# LIGHT, OREXIN, AND SAD: A MECHANISTIC STUDY USING A DIURNAL RODENT MODEL

By

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## ABSTRACT

Daytime light deficiency can modulate behavior, affective state, and cognition [1-5]. This is best illustrated by Seasonal Affective Disorder (SAD), a major depressive disorder with symptoms occurring in the fall and winter and remission in the spring and summer [1]. In order to study the neural mechanisms underlying the effects of light on behavior and cognition, our lab utilizes the diurnal Nile grass rat (*Arvicanthis niloticus*), as they provide an endogenously relevant and more translatable model to diurnal humans compared to nocturnal mice or rats. Grass rats housed in dim light during the day (dimLD, 50 lux) have shown increased anhedonia, behavioral despair, and impaired spatial learning/memory compared to those housed in bright light during the day (brLD, 1000 lux), as well as attenuation of the wakefulness-promoting and anti-inflammatory orexinergic system [6-11]. My previous work has also found that neuroinflammatory response is affected by seasonal lighting conditions; winter-like dimLD prompted sex- and corticolimbic brain region-specific activation of microglia and pro-inflammatory cytokine expression, compared to animals housed in summer-like brLD [12]. The behavioral and neural consequences of daytime light deficiency have been established, although the neural mechanism underlying and linking these effects remains unclear.

These dissertation experiments test the central hypothesis that the hypothalamic orexin system mediates seasonal lighting-induced changes in behavior and other neural responses relevant to SAD. We hypothesized that 1) orexin can be upregulated by bright light (aim 1), and 2) enhanced orexin would be sufficient to alleviate associated deficits in sleep and affective state, and buffer dimLD induced deficits in neuroplasticity and inflammatory response (aim 2).

The first aim establishes how orexin responds to light treatment using an early-morning bright light therapy (BLT) paradigm. The BLT group showed a higher level of wakefulness

during light treatment, better sleep quality at night, and improved entrainment of daily rhythms compared to the control group. The impact of BLT on the orexin system was sex- and brain region-specific with males showing higher OX1R and OX2R in the CA1, while females showed higher *prepro-orexin* but lower OX1R and OX2R in the BLA, compared their the same-sex controls. The neuroinflammatory and neuroplasticity markers also responded to BLT in a sex- and brain region-specific manner. BLT reduced TNF-α in the BLA of females, and upregulated CD11b in the mPFC and IL6 in the BLA in males. As for neuroplasticity markers, BLT upregulated BDNF in the BLA and CA1 in males, but downregulated BDNF in the CA1 and TrkB in all three brain regions in females.

The second aim directly tests the modulatory role of orexin on sleep/arousal, anhedonia, neuroplasticity, and neuroinflammatory response, through intracerebroventricular (ICV) infusion of orexin-A (OXA). OXA infusion promoted wakefulness in females during daytime, and improved sleep quality at night in males, and reduced anhedonia in both males and females. OXA treatment increased anti-inflammatory cytokines IL-4 and IL-10 in the mPFC of females, and in the CA1 of both sexes. Although no significant changes were found in neuroplasticity or pro-inflammatory markers, microglia proliferation and activated microglia phenotypes were decreased across brain regions in both sexes in the OXA group compared to the dimLD controls, while astrocyte number was decreased in the mPFC of females and BLA of males. Collectively, these findings suggest that orexin alleviates sleep disturbances and anhedonia, and reduces neural inflammation.

The findings of this study provide further understanding of the orexinergic system as a therapeutic target in affective disorders, as well as the mechanisms through which light influences the brain and behavior.

This dissertation is dedicated to my sisters, Katie and Erin. Your endless encouragement and belief in my abilities have pushed me through this journey, and for that, I will always be grateful.

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## **CHAPTER 1:** Introduction

Light can have a profound impact on mental health through its influence on mood, cognitive function, and behavior across species. One of the most dramatic examples of the effects of light on affective behavior, or rather lack of light, is seasonal affective disorder (SAD). SAD is a seasonally recurring major depressive disorder, with depressive symptoms occurring in the fall and winter and alleviating in the spring and summer [1]. According to the American Psychiatric Association approximately 10 million Americans experience SAD, with patients reporting symptoms lasting as long as 40% of the year [13]. In addition to anhedonia, depressed mood, and anxiety, patients can experience sleep disturbances including poor sleep quality at night and excessive sleepiness during the day [14, 15]. SAD patients also experience impaired cognitive function, with deficits in both working and spatial memory during the winter months [14, 15].

Theories on the etiology of SAD range in the importance of the role that circadian system plays. One of the prevailing explanations, the phase-shifting hypothesis, posits that SAD depressive episodes are caused by a phase delay, misaligning one's circadian rhythm and habitual sleep time [16]. While circadian misalignment is certainly a major component, as evidenced by the successful use of bright light therapy (BLT) in the treatment of SAD to induce a phase advance, the phase shift hypothesis does not consider neurotransmitter dysfunction or other biological mechanisms [17-23]. For example, heightened pro-inflammatory response has been implicated in SAD, possibly contributing to fatigue symptoms, sickness behaviors, and cognitive deficits [24]. PET and MRI scans have suggested that patients with SAD have deficits in serotonin transporter protein function, while other studies show overproduction of or issues in rhythmicity of melatonin [25-27]. It is unclear if these neural components of the disorder are

driving its development and behavioral presentation, or are rather downstream effects of a larger unknown network.

Instead, seasonality could be viewed as a dimensional factor; a dual-vulnerability hypothesis suggests that SAD could result from interactions of separate circadian and depression factors, each with distinct pathophysiological mechanisms [28, 29]. It is generally agreed that the initial causal factor in SAD is daytime light deficiency, as indicated by the therapeutic effects of bright light therapy. While decreased day-length can reliably invoke depressive behavior in both human and animal models, most modern humans are surrounded by artificial lights that prevent them from experiencing the full effects of severe changes in photoperiod [30-35]. People tend to spend the vast majority of their time indoors, making changes in light *intensity* a more relevant issue, particularly during seasonal changes. In a longitudinal study exploring seasonal daylength vs light intensity, healthy individuals did not experience significant differences in the total time spent exposed to light during the summer and winter [36]. They did however experience significant differences in exposure to bright light (1000 lux), averaging 2.6 hours during the summer and 0.4 hours during the winter [36]. Daytime light deficiency driving SAD is further supported by the use of bright light therapy for successful and lasting treatment, regardless of seasonal changes such as photoperiod, temperature changes, and physical activity changes [37-47]. Bright light is effective as a therapeutic tool, as it is one of the most salient cues for resetting circadian rhythm [16, 48, 49]. While the behavioral outcomes of SAD and light therapy are well known, the neural mechanisms underlying its pathophysiology and treatment are yet to be fully understood, due in part to lack of a reliable animal model to study these effects.

**An animal model of SAD**

To explore the neural mechanisms underlying light-dependent responses, our lab utilizes a diurnal rodent, the Nile grass rat (*Arvicanthis niloticus*). Grass rats are a valuable model because, unlike commonly used nocturnal laboratory rodents, grass rats show daily behavioral and physiological rhythms and responses to light in a similar manner to diurnal humans [50]. This relevancy and translational ability are critical when studying neuroprotective therapies related to arousal and sleep, as the mismatch between active and inactive phases in nocturnal vs diurnal models could result in clinical inefficacy [51]. Similar to humans with SAD, diurnal rodents show depressed-like behaviors when exposed to shorter daylengths [52-54] and dim light intensity (dimLD, ~50 lux) mimicking winter-like light conditions [6, 8-10, 55, 56].

DimLD prompts behavioral despair; in the forced swim test, grass rats housed in dimLD had longer periods of immobility and exhibited less escaping/climbing attempts [54]. Similarly, grass rats in winter-like light intensity have increased anhedonia, as demonstrated through a much lower sweet solution preference (SSP) compared to those housed in summer-like bright light (brLD, ~1000 lux) [54]. In the open field test, dimLD animals had fewer center entries and spent significantly less time at the center of the testing apparatus [8]. Consistent with this anxiety-like behavior, animals in dimLD also exhibit more marble burying compared to animals in brLD [8]. In the hippocampal-dependent Morris water maze, dimLD animals were slower to locate the platform, suggesting impairments in retention of spatial memory [9]. In addition to cognitive deficits, male grass rats housed in dim light show deficits in sociosexual behavior[56]. Beyond these mood and cognitive effects, the grass rats also show sensitivity in their locomotor activity and entrainment of daily rhythms in response to different daytime light spectrums [54, 57].

**Effects of daytime light deficiency on diurnal grass rats: *Circadian rhythm and wakefulness/sleep***

Organisms maintain homeostasis through internal rhythms, such as circadian rhythms, which have evolved to synchronize physiological and behavioral patterns with environmental changes [50, 58]. Circadian rhythms, typically following a 24-hour cycle, are endogenous but rely on external cues, known as zeitgebers, to align with the environment [59, 60]. Light acts as a primary zeitgeber, influencing daily behavior through both acute effects on activity and circadian entrainment [59-61]. The former effect, masking, involves an immediate response to a stimulus that can reinforce or temporarily override the influence of the circadian system [62-65]. Light prompting arousal in diurnal species and rest in nocturnal are examples of positive and negative masking, respectively [63, 66]. Circadian rhythms synchronize to a daily light/dark (LD) cycle through photic entrainment, which is thought to operate via two mechanisms: non-parametric entrainment, involving discrete light pulses, and parametric, involving continuous light exposure [61]. Photic cues are able to induce a shift in endogenous activity, resulting in a phase shift depending on the timing of exposure [67]. Exposure to light in the early subjective night (start of the endogenous inactive phase) causes a phase delay, with activity starting at a later hour, while exposure to light in the late subjective night (end of the endogenous inactive phase) causes a phase advance, with activity starting at an earlier hour [67].

The suprachiasmatic nucleus (SCN) of the hypothalamus serves as the master circadian clock, maintaining rhythmicity even when isolated from the body [68, 69]. Retinal photoreceptors transmit light signals via the retinohypothalamic tract to the SCN, aiding in regulation of hormone secretion, sleep, behavior, and other homeostatic mechanisms [70, 71]. The SCN synchronizes peripheral tissue clocks and extra-SCN oscillators in the brain through

clock genes such as PER1 and PER2, that drive molecular oscillations [72-74]. While individual cells can maintain independent rhythms, the SCN orchestrates these patterns for coordinated circadian functioning [74]. SCN activity and response to light is generally conserved between diurnal and nocturnal species, though the downstream functions differ [75-77]. Extra-SCN oscillators in downstream pathways from the SCN exhibit rhythms that are based on the animal's chronotype, such as light promoting activity or sleep in diurnal or nocturnal species, respectively [66, 76, 78].

Grass rats, similar to humans, have fundamentally diurnal circadian rhythms [79, 80]. This is reflected in their locomotor activity, as measured through wheel-running; grass rats are most active throughout the light period [80]. This is consistent with their sleep bouts, a measure of sleep consolidation, which are longest at night and shortest during the day [80]. Light pulses in the early night induce a phase delay, while light pulses at the end of the night or early morning induce a phase advance [80]. In the brain, SCN expression of clock genes PER1 and PER2 show peak expression at zeitgeber time (ZT) 10, in the opposite phase of nocturnal rodents [81]. When the SCN was lesioned, circadian rhythmicity and circadian regulated masking was disrupted, but light-induced masking response was not affected [82, 83]. Acute responses to light were still intact.

Manipulations in the intensity and type of daytime light can also affect sleep/wakefulness in the grass rats. Previous work demonstrated that grass rats housed in dimLD have greater variability in the timing of activity offset compared to those housed in brLD, although there were no significant differences other circadian parameters such as day/night activity ratio, total daily activity, or entrainment phase angle [55]. Additionally, there were no differences in PER2 protein product in the SCN between dimLD and brLD animals. This suggests that other mechanisms

besides circadian rhythm disruption are involved in behavioral responses to dimLD. In a more recent study, grass rats housed in bright daylight spectrum, which stimulates all photoreceptors, prompted activity onsets closer to lights-on compared to low-irradiance fluorescent light [57]. However, narrowband 480nm spectrum light, which maximizes melanopsin and minimizes S-cone stimulation, prompted activity offsets closer to lights-off compared to low-irradiance fluorescent light [57]. In addition to the type of daytime light, the timing of the light during the day has different effects on activity. Rather than full day-length exposure, pulses of the daylight spectrum and narrowband 480nm, as well as both combined with narrowband 365nm light, resulted in increased wakefulness and locomotor activity compared to the low-irradiance fluorescent light [57]. Combined, the cognitive and behavioral findings validate the grass rats as a reliable diurnal model of behavior in affective, homeostatic, and circadian responses to light, and present the opportunity to study neural responses.

**Neural effects of daytime light deficiency on diurnal grass rats: *Neuroplasticity***

Neuroplasticity is the ability of neurons to adapt, both morphologically and functionally, in response to internal and external stimuli[84]. This plasticity includes neurogenesis, synaptogenesis, and development of dendritic spines and branches, and is essential for learning, memory, and stress adaptation [84-86]. Disruptions in neuroplasticity are implicated in diseases and psychiatric conditions such as dementia and depression [87, 88]. Patients with major depressive disorder (MDD) often show decreased neuroplasticity in the prefrontal cortex (PFC) and hippocampus, as well as decreased neurotrophic factors [89, 90].

Synaptic plasticity could be a potential mediator through which seasonal light conditions influence affective state. Brain-derived neurotrophic factor (BDNF), a regulator of synaptic plasticity, neurogenesis, neuronal differentiation, and axonal growth and activity, is reduced in

hippocampal subregion CA1 of male grass rats housed in dimLD compared to brLD [9].This was reflected in immunoreactivity as well as mRNA and protein expression [9]. DimLD housing also prompts a reduction of CA1 apical dendritic spine density in both males and females [9, 10]. Morphological analyses of these spines revealed that there was a lower density of both mushroom and stubby spines in dimLD, suggesting that there were less mature synapses [9, 10]. However, both BDNF and dendritic spine density were rescued when animals were transferred from dimLD into brLD [9]. Both BDNF and neuroplasticity marker TrkB have been implicated in psychiatric disorders and offer potential therapeutic targets [91-94]. In grass rats, daytime light intensity modulates aspects of synaptic plasticity in a sex-specific manner [9-11]. While males and females show differing neuroplastic responses to lighting intensity, they show similar behavioral outcomes in affective state, sleep, and arousal [9-11, 95]. DimLD prompts sex- and brain-region specific responses not only in neuroplasticity, but also neuroinflammation.

