mean segment tortuosity in the isocortex which indicates twisting or weakening of vascular walls that can change hemodynamic blood flow and has been correlated to risk of vascular disease (Labeyrie et al., 2017; Lowerison et al., 2022).

Limitations and Future Directions

In this dissertation, I characterized and identified the role of disruptions to circadian rhythms via exposure to light at night on one glial cell population, and a biological mechanism contributing

to exacerbating stroke outcome. However, other cellular sources may contribute to an increase in pro-inflammatory cytokine response, including other peripheral infiltrating immune cells, reactive astrocytes, or collective neurovascular units in the brain. Indeed, exposure to light at night independent of neuronal injury and in otherwise healthy mice have significantly upregulated pro-inflammatory cytokine production in the CNS (Walker et al., 2020; Bedrosian et al., 2013), and the mechanism driving neuroinflammation after exposure to nighttime lighting remains unspecified. In Chapter 2, microglia-depleted mice exposed to dLAN after ischemia displayed normalized TNF-α concentrations relative to mice housed in dark night conditions which may suggest microglial crosstalk between other cellular sources to be a potential driving mechanism of this biological phenomenon which would require future investigation. Additionally in this chapter, we observed that exposure to dLAN in microglia-depleted mice significantly elevated corticosterone concentrations which was not an expected result. Microglial activation has been demonstrated in literature to be regulated through HPA axis and sympathetic nervous system response; activated from noradrenaline and inhibited by glucocorticoids (Sugama and Kakinuma, 2020). The interaction between circadian rhythm disruption, microglia, and the HPA axis, requires further characterization and evaluating exposure to nighttime light collecting corticosterone values across time of day could be of future interest.

Another limitation that requires future investigation reported in Chapters 2 and 3, is that experimental timelines were limited to the first 24 h after ischemic stroke to determine how acute exposure to LAN, and nighttime lighting of respective wavelengths affected infarct development. Indeed, the first several hours and days after ischemia are where the greatest changes to transcriptomes and injury development and progression occur (Li et al., 2020). However,

investigating the role of acute exposure to dLAN during initial infarct development and during the first 24 h requires characterization before investigating the role of circadian disruption during extended stroke recovery (e.g. 7-14 days). Microglia play a central role during the first 24 to 72 h after ischemic stroke, by inducing neuroinflammation and pathophysiological progression of infarct development (Zhang, 2019), however, the inflammatory process after ischemia is dynamic exploring and delineating inflammatory response immediately after dLAN exposure that has been observed after 24 h compared to neuroinflammation during recovery from exposure to dLAN could be of interest. Furthermore, this dissertation did not explore peripheral response that could provide further characterization of the effects of dLAN after injury.

I did not focus on the role of melatonin as a biological variable in this dissertation when investigating physiological changes associated with nighttime exposure and circadian disruption, because I sought to ascertain the effects of disruptions to circadian rhythms from suppressed melatonin after light exposure. Therefore, the species of *Mus* used in the experiments in this dissertation do not contain pineal melatonin (Goto et al., 1989). Melatonin, synthesized and released endogenously by the pineal gland is produced in the absence of lighting, peaking during the night and troughs during the light phase (Zawilska et al., 2009). This hormone has broad functions but is most well-known for stabilizing rhythms in the body (Saper et al., 2005), sleep-wake regulation (Arendt, 2000), regulating oxidative stress/reactive oxygen species production, apoptosis, mitochondrial function (Hardeland et al., 2011), and also has immunomodulatory properties that can influence immune function and response (Carrillo-Vico et al., 2013). Immunomodulation occurs bi-directionally, where melatonin administration into pineal glands can enhance aspects of immune response including promoting macrophage/microglial cellularity