**Neural effects of daytime light deficiency on diurnal grass rats: *Neuroinflammation***

Neuroinflammation encompasses the central nervous system's response to challenges to health and homeostasis. It involves a balance between pro-inflammatory response, focused on proliferation of apoptosis- and necrosis-inducing factors to clear threats to the neural environment, and anti-inflammatory response, focused on facilitating healing and repair. In a healthy system these responses are complementary, although exposure to chronic threat, both physical and psychological, often results in a cytotoxic cycle of prolonged pro-inflammatory response [96-99]. This heightened and chronic pro-inflammatory response is implicated in many psychiatric and neurodegenerative disorders, including depression and dementia [97, 98, 100-103].

The main modulatory cells involved in neuroinflammation are microglia and astrocytes. While microglia and astrocytes are typically associated with both pro- and anti-inflammation, respectively, they play a role in both types of responses. Following initial detection of a homeostatic challenge, activated microglia will begin to trigger reactive astrocytes. Astrocytes in the absence of microglia are less responsive during pro-inflammation and are thought to be involved in the initial support and amplification of this state rather than its induction [104, 105]. Both microglia and astrocytes undergo dynamic morphological changes directly related to their function, with phenotypes indicating neurotoxic or neuroprotective roles [96, 106-113]. These changes in branching complexity, process length, and soma size allow for direct contact with the neural environment and stimulate the release of chemokines and cytokines [114, 115].

The consequences of sustained pro-inflammatory cytokine release are reflected both psychologically and physically. Manipulation of immune modulators, particularly pro-inflammatory cytokine TNF-α, in both human and animal studies have been shown to induce psychiatric symptoms, sickness behavior, and sleep disruptions [116-124]. Inflammatory-induced deficits in cognition, affective state, and behavior contribute to the pathophysiology of depressive disorders, and are often associated with symptom severity [125-127].

Heightened pro-inflammatory response has been implicated in seasonal affective disorder, possibly contributing to fatigue symptoms, sickness behaviors, and cognitive deficits [24]. Patients with SAD are reported to have a higher resting pro-inflammatory profile [1, 24, 128]. They have been shown to have significantly higher levels of IL-6 and soluble IL-6 cytokine receptors compared to normal controls [24] [43]. Both pro- and anti-inflammatory cytokines exhibit seasonal patterns and are sensitive to environmental factors such as light [7-10]. Baseline expression of pro-inflammatory cytokine response is highest in the winter in humans,

particularly with TNF-α almost doubling in the winter and returning to lower levels in the spring [7, 8]. This is also observed in rodents, birds, and marsupials, compared to other seasons [7-9, 46, 47].

Daytime light deficiency has been demonstrated to prompt sex- and brain-region specific pro-inflammatory responses in grass rats compared to those housed in summer-like bright light [12]. This was reflected in microglia proliferation and morphology, with animals in dimLD exhibiting a hyper-ramified phenotype associated with chronic stress in dorsal hippocampal subregion CA1 and the basolateral amygdala (BLA) [12, 107, 129]. Expression of pro-inflammatory cytokines TNF-α and IL-6 were upregulated in the CA1 of females and BLA of males, however they were downregulated within the BLA of females [12]. While region-specific pro-inflammatory expression could be functionally beneficial, the observed sex differences in light-induced neuroinflammatory state could play a role in sex differences in the etiology of depression[130-134]. Light is an important factor in disease state and inflammation, though the exact mechanism through which light regulates neuroinflammatory response is unknown.

An emerging area of interest is in how BLT affects neuroinflammation. It was recently reported that BLT resulted in a reduction of peripheral pro-inflammatory cytokines TNF-α and IL-6 in patients with traumatic brain injury (TBI) [43]. This decrease in circulating pro-inflammatory cytokines suggests that BLT could influence pathways related to immune response, potentially interacting with neural systems affected by light intensity such as the orexinergic system.

**Neural effects of daytime light deficiency on diurnal grass rats: *Orexin***

A possible mechanism for the effects of daytime light deficiency on cognition and affective behavior observed in the grass rats could be through the light-sensitive and wake-

promoting orexin pathway. Orexin, or hypocretin, is a neuropeptide that plays a modulatory role in homeostasis, metabolism, stress adaptive responses, reward, and sleep/wakefulness [135-137]. It is produced in the lateral hypothalamus (LH) through proteolytic cleavage from the precursor protein *prepro-orexin* (PPO), and the axons from these orexin-containing cell bodies project throughout the central nervous system and the periphery [136, 138-140]. In the brain, these projections target regions involved in the sleep/wake cycle, reward, feeding, and the neuroendocrine system, as well as hindbrain and spinal regions involved in motor control. These pathways are modulated by ligands Orexin A (OXA) and Orexin B (OXB). OXA binds with affinity to G protein-coupled receptors OX1R and OX2R, while OXB binds with higher affinity to OX2R [136, 139]. OX1R and OX2R are found throughout the brain, and particularly in regions associated with stabilization of wakefulness and attention, as well as areas involved in affective state such as the PFC, hippocampus, and amygdala [136, 138-141] [142].

The central orexin system in grass rats is similar to that of humans, with secretion positively correlated to daylight exposure and daylength [143-147]. Bright daylight is associated with higher cerebrospinal fluid (CSF) OXA content in both humans and grass rats [143, 145]. Daily fluctuations in orexin are marked by a sharp increase in both CSF OXA and OXB with early morning light in diurnal non-human primates, with a gradual increase throughout the day before peaking and returning to minimum levels during the early dark period [148]. Fos-ir in orexin neurons in grass rats is high throughout the day, and low at night, similar to other diurnal species [144, 149]. This daily pattern in response to light is conserved in dogs and other diurnal mammals [150-152], and orexin expression correlates with wake and arousal in nocturnal species as well [153].

The activity of orexin neurons follows a pattern that is consistent with arousal, with maximal firing during active wakefulness [147, 153, 154]. These neurons have properties that help promote sustained activity, and are in a naturally depolarized state near their threshold that's thought to increase spontaneous responses to stimuli [155]. Conditions that require increased attention are associated with higher levels of orexin firing, even without active movement [153]. OXA expression also increases in response to positive emotions and social interactions, highlighting its role in motivated behavior and reward [156-159]. These neurons fire less during passive waking behaviors, such as grooming, and are silent during the inactive phase with the exception of REM sleep [147, 153]. Orexin expression occurs in a cyclical manner, for maintenance of wakefulness during the active phase and allowance of sleep during the inactive phase.

Orexin expression is influenced by both circadian and noncircadian drives[160]. Rats with lesioned SCNs show a weak expression curve of orexin when housed in their normal light/dark cycle, and further lose their pattern of expression when housed in constant light or constant dark [161]. Orexin is not wholly SCN mediated though, as the orexin neuronal population in the LH receives direct input from the retina in addition to input from the SCN [69, 162-164]. Grass rats show increased cFos expression in orexin neurons in the dorsal raphe (DR), but not the SCN, in response to light at ZT3 [165]. OXA cFos was also increased when grass rats were running on running wheels regardless of time of day, suggesting that spontaneous and rapid activation of orexin neurons can override circadian-driven daily expression [149]. Further noncircadian mechanisms of orexin are demonstrated by studies of sleep deprivation in non-human primates. CSF OXA levels rose in a linear fashion to wakefulness, but increasing the light phase by 5 hours further upregulated OXA well into the animals' typical sleeping period [152].

The orexinergic system rapidly adapted to the light extension and increased locomotion, and OXA was positively correlated with stress-induced activation of cortisol [152].

Dysregulation of orexinergic signaling is implicated in depressive disorders, resulting in deficits in cognition, affective state, and sleep in both humans and rodents [6, 135, 136, 166-168]. Circulating CSF levels of OXA in grass rats housed in dimLD are significantly less than that of animals housed in brLD [6]. This is reflected in the brain, with dimLD conditions leading to attenuated orexin in the LH and altered OX1R and OX2R throughout corticolimbic brain regions [6, 145]. Attenuation of orexin has also been demonstrated to underlie behavioral deficits associated with seasonal lighting conditions [6, 7]. OX1R antagonists in brLD conditions result in behavioral despair and anxiety-like behavior typically observed in dimLD animals [6]. This could be due in part to the loss of the neuroprotective properties of orexin on cognition, monoaminergic systems, and neurotrophic factors [137, 169, 170].

Orexin is also classically anti-inflammatory, and actively suppresses pro-inflammatory-mediated apoptosis [171-173]. OXA has been demonstrated to have a baseline buffering effect against pro-inflammatory cytokines both in vivo and in vitro, through attenuation of microglial production of TNF-α and IL-6 [172, 174]. In humans, preliminary trials suggest that intranasal administration of orexin dampens pro-inflammatory response in cardiac patients [175]. Orexin-knockout models are associated with increased expression of pro-inflammatory cytokines and activated microglia [172, 174]. Not only does orexin act to downregulate pro-inflammatory cytokines, but it also acts to upregulate production of anti-inflammatory cytokines [176-178]. Microglia have demonstrated the capacity to express orexin receptors, with an affinity for OX1R in mouse models of TBI [179]. Other inflammatory cells, such as astrocytes and oligodendrocytes, have also been found to express OX1R in both models of TBI and global

ischemia [180]. In vivo, exogenous administration of OXA following ischemic brain injury increased cortical microglial OX1R expression, and buffered TNF- α [172]. Similarly, pretreating isolated murine microglia cell lines in vitro with OXA decreased expression of pro-inflammatory cytokines following inflammatory threat from palmitic acid exposure [174]. This effect endured when the microglia were introduced to lateral hypothalamic neurons; there was increased neuronal survival with the presence of microglia that had been pretreated with OXA[174]. Orexin is able to affect neuroinflammation directly, through interactions with microglia, astrocytes, and cytokines, as well as indirectly through promotion of synaptic plasticity, neurotrophic factors, and other neuroprotective factors [140, 171, 181-184]. As such, it could be a key neuromodulatory factor in the effects of daytime light deficiency.

**Hypothesis and working model**

These dissertation experiments investigate the hypothesis that orexin modulates the effects of light on behavior, and neuroplastic and neuroinflammatory responses relevant to SAD. We aimed to first naturally upregulate the orexinergic system through early-morning BLT exposure, and then directly upregulate it in the absence of bright light through intracerebroventricular (ICV) administration of OXA. Orexin in this paradigm, whether enhanced by photic cues or directly administered, prevents dimLD driven attenuation of orexin throughout corticolimbic brain regions, and thus buffers downstream consequences (Fig. 1).

When light enters the retina, it is processed for both image-forming and non-image-forming effects. These non-image-forming effects tell us sensory information beyond a representation of the spatial environment, such providing a measure of environmental brightness for circadian response [185]. This light information is mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs), and travels through the retinohypothalamic tract to reach the

SCN and LH (Fig. 1) [186-189]. The SCN, the body's master pacemaker and light-entrainable oscillator, also sends input to the LH and its localization of orexinergic neurons [69, 162-164]. Summer-like bright light activates orexinergic neurons, while winter-like dim light attenuates their response[165]. In this framework, orexin is upregulated through either BLT or direct OXA infusion. Orexin then signals through its ascending and descending fibers, creating pathways to brain regions involved in wakefulness, mood, and cognition, reward, motor control, and neuroendocrine response (Fig. 1). These dissertation experiments focus on three corticolimbic brain regions in the orexinergic pathway that are involved in cognition and affective state: the medial prefrontal cortex (mPFC), basolateral amygdala (BLA), and dorsal hippocampal region CA1.

Orexin and light intensity in grass rats have been suggested to impact BDNF in the hippocampus, CA1 dendritic spine density, and hippocampal-dependent spatial learning/memory [9-11]. OXA administration could affect neuroplasticity directly through upregulation of BDNF-TrkB, and indirectly through buffering pro-inflammatory response and interactions with other mediators of plasticity [169, 190]. Within this pathway, the presence of orexin exerts anti-inflammatory effects on the grass rats, buffering dimLD induced pro-inflammatory response. Should orexin suppress microglia activation, this will theoretically downregulate pro-inflammatory cytokine expression and upregulate anti-inflammatory cytokine expression. The presence of orexin in this scenario interrupts the neurotoxic chronic pro-inflammatory cycle, buffering consequences for synaptic plasticity and neurogenesis.

As arousal and sleep behavior are heavily modulated by orexin, experimental dimLD animals could have similar behavioral effects to that of brLD [146, 191, 192]. This model provides both an early morning photic and non-photic circadian cue for entrainment of daily

rhythms. Previous research suggests that lighting-induced behavioral deficits are not wholly circadian driven in grass rats, though this could be dependent on the strength and duration of light exposure [55]. If the presence of orexin is not reflected in activity onsets/offsets and active duration, it may instead be reflected in improvement of sleep quality and sleep consolidation. Within this pathway, orexin buffers pro-inflammatory response, upregulates neurotrophic factors through direct and indirect effects, and improves arousal and sleep; these factors combined could result in improved affective state, reflected in decreased anhedonia consistent with bright-light housing [6].

Our group has previously demonstrated the effects of daytime-light deficiency on affective and cognitive behavior, central orexin, and neuroinflammation, though we have yet to directly study the mechanisms driving and linking this network. This pathway creates a framework through which to test the modulatory role of orexin in these effects.

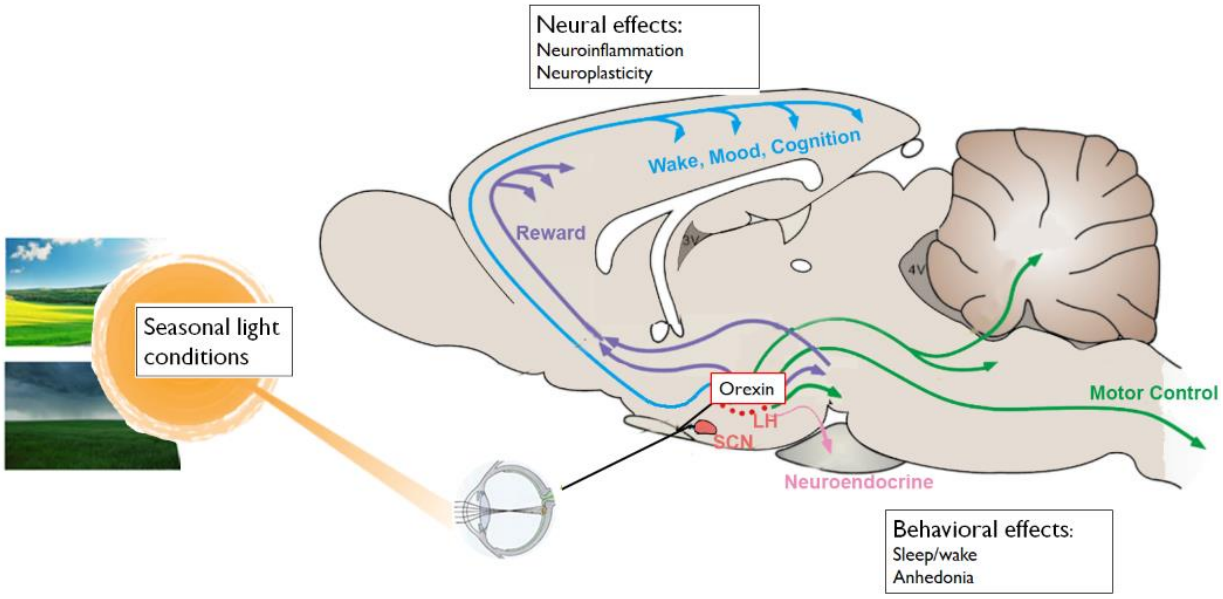


**Figure 1:** The orexinergic pathway in response to seasonal lighting conditions (diagrammed in the rat brain). Light passes through the retina, and travels via the retinohypothalamic pathway to the SCN and LH (red). The orexin neurons in the lateral hypothalamus are involved in the regulation of neuroendocrine response (pink), motor control (green), reward (purple), and

**Figure 1 (cont'd)**
wakefulness, mood, and cognition (blue). Orexin could be a modulator in the effects of daytime light deficiency and buffer the behavioral and neural outcomes associated with SAD. This diagram is adapted from Zhang et al., 2013 [193].

**Overview of dissertation experiments**

The effects of brLD and dimLD alone on neuroplasticity, neuroinflammation, sleep, and affective behavior have been established; these dissertation experiments focus on orexin-driven therapeutic interventions within dimLD conditions to buffer these responses. Given orexin's light sensitivity and role in mood and cognition, the present study poses the question if the behavioral and neural responses associated with SAD in dim light winter-like conditions are due to attenuated levels of orexin. These experiments are based off the hypothesis that enhanced orexin will be sufficient to alleviate deficits in sleep, affective state, and neuroplasticity, and buffer the pro-inflammatory response induced by dim lighting conditions.

I first established that orexin responds to BLT, by investigating the effects of early-morning BLT on central orexin, arousal/sleep, neurotrophic factor expression, and markers of proinflammatory state (Chapter 2). I utilized a photic-based therapeutic intervention, aiming to prompt natural upregulation of orexin. Male and female grass rats housed in dimLD received daily 1-hour exposure to early morning BLT, simulating daylight (full-spectrum white light, ~10,000 lux) throughout 4 weeks, while a control group received daily 1-hour narrowband red-light exposure. The resulting sex- and brain region-specific upregulation of orexin receptor expression and orexin precursor PPO suggested that orexin could be one of the main neural mechanisms through which BLT exerts its effects.

I then directly tested the modulatory role of orexin in these behavioral and neural responses, through ICV infusion of OXA or artificial cerebrospinal fluid (aCSF) with the use of a programmable minipump (Chapter 3). The OXA administration without BLT helps elucidate

which effects can be attributed to orexin alone, asking if orexin is *sufficient* to buffer these responses in the absence of bright light. Male and female grass rats housed in dimLD received delivery of OXA/vehicle throughout the beginning of the light phase (ZT0-ZT6), simulating natural release of orexin and the treatment regimen in human patients. These groups were compared to a control cohort of animals housed in summer-like brLD that received vehicle infusion. In addition to the factors measured in Chapter 2, this experiment quantified anhedonia as well as anti-inflammatory cytokine expression in order to get a more complete picture of behavioral and neural responses to orexin.

Together, these dissertation experiments examine orexin's role in the pathophysiology of SAD. The results of these experiments provide a foundation for further exploration of the orexinergic system as a therapeutic target in depressive disorders.

**CHAPTER 2:** How does orexin respond to bright light: determination of the effects of BLT on wakefulness/sleep, central orexin, and neuroinflammation

**Bright light therapy**

BLT is classically used in the treatment of SAD [4, 49, 194]. Operating through the delivery of a morning pulse of intense light, it is a non-pharmacological and noninvasive treatment that results in increased mood, cognitive performance, and daytime wakefulness, among other psychobiological benefits [1, 195]. Many SAD patients achieve full remission with bright light therapy alone, and it has been shown to be as successful as antidepressant medication [194]. Correct use of BLT results in more restful sleep at night, promotes higher vigilance states in the morning, and decreases excessive sleepiness throughout the day [196-198]. For this purpose, it is often used to counteract fatigue and disturbed sleep side effects of medications used to treat cancer and immune diseases [199-202]. Another promising use of bright light therapy is in the treatment of Alzheimer's and dementia. The circadian system naturally declines in responsivity to light stimuli as we age, possibly due to decreases in retinal function [203]. Elderly populations, particularly those housed in nursing homes, often receive little natural light and tend to lead a sedentary and largely indoor lifestyle [204]. Light therapy in both dementia patients and general nursing home populations has been shown to lessen sundown syndrome, agitation, and depressive symptoms, while improving sleep and slowing cognitive decline [38, 205-208].

The benefits of BLT are often dependent on the characteristics of the light itself [1, 209]. The optimal dose is 10,000 lux of full-spectrum or cool white fluorescent light, with a duration ranging from 30 minutes-2 hours daily for 2-4 weeks [195]. There are minimal side effects, including mild headache and eyestrain. It has been reported that men tend to perceive blue-

enriched light as brighter, and show improvements in sleep quality and performance on tests of mood and cognition following this full-spectrum blue-enriched light exposure; this suggests that there could be a sex-specific component to the mechanism of BLT [210]. However, the neural mechanisms underlying the therapeutic effects of BLT remain unclear [29, 211-213].

The leading hypotheses are based in chronobiological mechanisms [214]. Light can reach the brain directly through light-activated retinofugal pathways and has also been demonstrated to exert indirect effects through soluble gas neurotransmitters in retinal blood vessels [215, 216]. The photon-count theory postulates that shorter photoperiods or lack of light intensity, particularly in winter months and in vulnerable individuals, leads to a lack of sufficient light, or photons, to the retina. This is supported, in that there is a positive correlation between photoperiod and severity of depression symptoms; more compellingly, light intensity can predict remission [217, 218].

The phase-shift hypothesis states that insufficient light intensity disrupts an individual's sleep-wake cycle, leading to a phase delay, or a shift in endogenous rhythms to a later time [16]. This rationale suggests that during winter months circadian misalignment can occur without consistent bright daytime light providing an entrainment cue to aid in synchronization to the environment [25]. This is supported in observations of circadian mediated biological processes such as cortisol and melatonin release, delayed onset/offset of sleep, and body temperature in patients with SAD [30, 219]. As the circadian system itself cannot be easily and directly measured in humans, markers of the circadian pacemaker such as melatonin, core body temperature, activity/rest cycle, and cortisol can be used [220]. Circadian phase position, or the phase of an individual's circadian rhythm compared to their environment, in clinical populations is often measured through dim light melatonin onset (DLMO), as it's relatively simple to

measure through saliva samples, can reliably be obtained before sleep, and masking of melatonin can be avoided by taking the samples under dim light [220-222]. Determination of the time at which melatonin levels rise above baseline marks the onset of the sleep cycle, which can be used to calculate phase position relative to time[221, 222]. When light therapy is administered in the morning the strong pulse of bright light often results in a phase-advance, shifting back the rhythms of the previously phase-delayed patients to a time that is synchronized with their environment [4, 16, 25]. The degree of phase advance following light therapy is positively correlated to remission of depressive symptoms [4, 16]. Bright light therapy scheduled in the evening induces a phase delay, and can be utilized in patients who instead experience a phase advance with SAD [16, 223]. These effects are not limited to SAD patients, as similar findings with phase shifts have been found in patients with dementia and Alzheimer's [224]. Phase angle in dementia patients following light therapy is positively correlated to sleep quality and performance on cognitive assessments [224].

There are discrepancies in findings and magnitude of positive results following light therapy, possibly due to the need to individually tailor treatment to an individual's circadian phase. Sleep and biological rhythms are often irregular in dementia patients, and release of homeostatic sleep factors, such as melatonin, occurs later in the day compared to controls [203, 225-227]. Melatonin can sometimes worsen sundown syndrome, especially when combined with light therapy [42]. There is a distinct lack of control for the characteristics of the light administration, and it is unclear if light is more beneficial if administered in the morning or evening [228]. BLT paradigms in diurnal rodents, such as fat sand rats or Sudanian grass rats, have been shown to alleviate depression-like behaviors, though these studies rely on manipulation of daylength rather than manipulation of light intensity for winter-like conditions

[229, 230]. The available studies that explore the neural basis of light therapy often differ in their method for not only light administration but also light intensity and duration [46, 47]. There is a need to establish what is driving the neurobiological mechanism, in order to better optimize lighting parameters.

**Determination of effects of BLT on wakefulness/sleep and daily rhythms**

One of the core symptoms of SAD and other major depressive disorders is abnormalities in sleep [231]. Insomnia and prolonged initiation of sleep are often reported, as well as both phase delays and advances in intrinsic circadian rhythms [25, 223, 232-236]. Common antidepressant medications can initially worsen sleep disturbances, and disordered sleep symptoms can still remain even after improvements in mood [237-239]. BLT has proven to be effective for treating sleep abnormalities and fatigue symptoms, through promoting daytime wakefulness, vigilance, and alertness [210, 240, 241]. Patients receiving BLT show a reduction in nighttime wakefulness and an increase in sleep bout duration, suggesting a better quality of sleep [38, 242].

Sleep/wakefulness and locomotor activity were continuously recorded in the current study, in order to explore the effects of BLT on both circadian rhythm and homeostatic driven sleep behavior. This study utilized a combination of piezoelectric sensor plates and above-cage activity sensors, as this allows for differentiation between inactivity due to the animal being in a quiet alert phase and inactivity due to sleep. It is predicted in the current study that animals receiving BLT will show better entrainment of daily rhythms to their light cycle, as measured through general locomotor activity onsets/offsets, active duration, and amplitude of activity. Sleep quality, as measured through sleep bout length and frequency, is also expected to improve due to the early photic cue and actions of orexin on sleep and wakefulness.

**Determination of effects of BLT on central orexin, neuroplasticity markers, and pro-inflammatory markers**

Our previous work has found that daytime light deficiency leads to altered expression and downregulation of orexin receptors in corticolimbic brain regions [6, 145]. Prior studies have also demonstrated almost a two-fold increase in hypothalamic PPO between dimLD and brLD in males, but not in females[145]. Sex differences in baseline orexin levels have been reported, and are thought to functionally contribute to sex-specific stress responses [243]. Expression of OX1R and OX2R were examined in corticolimbic brain regions implicated in mood and cognition (the mPFC, CA1, and BLA) in the current study, as well as hypothalamic PPO mRNA expression. It is expected that BLT will provide a photic cue similar to brLD, encouraging upregulation of hypothalamic PPO and expression of orexin receptors in a possibly sex-dependent manner.

Expression of neurotrophic factors BDNF and TrkB were measured in order to help validate neuroprotective effects of BLT and enhanced orexin. Orexin has been shown to increase BDNF-TrkB expression in the hypothalamus and ventromedial prefrontal cortex [190, 244]. Administration of OXA can increase BDNF protein levels in the brain, however some studies have shown that this effect is lost with an OX1R antagonist [169]. This suggests orexin receptors play a necessary role in orexin's neuroplastic effects. As such, control animals in dimLD in brain regions with heightened pro-inflammatory response are expected to have attenuated expression of neuroplasticity markers [245-247].

Expression of microglia activation marker CD11b and cytokines TNF-α and IL-6 were also analyzed. These cytokines were selected due to their role in apoptosis signaling, cognition, and sleep/wakefulness. Sustained release of pro-inflammatory cytokines such as TNF-α and IL-6 is detrimental and associated with a loss of synaptic plasticity, demyelination, and

downregulation of BDNF-related signaling pathways [247-252]. Administration of TNF-α increases feelings of anxiety, depression, and fatigue in humans, and worsens memory task performance [120-123]. In mice, increased hypothalamic TNF-α has been demonstrated to suppress clock genes and clock-controlled genes, affecting sleep behavior and locomotion during their active phase[124]. Both TNF-α and IL-6 have demonstrated seasonal expression and sensitivity to dimLD; furthermore, it was recently reported that BLT resulted in a reduction of peripheral TNF-α and IL-6 in patients with TBI [12, 43]. It is predicted that BLT will buffer the expression of CD11b, TNF-α, and IL-6 in a brain region- and sex-dependent manner, similar to previous findings [12].

Overall, the animals housed in dimLD receiving early-morning BLT are expected to have a similar neuroinflammatory status to those housed in summer-like bright light[12]. It was predicted that the hypothalamic orexin/hypocretin system could be one of the neural pathways underlying the therapeutic effects of BLT, reflected in expression of pro-inflammatory and neuroplasticity markers.

**Experimental approach**

***Subjects and housing conditions***

Male and female adult grass rats (*Arvicanthis niloticus*) at 4-6 months old were obtained from the long-standing breeding colony at Michigan State University [253]. Animals were housed under 12:12 hr light/dark cycle (lights on at 06:00 am) in Plexiglas cages (43 × 23 × 20 cm) with a metal hut provided for enrichment. Food (PMI Nutrition Prolab RMH 2000, Brentwood, MO, USA) and water were available *ad libitum*. In the colony room, light was provided by fluorescent lights mounted to the ceiling, with light intensity of ~300 lux at the center of the room. Experimental animals were randomly assigned into two groups ($n$ = 6/group

for males, *n* = 8/group for females) to receive 1 hr full-spectrum white light simulating bright light therapy (BLT) or red light as control, from 06:00 to 07:00 daily for 4 weeks (Fig. 2). Lighting conditions were tightly controlled by light fixtures (Multispectral Luminaire, Telelumen LLC, CA) mounted 12.7 cm above each cage and illuminated the entirety of the cage. During the 12 hr light phase, both groups were exposed to simulated dim fluorescent light at 50 lux as done in our previous studies [9, 55]. The BLT group received 1 hr full-spectrum white light (~10,000 lux, total irradiance: 7.92 mW/cm$^2$, melanopic irradiance: 1.51 mW/cm$^2$); the control condition received narrowband red light (780 nm, ~180 lux, total irradiance: 1.51mW/cm$^2$ with no melanopic irradiance), overlayed on the dim housing light. Red light serves as an ideal control, as rodents lack the photoreceptors or spectral sensitivity to detect red light [254, 255]. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) and were approved by the Institutional Animal Care and Use Committee of Michigan State University.