and proliferation (Kaur and Ling, 1999), promoting and restoring cytokine production concentrations in immunosuppressed mice exposed to hemorrhage (Wichmann et al., 1996), but also modulates exacerbated immune response by reducing the number of circulating neutrophils, macrophages, monocytes, and microglial activation after focal ischemia (Lee et al., 2007). In the context of prolonged exposure to light in the night or nighttime lighting, melatonin concentrations are suppressed which can disrupt melatonin signaling and physiology that can result in phase shifts (Boivin et al., 1996). Administration of melatonin after ischemic stroke reduces infarct volume in Sprague Dawley rats which contain pineal melatonin (Pei et al., 2003), however, whether this reduction occurs in mice with or without intact pineal melatonin rhythms, and whether circadian disruption and nighttime lighting that results in dysregulated immune response influences this finding, remains unspecified and requires future investigation.

When interpreting the data presented in this dissertation as translatable in the clinical setting, I characterized one biological component, light at night, as a variable contributing to adverse health consequences of disruptions to circadian rhythms. Disrupted sleep is another biological consideration which can contribute to secondary progression of injury in hospital settings, however, we sought to identify the role of light at night independent of sleep. Studies investigating the role of dLAN have demonstrated that dLAN does not affect sleep architecture in nocturnal rodents (Borniger et al., 2013). Ultimately, several mechanisms contribute to ischemic injury and formation in the clinical setting, including melatonin suppression from nighttime lighting exposure from hospital settings, frequent waking after procedures resulting in sleep disturbances, and nighttime lighting, all of which likely all contributes to the detrimental health risks associated with disrupted circadian rhythms.

Implications and Clinical Applications

Circadian rhythms are modulated through external cues such as light and are important in adapting to changes in the environment and maintaining internal physiology. The results in this dissertation suggest that exposure to dim white nighttime lighting conditions can have significant implications affecting physiology and health after ischemic injury and in otherwise healthy aged populations. Proper alignment of circadian rhythms is important for optimal health, however, exposure to nighttime lighting is only becoming more ubiquitous to modern life, affecting over 80% of the world globally and 99% of the United States and European populations.

Strategies for preventing or reducing the negative consequences associated with dim white light at night can include shifting the wavelengths of lighting for night shift workers in environments that expose workers or patients who require medical intervention. Although studies in this dissertation have characterized the harmful effects of nighttime lighting, light conditions are critical, necessary, and often unavoidable in shift-working populations. Adequate lighting sources are essential for visual tasks to reduce errors, and light is important in improving alertness, cognition, and performance (Smith et al., 1998; Kretschmer et al., 2012). Literature has well-characterized the role of red wavelength lighting in minimally disrupting circadian rhythms in preclinical and clinical settings (Satlin et al., 1992). However, red light during the night setting can impose challenges to night shift work staff by obscuring visual tasks or procedures, including those requiring fine color discrimination. In Chapter 3 I established that eliminating blue-wavelengths in white LED lighting prevents the increased infarct sizes, functional deficits,

and poor outcomes associated with dLAN, resulting in comparable injuries to dark nights or red LAN. Eliminating blue-wavelengths within white LED light creates the appearance of "orange" lighting that can potentially improve color perception without adverse consequences associated with more full-spectrum white lighting. However, future investigation evaluating lighting intensities necessary to not profoundly disrupt circadian rhythms while maintaining visual acuity could be of interest. Patients resting with their eyes closed can also be affected by lighting intrusions through the eyelids (Robinson et al., 1991). Therefore, reducing light exposure directly through eye masks or with goggles that filter out blue-wavelength light can also be a viable alternative source which requires future examination. Lastly, other studies have demonstrated that the acute effects of dLAN can be reversible in some instances, pertaining to mood disorders associated with disruptions to circadian rhythms (Bedrosian et al., 2013). A caveat to this statement is that reversing exposure to artificial light may not be applicable to secondary damage after injury that likely is permanent. Nevertheless, adhering to consistent schedules and reducing unnecessary sources of illumination, e.g., blackout curtains to reduce street lighting, reducing prolonged exposure to smartphone or computer devices late into the night, or avoiding leaving the TV illuminated in the bedroom while sleeping, can all improve health outcomes.