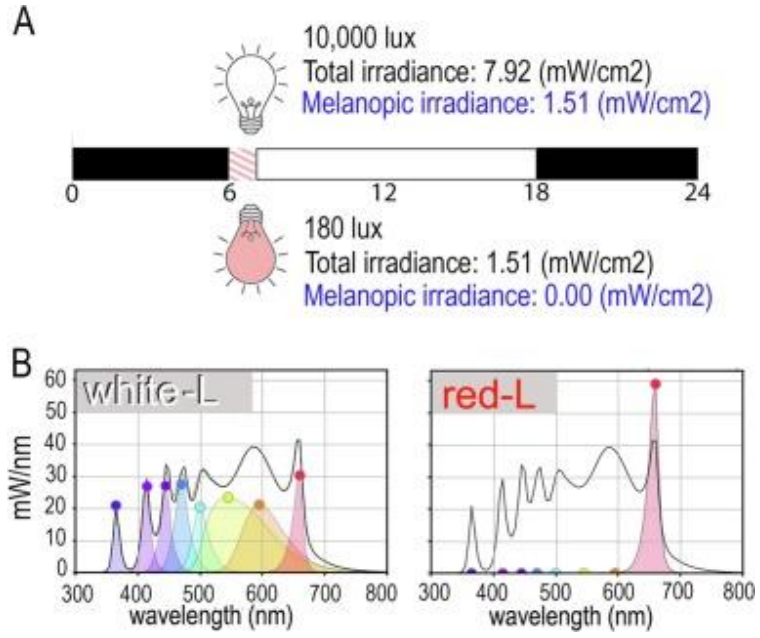


**Figure 2**: A, Experimental Paradigm: Grass rats housed in daily 12:12 h light (50 lux): dark cycle received 1 hr early-morning exposure to full-spectrum white light (10,000 lux) or

**Figure 2 (cont'd)**
narrowband red light (680 nm, 180 lux) to simulate bright light therapy or control condition, respectively. B, Spectral power distribution of the white or red light used in the study.

***Monitoring sleep/wakefulness***

Sleep/wakefulness was monitored continuously during the last 2 weeks of light treatment using a piezoelectric system (Signal Solutions, Lexington, KY). Animals were singly housed, and a piezoelectric sensor plate was placed beneath each cage. This detection method is noninvasive and achieves >90% accuracy for sleep/wake classification based on validation with simultaneous recording through EEG/EMG [256]. Piezo signals were acquired in the same manner as previous studies have described [95, 256-259]. These signal outputs were converted into FeatVec files (FeatVecMaker, Signal Solutions version 2.08218) and analyzed using SleepStats (version 2.28).

***Monitoring in-cage locomotor activities***

In-cage locomotor activities were monitored in animals throughout the entire 4 weeks using an Actimeter system, an internet of things (IoT) sensor platform (Geocene Inc., Vallejo, CA)[95, 259]. The passive infrared sensors were positioned directly above the cage lid and angled to provide 100% cage area detection for movement. The system was set for 20 second sample intervals with data outputted as fractional from 0.00 to 1.00. Meaning, a data point of 0.2 indicates 20% of activity during the 20 second interval. The locomotor activity data was processed and analyzed using ClockLab (Actimetrics, IL) to produce actograms and hourly activity profiles throughout the 4-week recording period. Clock time for activity onset/offset, duration of the active phase, and amplitude of activity was analyzed and compared using data collected in the last week of recording. Active duration was calculated as the time of activity offset minus the time of activity onset during the final week of light treatment.

***RT-qPCR***

Following the hour of BLT or red-light exposure on day 28, animals were euthanized with CO2 followed by rapid decapitation. Whole brains were flash frozen and stored at -80 °C. Brains were then sectioned at 200 µm via cryostat and thaw mounted onto slides. The mPFC, BLA, CA1, and LH were punched from the slices using a 1-mm micropuncher (Harris Micropunch, Hatfield, PA). RNA was extracted with Qiagen RNeasy Plus Mini kits (Qiagen, Valencia, CA, Cat# 74134), and quantified using a Qubit Flex Fluorometer (Thermo Fisher Scientific). The RNAs were converted to cDNA using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA, Cat# 4368814).

The RT-qPCR was conducted using a SYBR green Master mix consisting of 5 ng cDNA and 0.25 uM of each primer set. Reactions were performed in triplicate. Primers for pro-inflammatory markers, neuroplasticity markers, and orexin markers have been designed based on corresponding sequences in Nile grass rats [260]. Forward and reverse primer sequences used for pro-inflammatory markers CD11b, TNFa, IL-6, “housekeeping” gene HPRT1, neuroplasticity markers BDNF and TrkB, and PPO, OX1R, and OX2R are listed in Table 1. CT values were automated by the QuantStudio-5 Real-Time PCR System analysis software (Applied Biosystems, 272511214). The ΔΔCT method was used to calculate the level of gene expression in each condition relative to the white-light BLT group.

| Primer | Forward Sequence | Reverse Sequence |
|---|---|---|
| CD11b | GCA GAC TTG CAA GGG TTC AG | GGA GGT ATC TTA CTC TTC GCT |
| TNF- α | GGT TTT CTC CAC CAA GGA AGT TTT C | TCT GCT TGC TGC CTG TGC |
| IL-6 | TCT ACT AGA GCC TAG TGA GCT CTG C | ACA TGA GTC AGA TAC CCG ACG A |
| HPRT1 | CTC ATG GAC TGA TTA TGG ACA GGA | GCA GGT CAG CAA AGA ACT TAT AGC C |
| BDNF | GTC CCG CTA TCA AAA CCG CA | GCC TTC CTT CGT GTA ACC CCA |
| TrkB | TGC ACA TCG CTC AGC AAA TCG | ATC GGA TGG GCA ACA TTG TGT G |
| *Prepro-orexin* | TCG CCA GAA GAC GTG TTC C | CCA GCT CCG TGC AAC AGT T |
| OX1R | GCC GGA TTA CCT CTA CCC G | GCT ATG AGG AAC ACG GCC AC |
| OX2R | GAC CAG TCC GTG ATG TCC AG | CAG ATG ACC AGT TGC GAC GA |

**Table 1:** Forward and reverse primer sequences for CD11b, TNF- α, IL-6, HPRT1, BDNF, TrkB, *prepro-orexin*, OX1R, and OX2R.

***Statistical analysis***

A mixed-model analysis with an autoregressive lag1 structure was used to analyze the behavioral time-series data. As grouping variables only contained two levels, two-tailed t-tests were used to further analyze differences between groups at individual time points of interest. The effect of early-morning light treatment on the behavioral parameters and expression of PPO and orexin receptors, neuroinflammatory markers, and neuroplasticity markers was analyzed using One-Way ANOVAs and unpaired two-tailed t-tests using SPSS (IBM Version 27). Sex was not included as a factor in the statistical analyses, as male and female cohorts were run independently, and the qPCR was carried out several months apart. Data sets were examined for normality (Shapiro-Wilk's tests), homogeneity of variance (Levene's tests), and outliers (Grubb's Test for a Single Outlier). Unless specified otherwise, data analyzed met the assumptions for normal distribution and equal variances. Statistical significance for all tests is indicated by $p < 0.05$.

## Results

### *Effects of BLT on wakefulness*

During each daily 1-hour early morning light treatment, the white-light BLT group had significantly more wakefulness compared to the red-light control group in both females (Fig. 3A, $t_{14} = 6.818$, $p < 0.001$) and males (Fig. 3A', $t_9 = 5.455$, $p < 0.001$). There was no significant difference in wakefulness between the light treatment groups during the hour before or the hour after the light treatment in either sex (Fig. 3A and A', ps > 0.05). The temporal dynamic of BLT's wakefulness-promoting effects during the hour of treatment was further analyzed at 15-minute intervals (Fig. 3B, B'). Collapsed across sex, the effects appear to be more prominent during the first 30 min of bright-light exposure. This was mostly driven by the males, (Fig. 3B'), though, which showed a significant difference between the BLT and control groups only during the 1st ($t_9 = 10.601$, $p < 0.001$) and 2nd 15-minute intervals ($t_9 = 2.658$, $p = 0.026$), but not in the last two intervals (ps > 0.05). In females, however, significant differences between the BLT group and controls were detected in all 4 intervals (Fig. 3B, ps < 0.05).

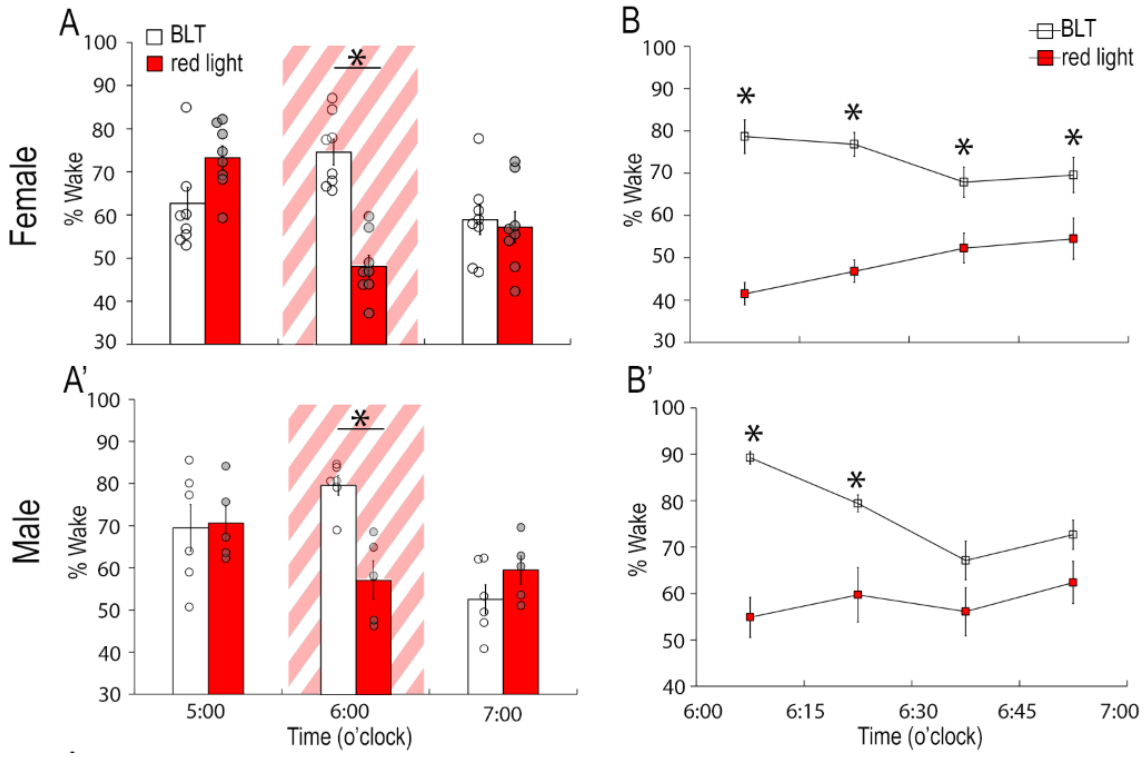


**Figure 3**: BLT promotes wakefulness. Higher percent of wakefulness was observed in the BLT group compared to the red-light control group during the hour of treatment, but not in the hour before or after treatment, in both females (A) or males (A'). The percentage of time in wakefulness was further compared in 15-minute intervals during the hour of light treatment in females (B) and males (B'). The shaded area represents the hour of light administration. Data are shown as Mean ± SEM, n = 5–6/group for males, n = 8/group for females. * $p < 0.05$.

***Effects of BLT on sleep***

A clear day/night difference in percentage of time spent sleeping during the last week of treatment was observed with higher sleep at night in both females (Fig. 4A, $F_{1, 28} = 156.66$, $p < 0.001$) and males (Fig. 4A', $F_{1, 18} = 110.17$, $p < 0.001$), which was expected given the diurnal nature of Nile grass rats (Fig. 4A, A'). There was no significant effect of light condition or interaction between light and time in the percentage of sleep during the day or night (ps > 0.05). As female and male cohorts were run independently, sex was not used as a factor in statistical analyses; these data reflect effects of condition within each sex. Further analysis at 30-minute

intervals revealed treatment group differences in the percentage of time spent sleeping at specific times of day or night in both males (Fig. 4B', $F_{1, 261} = 197.95$, $p < 0.001$) and females (Fig. 4B, $F_{1, 285} = 254.6$, $p < 0.001$). In males, the BLT group had a significantly higher % sleep soon after midnight (0:30–1, $t_9 = 2.263$, $p = 0.032$); while in females, the BLT group had higher % of sleep during the early night (18:30–19:30, 20–20:30, ps < 0.05), compared to their respective same-sex controls.

Consistent with the percentage of time spent sleeping, sleep bout length also showed a day/night difference with longer sleep bouts at night in both females (Fig. 5A, $F_{1, 28} = 97.2$, $p < 0.001$) and males (Fig. 5A', $F_{1, 18} = 78.39$, $p < 0.001$). There were no effects of light condition or interaction between light and time in sleep bout length for either sex (Fig. 5A, A', ps > 0.05). The distribution of bout lengths presented differently in males and females, though (Fig. 5B, B'). In males (Fig. 5B'), the BLT group had a clear shift toward longer sleep bouts at night, with fewer shorter bouts (120–240 s, $p = 0.05$; 240–480 s, $p = 0.05$) and significantly more longer bouts (1920–3840 s, $p < 0.05$; >3840 s, $p = 0.04$). This is reflected in sleep bout analysis in 30-minute intervals, with males that received BLT having more consolidated sleep in the middle of the night compared to controls (Fig. 6A'; 21:30-22:00, $p = .0.02$; 23:00-23:30, $p = .0.12$; 23:30-0:00, $p = 0.04$; 0:30-1:00, $p = 0.03$; 1:30-2:00, $p = 0.018$). In females, although there were no significant differences between light treatments in the distribution of sleep bout percentages during the day or night (Fig. 5B), further analysis of sleep bout length at 30-minute intervals revealed longer sleep bouts in the beginning of the night in BLT females (Fig. 6A; 18:30-19:00, $p = 0.01$; 20:00-20:30, $p = 0.01$).

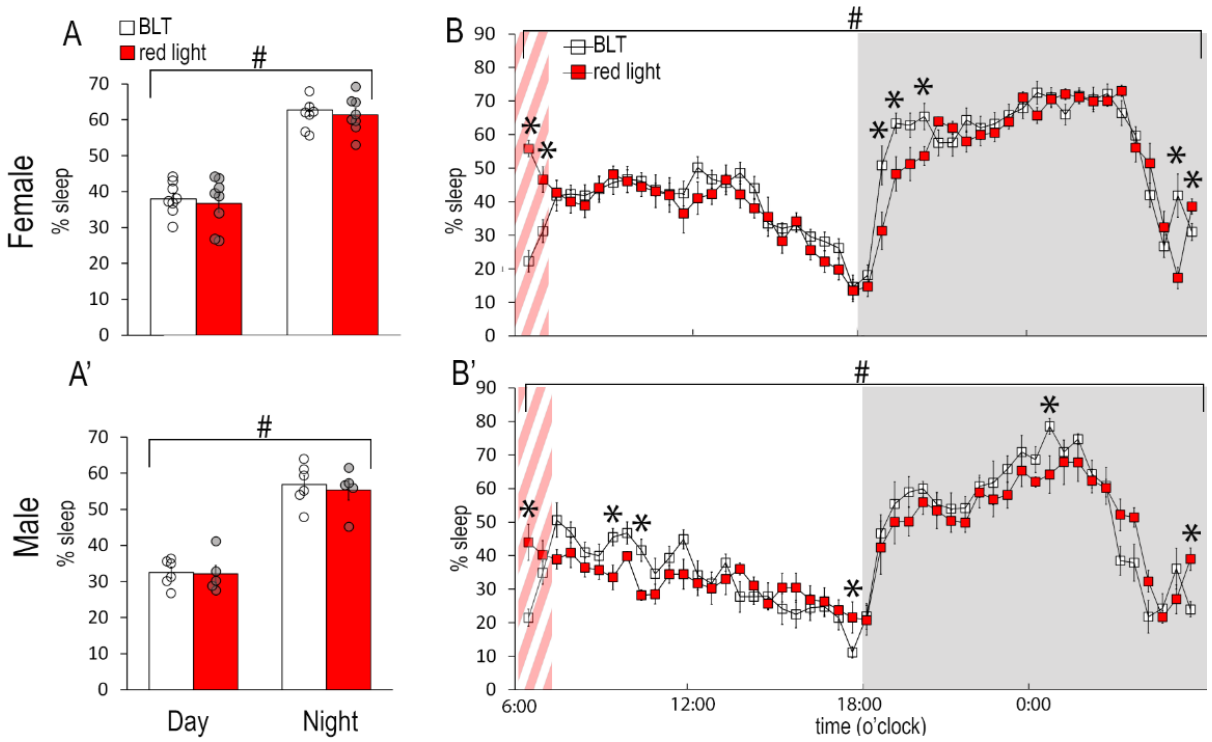


**Figure 4**: Effects of BLT on the amount of sleep. Overall % of time spent sleeping during the day and night was not significantly different between the BLT light and control red light group in either females (A) or males (A'). Analysis in 30-minute intervals revealed higher sleep % during morning and at midnight in males (B') and during early night in females (B) in the BLT and control red light group. The red and white shaded area represents the hour of light administration. Gray shaded areas indicate dark phase. Data are shown as Mean ± SEM, n = 5–6 for males, n = 8 for females. * indicates significant effect of light condition, # indicates significant effect of time, $p < 0.05$.

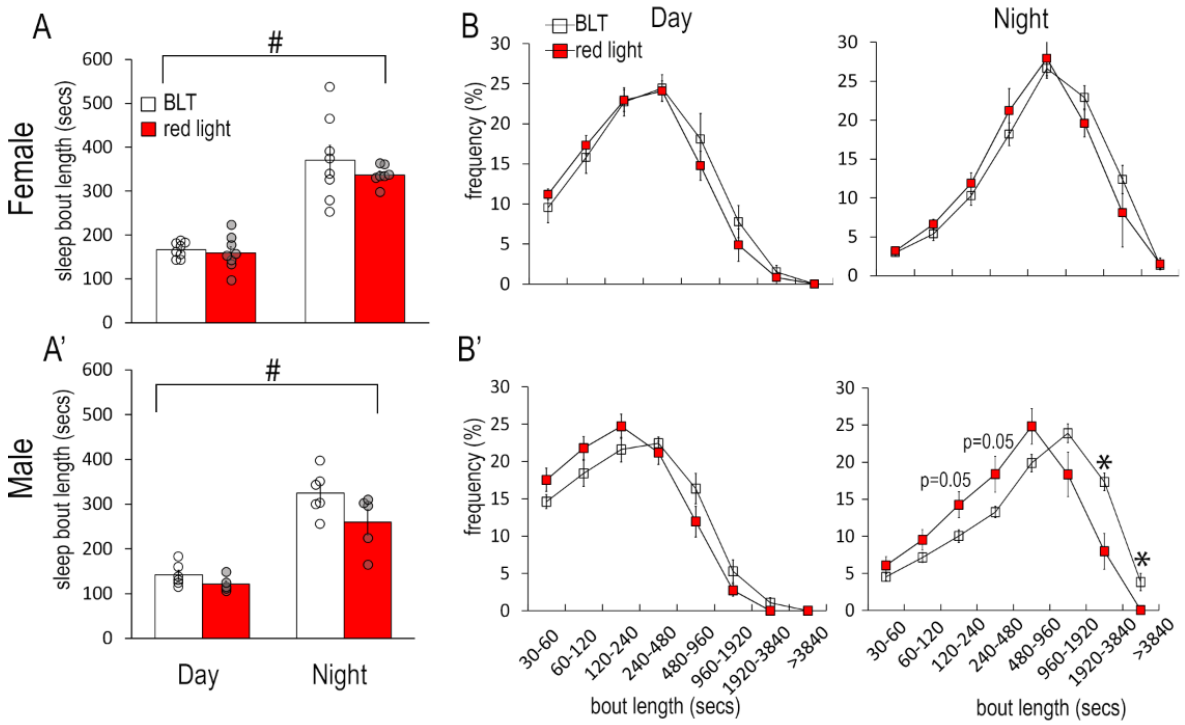


**Figure 5:** Effects of BLT on the quality of sleep. Overall sleep bout lengths during the day and night were not significantly different between the white light and the red-light control groups in either females (A) or males (A'). Analysis of the frequency of different sleep bout length revealed that BLT resulted in higher frequency of longer bout length at night in males (B') but had no significant effect in females (B). Data are shown as Mean ± SEM, n = 5–6 for males, n = 8 for females. * indicates significant effect of light condition, # indicates significant effect of time, $p < 0.05$.

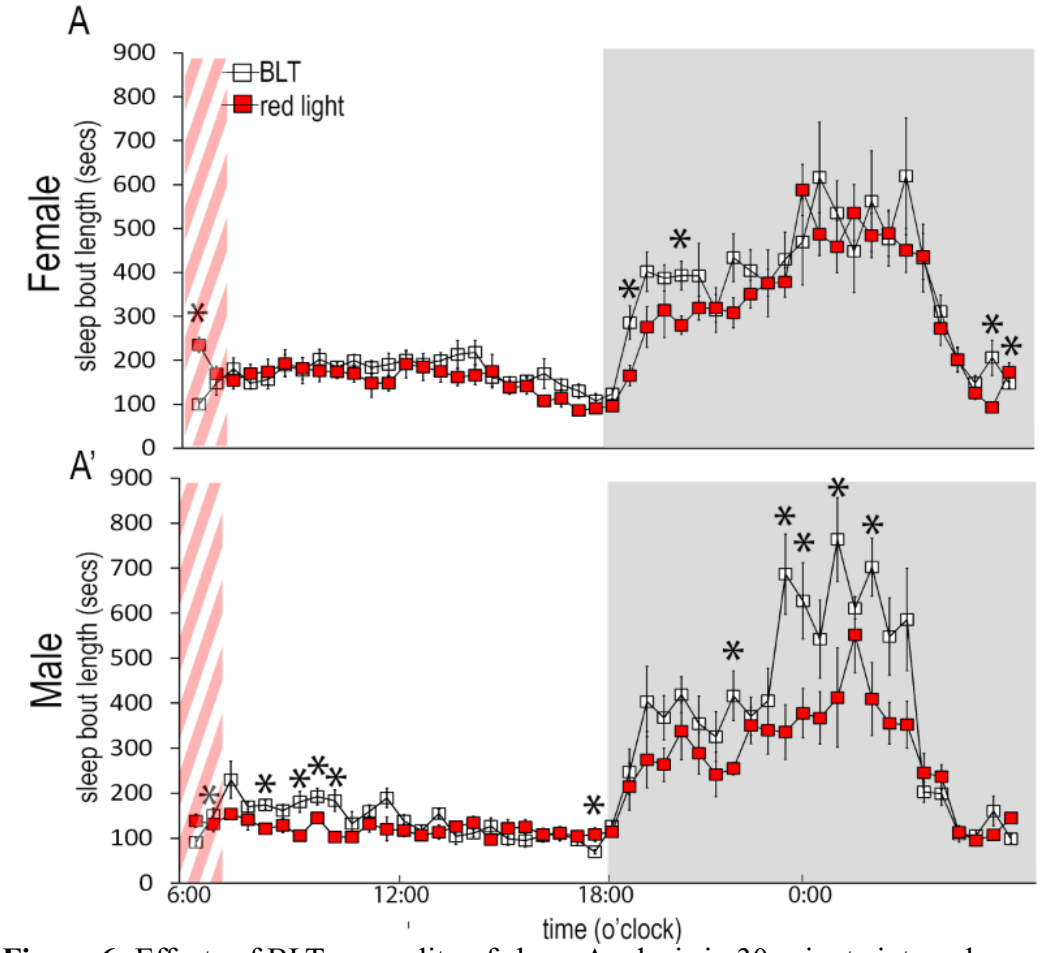


**Figure 6:** Effects of BLT on quality of sleep. Analysis in 30-minute intervals revealed higher sleep bout duration during morning and the middle of the night in males (A') and during early night in females (A) in the BLT light and control red light group. The red and white shaded area represents the hour of light administration. Gray shaded areas indicate dark phase. Data are shown as Mean ± SEM, n = 5–6 for males, n = 8 for females. * $p < 0.05$.

***Effects of BLT on daily rhythms of locomotor activity***

In-cage locomotor activity was also monitored for the female cohort throughout the 4-week treatment period (Fig. 7). A clear diurnal pattern was observed in both the BLT (Fig. 7A) and the control groups (Fig. 7B). Quantitative analysis of the hourly activity profile revealed no significant treatment effect during the first 3 days of the treatment (Fig. 7C). During the next 4 days (Fig. 7D), the BLT group was significantly more active during the 1 hr treatment session ($t_{12} = 2.487$, $p = 0.029$) and the hour right after ($t_{14} = 2.469$, $p = 0.03$). This arousing effect of BLT

was also observed in week 4 (Fig. 7E). The BLT group also had significantly earlier activity offset (Fig. 7F, $t_{13} = 2.516$, $p = 0.026$) and shorter active duration (Fig. 7G, $t_{13} = 2.004$, $p = 0.039$) than the control group.

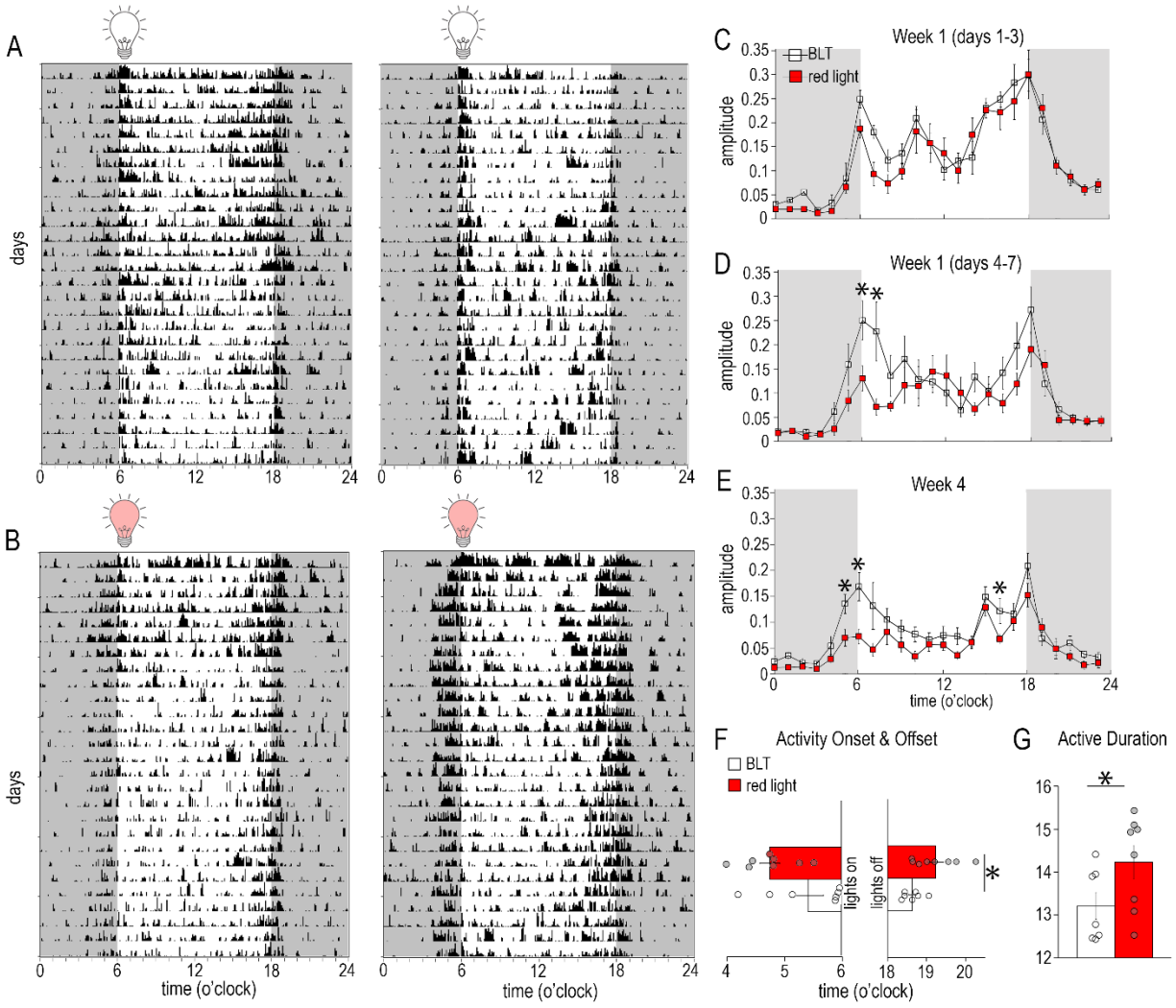


**Figure 7:** Effects of BLT on daily rhythms of in-cage locomotor activity in females. Representative actograms showing daily in-cage locomotor activity over 4 weeks in animals receiving BLT (A) and red light (B). Hourly activity profile was compared between the two group during the first 3 days (C) and the rest 4 days of week 1 (D), and during the total of week 4 (E). Activity onset and offset (F) and active duration (hours, G) were also compared in week 4. Gray shaded areas indicate dark phase. Data are shown as Mean ± SEM, n = 8. * $p < 0.05$.

***Effects of BLT on central orexin***

In males, there was no significant difference between light conditions in hypothalamic PPO mRNA levels, but in females, significantly higher PPO was observed in the BLT group (Fig. 8, $t_{14} = 2.378$, $p = 0.03$). In females, the BLT group had significantly less expression of OX1R ($t_{14} = 3.928$, $p = 0.002$) and OX2R ($t_{14} = 3.172$, $p = 0.007$) in the BLA (Fig. 9A, B). Males in the BLT group however had higher OX1R expression in the BLA ($t_{11} = 2.213$, $p = 0.05$) and the CA1 ($t_{10} = 2.536$, $p = 0.03$), as well as higher expression of OX2R in the CA1 ($t_{11} = 2.596$, $p = 0.02$), compared to their controls (Fig. 9A', B').

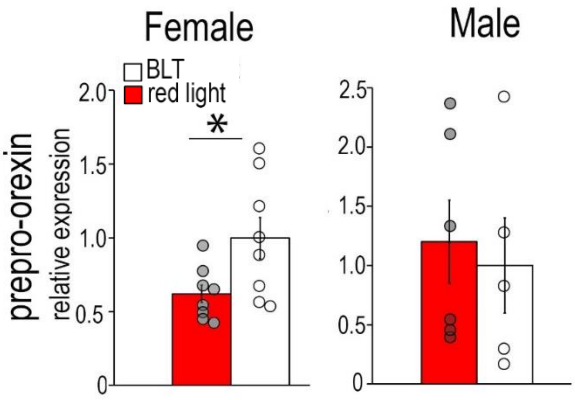


**Figure 8:** Expression of *prepro-orexin* in the lateral hypothalamus of female and male grass rats exposed to BLT or red light. Data are shown as Mean ± SEM, n = 6 for males, n = 8 for females. * $p < 0.05$.

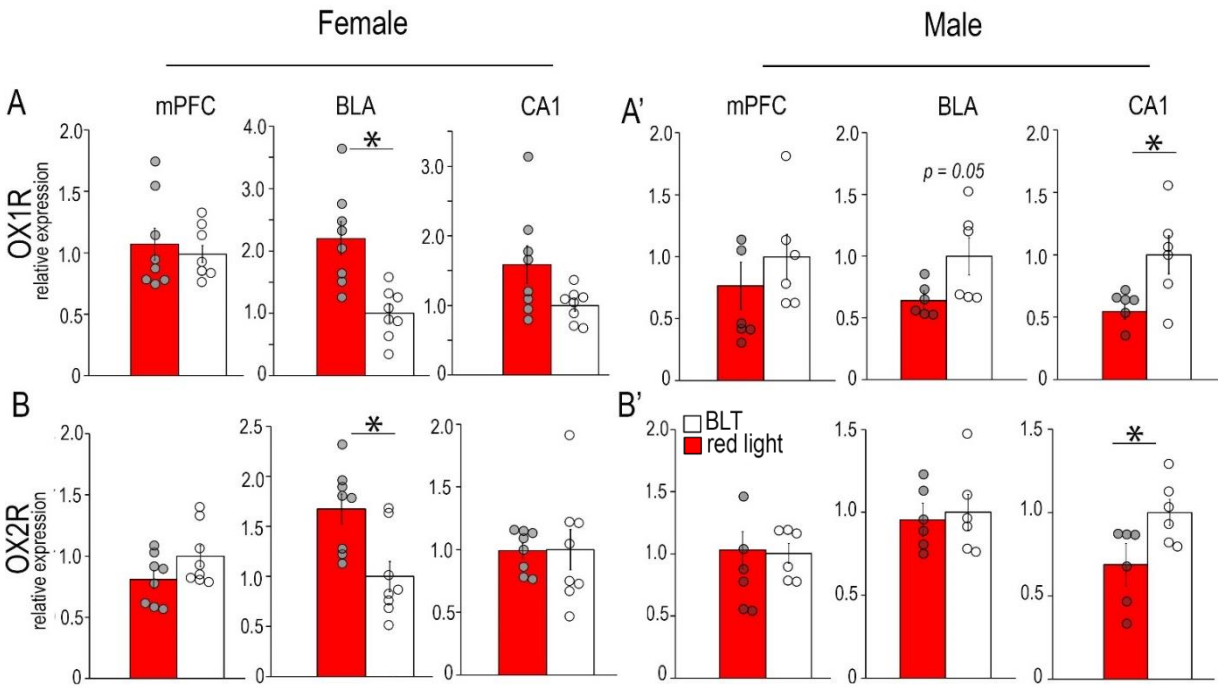


**Figure 9:** Expression of OX1R and OX2R in the mPFC, BLA, and CA1 of female (A, B) and male (A', B') grass rats exposed to BLT or red light. Data are shown as Mean ± SEM, n = 8 for females, n = 6 for males. * $p < 0.05$.

***Effects of BLT on synaptic plasticity markers***

In females (Fig. 10), the BLT group had significantly lower expression of BDNF in the CA1 ($F_{1,14} = 7.638$, $p = 0.01$) and lower expression of TrkB in the mPFC ($F_{1,12} = 17.878$, $p = 0.001$), BLA ($F_{1,14} = 20.636$, $p < 0.001$) and CA1 ($F_{1,12} = 9.907$, $p = 0.008$) compared to the controls. In males (Fig. 11), the BLT group had higher BDNF expression in the BLA ($F_{1,10} = 7.937$, $p = 0.01$) and CA1 ($F_{1,10} = 12.456$, $p = 0.006$), but no significant differences between groups were found in their expression of TrkB (mPFC: $F_{1,10} = 4.248$, $p = 0.06$; BLA: $F_{1,12} = 0.130$, $p = 0.72$; CA1: $F_{1,10} = 4.780$, $p = 0.053$).

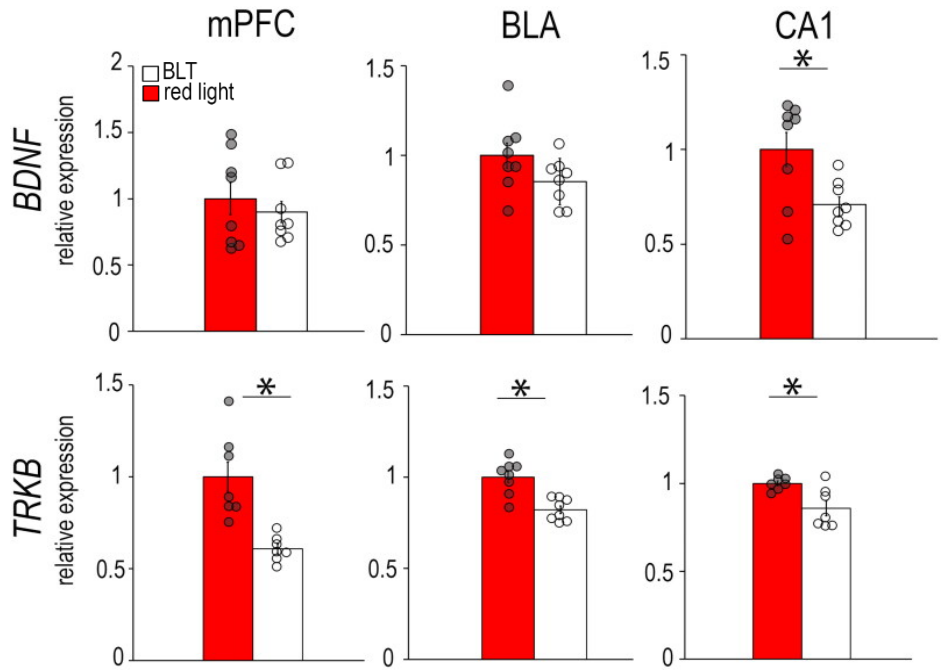


**Figure 10:** Levels of mRNAs for neuroplasticity markers BDNF and TrkB in the mPFC, BLA, and CA1 of female grass rats exposed to BLT or control red light. Data are shown as Means ± SEMs, n = 8/group. *p < 0.05.

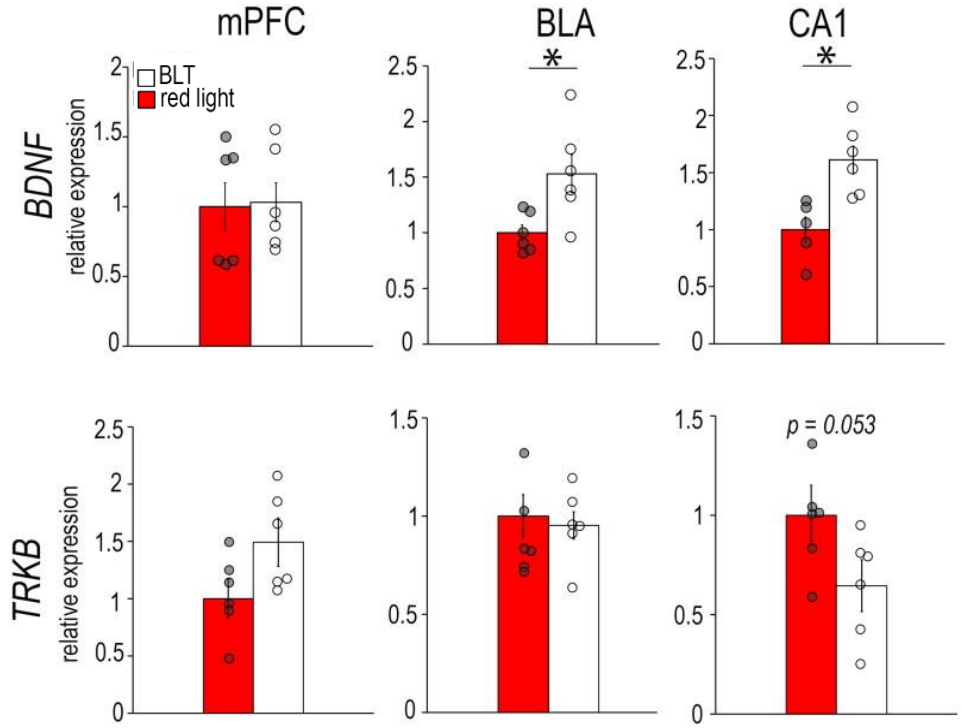


**Figure 11:** Levels of mRNAs for neuroplasticity markers BDNF and TrkB in the mPFC, BLA, and CA1 of male grass rats exposed to BLT or control red light. Data are shown as Means ± SEMs, n = 6/group. *p < 0.05.

***Effects of BLT on neuroinflammatory markers***

In the female mPFC (Fig. 12), there were no significant differences between the BLT and control groups in the expression of the microglia marker CD11b ($F_{1,14} = 1.937$, $p = 0.18$), or the pro-inflammatory cytokines TNF-α ($F_{1,12} = 0.603$, $p = 0.45$) and IL-6 ($F_{1,12} = 4.583$, $p = 0.053$). In the female BLA, the BLT group had significantly lower expression of TNF-α compared to controls ($F_{1,14} = 4.797$, $p = 0.04$), but the groups did not differ in expression of CD11b ($F_{1,14} = 1.189$, $p = 0.29$) or IL-6 ($F_{1,14} = 3.353$, $p = 0.08$). In the female CA1, the groups did not differ in any of the inflammatory markers (CD11b, $F_{1,13} = 0.022$, $p = 0.88$; TNF-α, $F_{1,13} = 1.508$, $p = 0.24$; IL-6, $F_{1,14} = 0.12$, $p = 0.73$).

In the male mPFC (Fig. 13), BLT significantly increased CD11b ($F_{1,10} = 7.293$, $p = 0.02$) and IL-6 expression ($F_{1,9} = 7.488$, $p = 0.02$), but not TNF-α ($F_{1,10} = 3.677$, $p = 0.08$), when compared to controls (Fig. 13). Within the male BLA, BLT also significantly upregulated IL-6 ($F_{1,10} = 13.231$, $p = 0.005$), but not the other inflammatory markers (CD11b, $F_{1,10} = 2.371$, $p = 0.15$; TNF-α, $F_{1,10} = 4.465$, $p = 0.06$). In the male CA1, there were no significant differences between BLT and control groups in inflammatory marker expression (CD11b, $F_{1,10} = 0.001$, $p = 0.97$; TNF-α, $F_{1,10} = 4.629$, $p = 0.056$; IL-6, $F_{1,10} = 2.143$, $p = 0.17$).

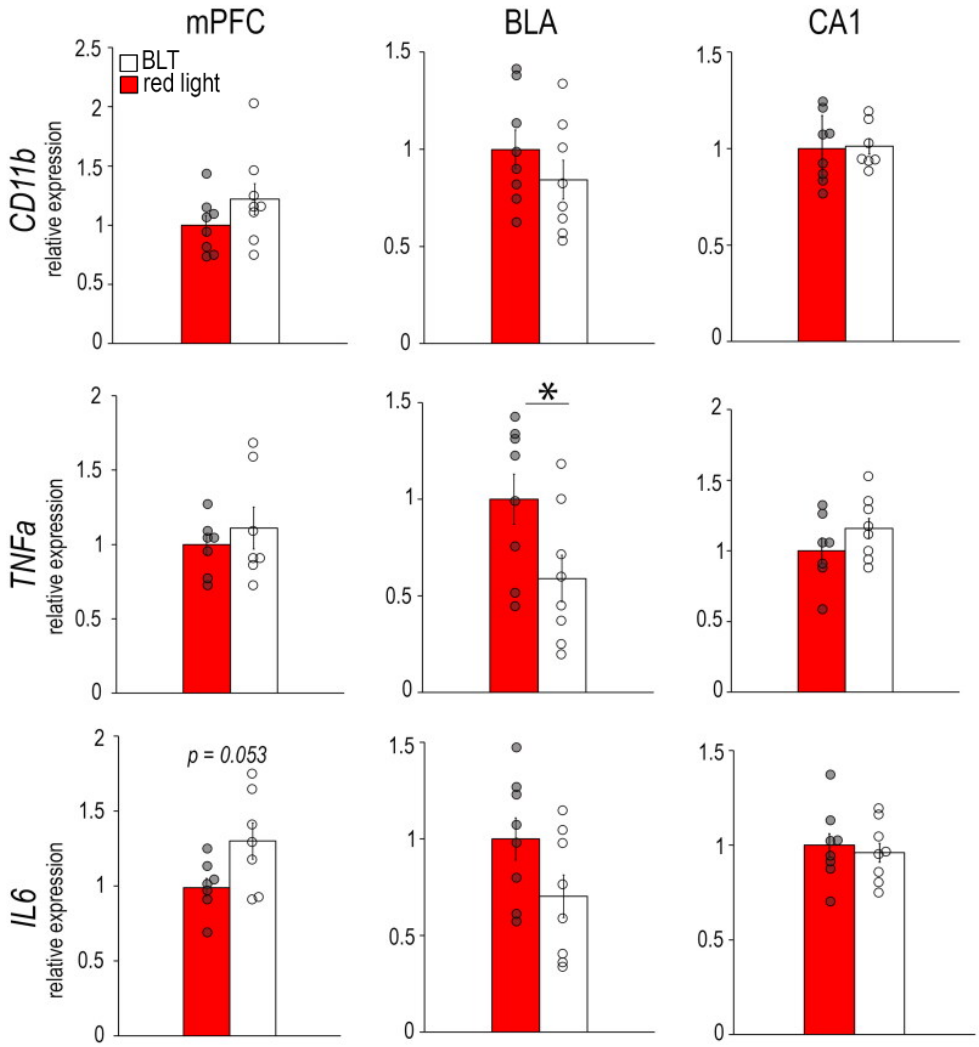


**Figure 12**: Levels of mRNAs for the microglia marker CD11b and pro-inflammatory cytokines TNF-α and IL6 in the mPFC, BLA, and CA1 of female grass rats exposed to BLT or control red light. Data are shown as Means ± SEMs, n = 8/group. *p < 0.05.

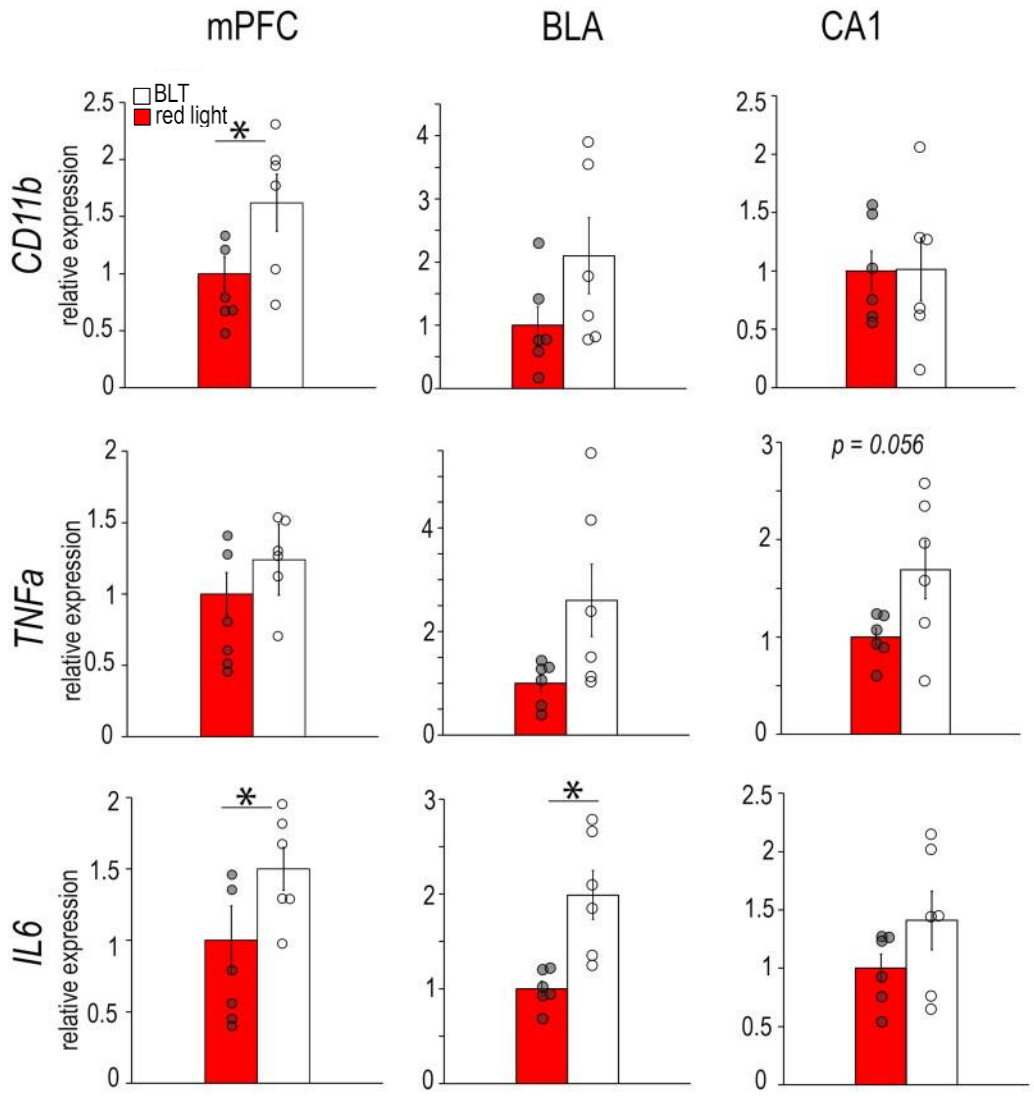


**Figure 13:** Levels of mRNAs for microglia marker CD11b and pro-inflammatory cytokines TNF-α and IL6 in the mPFC, BLA, and CA1 of male grass rats exposed to BLT or control red light. Data are shown as Means ± SEMs, n = 6/group. *p < 0.05.

## Discussion

### *Light exposure during BLT*

The non-image forming effects of light are mediated by ipRGCs [261-264]. The ipRGCs capture light using the photopigment melanopsin, which is maximally sensitive to blue light at 480 nm wavelengths[263]. Therefore, blue-enriched light or bright full-spectrum white light (10,000 lux) are commonly used in BLT, and have been shown to be an effective treatment for

depression [265]. In the present study, we utilized a multispectral emulator that was programmed to emit full-spectrum white light at 10,000 lux, the same intensity often recommended and utilized for BLT in humans [49]. Dim red light was used as the control condition, as it is often used as the control in clinical trials assessing the efficacy of BLT in treating seasonal or non-seasonal depression [41, 266-270]. In contrast to human subjects involved in a research study or conducting at-home treatment who are required to sit in front of the light source, light exposure to our grass rats was voluntary because the animals had free access to a metal hut in their home cage where they could be shielded from light. This may have contributed to individual differences in behavioral and brain outcomes but, as entire groups, there were significant effects of BLT in numerous behavioral and orexin system parameters.

***BLT-induced wakefulness and arousal***

During the daily one-hour light treatment, the BLT group showed more wakefulness when compared to that found in the red-light control group (~80% in BLT *vs*. ~60% in control, Fig. 3). In addition to wakefulness, BLT also had an arousing effect reflected by an almost 3-fold higher locomotor activity compared to the controls (Fig. 7). These findings are consistent with what have been found in humans, in that BLT promotes wakefulness, vigilance, and alertness [210, 240, 241]. In sleep-deprived adult humans, early morning BLT improves alertness, cognition, and mood, but only during and immediately after the treatment session [271]. This is consistent with our findings that the BLT group was more awake and active than the controls only around the treatment hour (Fig. 3, 7).

***BLT and nighttime sleep quality***

We found a clear day/night difference in the percentage of time spent asleep and in sleep bout length for grass rats of both sexes regardless of their treatment condition, reflecting the

well-known diurnal nature of these animals. These results are consistent with EEG data obtained from another closely related diurnal rodent, Sudanian grass rats [272]. In our male grass rats, following the increased wakefulness during the BLT exposure, there seemed to be a rebound in the percentage of time spent sleeping and in their sleep bout length in the morning, which could be due to a homeostatic drive. However, this rebound of sleep was not observed in females, suggesting a possible sex difference in sleep maintenance. Although there was no significant difference in the total amount of sleep between the BLT and control groups, longer nighttime sleep bouts were observed in BLT-treated males and females compared to the controls. Furthermore, in males there was a clear shift towards longer sleep bouts in the BLT group at night. These findings collectively indicate that BLT leads to more consolidated sleep, an indicator of better sleep quality [273-275]. BLT improves human sleep in a similar manner, such that sleep at night becomes less fragmented or interrupted [38, 242].

***BLT and the entrainment of daily rhythms***

In-cage locomotor activity was monitored in females throughout the entire 4 weeks of the study (Fig. 7). It was somewhat unexpected that during the first 3 days of the treatment, there was no significant difference between the BLT and control group (Fig. 7C). In previous studies, we found light-induced increases of activity during 1-hour light exposure in both male and female grass rats [78]. It is possible that the BLT-induced arousal was masked by novelty-induced arousal. Indeed, differences between studies in the colony and experimental treatment room used, involving a change from lights in the ceiling of the room to light fixtures above each individual cage, as well as a change from group housing to single housing, could all have potentially been arousing to the animals and led to the initial increased activity in both BLT and control groups found here. During the last 4 days of the first week, when the novelty effects were

likely wearing off, the arousing effect of BLT emerged (Fig. 7D) and persisted into week 4 (Fig. 7E). During week 4, the animals were awake ~80% of the time during the BLT administration, but they were only active/moving around in the cage ~20% of that time. Thus, the animals were in a quiet alert phase most of the time, indicating that the sleep/wake data we obtained were not strictly activity driven.

When circadian parameters were analyzed in the final week of treatment, there was a delay in activity onset in most animals (5 out of 8), an advance in activity offset, and a more consolidated activity phase in the BLT groups compared to their same-sex controls (Fig. 7F, G). The effect of light exposure on circadian entrainment has previously been studied in grass rats [80, 276]. Although the duration of light varied from 20 minutes to 4 hours, when exposed in the early subjective day, the majority of the animals showed a phase advance based on their activity onset. The delayed activity onset observed in the present study may seem to contradict these previous findings, but numerous methodological differences should be considered, such as measuring wheel running vs. in-cage locomotor activity, a single light pulse vs. daily exposure, light intensity <400 lux vs. 10,000 lux, and more importantly the animals being housed in constant dark vs. a 12:12 light/dark cycle. While a single light pulse is effective for assessing the non-parametric aspects of entrainment, or the ability of the body's clock to change phase in response to a zeitgeber, the paradigm used in the present study of daily bright light exposure to animals under a dim light/dark cycle is likely revealing a combination of parametric and non-parametric entrainment, as the result of continuous or pulsed light exposure, respectively [48]. When humans are housed under constant dim light, a brief light pulse shifts their daily rhythm in melatonin, in a way similar to the non-parametric phase-response curve obtained in laboratory rodents [277, 278]. Our results suggest that when humans receive daily BLT under a regular

daily light/dark cycle, the response of their circadian phase could differ from that predicted from non-parametric entrainment.

These findings are not consistent with the phase-shift theory. This theory postulates that the underlying mechanism of SAD is due to misalignment of circadian rhythm, resulting in a phase delay, and that BLT works by inducing a phase advance. The current study instead saw a phase *delay* in the BLT treated animals compared to the red-light controls. Average activity onset in the BLT group was approximately 30 minutes before lights-on, while the red-light dimLD control animals had average onsets nearly an hour before lights-on. Similarly, average activity offset was significantly closer to lights-off compared to the red-light controls. The active phase was shorter and more consolidated around the 12:12 light/dark cycle in the BLT group, as seen in their actograms and duration of daytime activity, suggesting better entrainment of circadian rhythm. The red-light controls had disorganized activity rhythms comparatively, and increased activity during the dark phase compared to the treatment group. Phase advances in patients with SAD have been reported, though not as commonly as phase delays [25]. A recent longitudinal study with SAD patients utilizing actigraphy bracelets suggests that individuals with morning chronotypes are more likely to experience phase advances during winter months, while evening chronotypes are more likely to experience phase delays [279]. A more personalized approach may be necessary if one is using BLT with the intent to induce a phase shift, considering individual variations in chronotype. The findings from the current study suggest that while BLT affects circadian rhythm, the phase-shift theory does not fully explain the mechanisms underlying the observed behavioral responses from daytime light deficiency.

***BLT and central orexin***

To explore the brain mechanisms underlying the observed behavioral responses to BLT, we examined the central orexin neuropeptide system. The orexin system is involved in a wide range of behavioral and physiological processes such as sleep/wakefulness, feeding and energy homeostasis, reward, emotion, mood and cognition [280-284]. Thus, the orexinergic system could be an excellent target for BLT to achieve its therapeutic effects on mood disorders and cognitive impairments [38, 44, 46].

Previous work from our group focused on the impact of daytime light conditions on the central orexin system. In male grass rats, we found that light exposure during the subjective day activates orexin-immunoreactive neurons in the lateral hypothalamus [165]. In both male and female grass rats, brighter light during the day mimicking naturalistic intensity levels is associated with higher levels of orexin peptide in the hypothalamus and cerebrospinal fluid [6, 145]. These findings collectively suggest that orexin neurons are sensitive to light. In the present study, we examined the response of the orexin system to BLT and found sex- and brain-region specific responses in the expression of orexin and its two receptors. A significantly higher expression of PPO mRNA in the BLT group was found in the hypothalamus of females, but not in males (Fig. 8). However, BLT led to lower expression of OX1R and OX2R in the female BLA, but higher expression in the CA1 of males.

The higher expression of hypothalamic PPO mRNA in the BLT females was somewhat unexpected, as our previous study comparing female grass rats housed in bright naturalistic (1,000 lux) vs. dim (50 lux) fluorescent light during the daytime found no difference in PPO [145]. This suggests that the much higher light intensity or the melanopic irradiance in our BLT paradigm could be required for upregulating PPO in female grass rats. On the other hand, our

previous study found that hypothalamic PPO in males almost doubled when animals were housed under bright vs. dim light, but here this was not significantly different between BLT and controls, suggesting that sustained bright light exposure throughout the day or at a later time of the day might be necessary to upregulate PPO in males. The intensity (1,000 lux vs. 10,000 lux) and duration/timing (12 hours vs. 1 hour) of light exposure also likely contributed to differences between our studies in orexin receptor expression, such that the mPFC previously had the most prominent changes in OX1R and OX2R protein following bright light housing but OX1R and OX2R mRNA expression was here unaffected by BLT [145]. In male grass rats, BLT instead upregulated orexin receptor expression in the CA1. Our previous studies demonstrated that the OX1R pathway in the CA1 mediates the impact of lighting conditions (i.e., bright vs. dim light during the daytime) on spatial memory in male grass rats [11]. Thus, upregulation of orexinergic pathways in the male dorsal hippocampus could underlie some of the beneficial effects of BLT on cognitive function [208]. Although OX1R and OX2R expression in their CA1 was not different from controls, female grass rats in the BLT group had lower OX1R and OX2R in the BLA. Downregulation of receptors has been found in many G-protein coupled receptors following prolonged agonist exposure [285]. Therefore, the lower OX1R and OX2R mRNA expression in the female BLT group could be due to increased orexin peptide ligand release and binding. The fact that there were sex- and brain region-specific responses of the central orexin system to manipulated light conditions in the present study is consistent with our previous study in Nile grass rats manipulating daytime light intensity [145], and with findings in humans and other animal models highlight sex-specific role of the orexin system in affective and motivated behaviors (reviewed in [243, 286]).

***BLT and neuroplasticity***

We previously found significantly lower CA1 apical dendritic spine density in both males and female grass rats housed in winter-like dim daylight when compared to grass rats housed in summer-like bright daylight [9, 10]. These findings suggest that synaptic plasticity could be a potential mediator through which seasonal light conditions influence affective state. The present study examined expression of two synaptic plasticity markers, BDNF and TrkB, and revealed that BLT effects were generally consistent with those from summer-like bright light housing. In females, the BLT group had lower BDNF mRNA expression in the CA1 and lower TrkB mRNA expression in mPFC, BLA, and CA1 (Fig. 10). When BDNF and TrkB protein levels were previously compared between grass rats housed in bright or dim daytime light conditions, the differences did not reach statistical significance despite mature BDNF and the phospho- over total-TrkB ratio in the CA1 being ~30% lower in the bright light group [10]. In males, we here found that BLT increased BDNF expression in the BLA and CA1 (Fig. 11). Consistently, we previously found higher levels of BDNF immunoreactivity in the CA1 of males housed in bright light [9, 10]. Although we were unable to directly test for sex differences in the present study, they are suggested by our results and are consistent with the sex-specific responses in BDNF and TrkB reported by others. Social isolation and maternal deprivation significantly reduce BDNF in male mice and rats, but not in females [287-289]. Environmental enrichment, however, increases BDNF in females, but not in males [290, 291]. Lastly, reducing forebrain BDNF leads to a higher phospho- over total-TrkB ratio in male but not in female mice [292]. Gonadal hormones, particularly estrogens, likely play a role in these sex differences [293-296], which could contribute to sex differences in the pathophysiology of depression. In human postmortem tissue, depressed men have less hippocampal BDNF compared to non-depressed men, while no

difference was found between depressed and non-depressed women [297]; this is consistent with the light (BLT or summer-like bright light)-induced changes of BDNF in our male and female grass rats.

Relationships among sleep, synaptic plasticity, and depression have been well established [298-300]. Numerous studies have examined the impact of sleep deprivation on hippocampal BDNF expression, but results are equivocal probably due to the differences in species studied (mice vs rats), age of the animals, and methodological details including the procedures inducing sleep deprivation and their durations (reviewed in [301]). It is also noteworthy that those studies almost exclusively involved male subjects. Our present and recent findings [259] help fill this important gap in knowledge, by showing a potential association between improved sleep quality and the central expression in BDNF and TrkB in both males and female grass rats following BLT.

Orexin has been shown to upregulate expression of neurotrophic factors, such as BDNF [184, 302-304]. In the current study, we found that BLT increased BDNF in the male CA1, but downregulated it in females (Fig. 10, 11). This pattern of changes in BDNF is consistent with BLT-induced changes in OX1R in the grass rat CA1 [259]. Further analyses confirmed that there is a significant positive correlation between OX1R and BDNF mRNAs in the CA1 of both males ($r = 0.770$, $p = 0.006$) and females ($r = 0.579$, $p = 0.01$). This is intriguing, and the possible causal role of orexin in light-dependent neuroplastic and neuroinflammatory responses will be tested in the grass rat SAD model in Chapter 3.

***BLT and neuroinflammation***

Brain region- and sex-specific responses in neuroinflammatory markers were found in our previous study comparing grass rats chronically housed in either a winter-like dim daylight

condition or a summer-like bright daylight condition (~1000 lux) [305]. It was reasonable to expect that animals housed in the winter-like dim light but exposed to daily early morning BLT would show a restoration of the neuroinflammatory status seen in animals housed in summer-like bright light. That was not always the case. For example, our prior study found lower IL-6 expression in the BLA of males housed in brLD than those housed in dimLD [305], but here we found IL-6 upregulated in males by BLT. Furthermore, while we previously found that females housed in brLD had lower TNF-α expression in the CA1, the present study found that BLT had no significant effect there on TNF-α. These few inconsistencies are likely due to differences between our studies in the lighting paradigms used to address the specific questions asked, including the light intensity (1,000 vs. 10,000 lux) and the timing/duration of light exposure (all day vs only 1 hour in the early morning). It has been shown that combined BLT and antidepressant treatment decreases the proinflammatory profile (as defined by peripheral circulating neutrophil count) in SAD patients [306]; BLT alone reduces peripheral proinflammatory cytokines including IL-6 and TNF- α [43].

Sleep is known to affect neuroinflammation. In the current study, BLT led to longer nighttime sleep bout length in both sexes, indicating better sleep quality or less sleep fragmentation in these animals. In male C57BL/6J mice, an acute sleep disturbance (1 day) was sufficient to induce memory impairments and neuroinflammation as indicated by higher IL-6 protein levels and number of microglia in the CA1 [307]. In male Wistar rats, chronic sleep restriction (only 6 hours permitted per day over 21 days) also caused memory impairments and increased hippocampal IL-6 and TNF-α [308]. In humans, sleep duration and sleep disturbance affect inflammatory cytokine levels [309-311]. For instance, a recent study reported that sleep restriction from 8 to 6 hours per night in a small sample of both men and women led to enhanced

monocytosis; this effect was attributed to alterations in the epigenome of hematopoietic stem and progenitor cells, which in turn primed these cells for heightened inflammatory responses [312]. There is also a link between poor sleep quality and neuroinflammation, suggested by their comorbidity in dementia, and neuroinflammation has even been proposed as a mediator of the relationship between sleep disturbance and neuropathology in dementia [313].

In addition to regulating sleep/wakefulness, we also found that BLT alters expression of hypothalamic orexin and its receptors [259]. The central orexin system is another potential pathway through which BLT modulates neuroinflammation [140, 171, 181-184]. Orexin administration attenuates production of TNF-α and IL-6 [172, 174], whereas orexin-knockout mice show increased TNF-α, IL-6, and activated microglia [172, 174]. In humans, preliminary trials suggest that intranasal orexin dampens pro-inflammatory response in cardiac patients [175]. Our findings of region-specific changes in IL-6 and TNF-α expression within the brain of diurnal grass rats, combined with our findings in the orexinergic system, suggest that orexin and neuroinflammation could be potential mechanisms underlying the effects of BLT, which could be further tested in clinical studies.

**Conclusion**

In the current study, we investigated the impacts of BLT on sleep/wakefulness, daily rhythms, the central orexin system, and neuroinflammatory markers using a diurnal rodent, the Nile grass rat. This study contributes critical information about the behavioral and neural effects of BLT, which are not well understood despite BLT's widespread use in treating human psychiatric and neurological disorders [38, 46, 314]. Utilizing a diurnal rodent, as opposed to common nocturnal laboratory rodents, allows for more comparable behavioral outcomes and a potentially translatable mechanistic understanding of diurnal humans. It is particularly valuable

to use a diurnal model when studying the effects of light on sleep/arousal, daily rhythms, and the wakefulness-promoting orexin system. We demonstrated that early morning BLT given daily for 4 weeks leads to changes in the expression of orexin, neuroinflammatory, and neuroplasticity markers in corticolimbic brain regions involved in regulating affective and cognitive functions. Consistent with our previous findings in grass rats housed in winter- or summer-like lighting conditions [145] [305] the effects of BLT are brain region-specific and differ between males and females. The results from the present and follow-up studies will contribute to more effective and personalized treatment protocols for BLT and to novel light-based therapeutics for preventing and treating mood and cognitive disorders. The present study serves as the first step toward a better understanding of the neural mechanisms through which BLT improves sleep, mood, and cognitive function, using a diurnal rodent, particularly through the orexinergic system.

**CHAPTER 3:** Testing the hypothesis that orexin modulates wakefulness/sleep, anhedonia, and neuroinflammation

Emerging research suggests a complex interplay between orexin and mood regulation, particularly in the context of depression and sleep disorders [135, 136, 146, 166, 181, 192, 315]. While orexin's exact mechanisms in these conditions remain under investigation, studies indicate its involvement in modulating emotional responses, stress resilience, and the regulation of sleep architecture. Understanding the intricate relationship between orexin, depression, and sleep holds promise for the development of novel therapeutic interventions targeting these interconnected systems to improve both mental health outcomes and sleep quality.

**Determination of effects of OXA on wakefulness/sleep and daily rhythms**

Orexin is a prominent regulator of the wake/sleep cycle, due to its arousal and wakefulness-promoting effects [316]. Loss of orexin neurons and orexinergic signaling is associated with narcolepsy, where patients are unable to maintain a wakefulness state [317]. Daytime fatigue and nighttime sleep disturbances are associated with both narcoleptic and depressive patients, suggesting a disruption of orexin signaling in depression [318]. Intranasal OXA administration in humans and non-human primates has been demonstrated to promote wakefulness, improve short term memory and attention deficits associated with sleep loss [192, 319, 320]. Similarly, ICV injection of OXA alone increases arousal and wakefulness in healthy rats [321]. Daily intranasal OXA administration in male grass rats improved spatial memory and increased phosphorylation of $CA^{2+}$/calmodulin-dependent protein kinase II α and glutamate receptor 1 in the CA1[11]. In the current experiment, sleep/wakefulness and locomotor activity were recorded during treatment, similarly to Chapter 2. These data allow for measurements of the effects of OXA administration on arousal, sleep quality, and daily rhythms. It is predicted that

the presence of OXA in the current experiments will improve daytime wakefulness and nighttime sleep bouts through a non-photic circadian cue compared to those receiving aCSF. The OXA group is expected to resemble the sleep and activity of the brLD controls as treatment progresses throughout week 4.

**Determination of effects of OXA on anhedonia**

Anhedonia, or lack of interest in rewarding and enjoyable activities, is described by the DSM-V as the "decreased ability to experience pleasure from positive stimuli" [322]. While anhedonia alone is not a whole picture of affective state, it is a major component of major depressive disorders [323]. It is often tested in rodent models via sweet-solution preference (SSP) to understand psychological stress, as well as assess antidepressant medication[324-326]. Grass rats housed in dimLD show anxiety-like behavior, behavioral despair, and anhedonia compared to those housed in brLD [6, 8, 54, 55].

The current experiment aims to assess affective state during OXA administration, however it is critical to use a noninvasive behavioral test when studying *baseline* neuroinflammatory state, particularly if that task involves repeated stress or handling [327-329]. Neuroinflammatory state is sensitive to physical and psychological stressors and is reactive to activation of the HPA-axis [330-333]. Microglia activation occurs quickly and is still detectable up to a week following acute stress or injury [249, 334-337]. While several behavioral tests assessing different aspects of affective state would more definitively show antidepressant effects of OXA in the current study, the activity data and brain tissue would not be able to be utilized. The use of SSP and monitoring of sleep/arousal allows use of the animals' brains, creating the opportunity to directly compare resting neuroinflammatory state to behavior within subjects if needed. Furthermore, the effects of dimLD on behavioral despair have already been well

established in our group; adding a forced swim test to the current project's approach would provide further support to the SSP and sleep behavior, but at the cost of repeating the entire experiment (n = 68) specifically for this behavioral task.

The SSP protocol for the current experiments was selected due to its non-invasive nature. Testing occurred over 72 hours during the last 4 days of OXA/vehicle administration, to ensure behavior was reflective of the full extent of treatment. The saccharin was only available in the last 24 hours (morning Day 27- morning Day 28), as introducing and removing the saccharin could cause changes in food-seeking locomotor activity or could be perceived as loss of a reward [338-340]. Animals had access to food *ad libitum* throughout testing, to prevent acute stress from food deprivation [338].

As anhedonia is a common symptom in SAD and other depressive disorders, it was predicted that control animals in dimLD will show less saccharin preference than those treated with OXA [231, 341, 342]. Subjects in the OXA treatment group could demonstrate improvements in affective state comparable to groups housed in brLD [6, 54, 55]

**Determination of effects of OXA on expression of neurotrophic factors and inflammatory cytokines**

Neuroinflammatory response often exerts its cytotoxic effects through reductions in synaptic plasticity, by altering signaling of neurotrophic markers such as BDNF and TrkB [247, 250-252]. This is in direct contrast to orexin, which is associated with upregulation of markers of neuroplasticity [140, 183]. For similar reasoning in Chapter 2, this experiment examines the mRNA expression of BDNF and TrkB throughout corticolimbic brain regions. Doing so will also help create a more comprehensive understanding of neuroinflammatory state, as pro- or anti-inflammatory expression is likely to be negatively or positively correlated with downstream

effects on plasticity, respectively. Consistent with earlier findings, neuroplastic responses in the brain are expected to be region- and sex- specific [95] [12, 113].

Neuroinflammatory state is a balance of pro- and anti-inflammatory mediators, and examination of both types of cytokines will yield a more complete picture of the neural environment if there's disruption of this balance. Similar to Chapter 2, this experiment examines the expression of microglia marker CD11b and pro-inflammatory cytokines TNF-α and IL-6 throughout the mPFC, BLA, and CA1. In addition, this experiment quantifies anti-inflammatory markers IL-4 and IL-10. IL-4 is reliably expressed throughout corticolimbic brain regions and modulates depressive behavior in rats by inhibiting pro-inflammatory cytokine synthesis and microglia activation [343-345]. IL-10 also has neuroprotective properties and has demonstrated seasonal expression, peaking in the spring and summer [346, 347]. OXA has been demonstrated to suppress pro-inflammatory cytokines while upregulating anti-inflammatory cytokines, both in vivo and in vitro [174, 348] [349] [172, 174]. It is predicted that direct OXA administration will buffer pro-inflammatory marker expression, and instead upregulate expression of IL-4 and IL-10. As cytokine expression has demonstrated sex- and brain-region specific responses in previous work from our lab, it is also expected to in the current project in a similar manner to dimLD and brLD housing [12, 95].

**Determination of effects of OXA on microglia and astrocytes**

Both microglia and astrocytes in normal physiological conditions aid in the protection and survival of neurons, but dysfunction of these cells in response to a prolonged threat or stressor negatively impacts neuronal survival [350, 351]. Over-active microglia and astrocytes, as defined by proliferation, accelerate the process of neuronal death [350, 352]. During activation these glial cells both have the capacity to express OX1R, suggesting that orexin can play a direct

modulatory role in their inflammatory response [179, 180]. OXA administration has been shown to abolish increased proliferation of astrocytes [353] and microglia [172, 354], as well as change morphological properties of microglia.

Microglia have been demonstrated to be brain region-dependent in proliferation and morphological state, thought to represent adaptive functional responses specific to the neural area [355, 356]. In a healthy central nervous system, resting microglial cells have a ramified morphology characterized by long, thin branching [96, 108]. Upon pro-inflammatory stimuli, microglia can take on a wide range of phenotypes, changing morphological properties depending on the nature and duration of the stressor. Hyper-complex and hyper-ramified microglia are associated with chronic stress and prolonged inflammatory response [107, 129, 357]. These microglia have exaggerated processes and branching beyond basal ramification state and have a large or irregularly shaped soma [96, 107, 111]. Quantitative morphology is necessary to distinguish between the phenotypes, especially between resting ramified and hyper-ramified [358, 359]. OXA administration is expected to be anti-inflammatory and neuroprotective, through decreasing pro-inflammatory related microglial phenotype as well as microglia and astrocyte proliferation.

**Experimental approach**

***Subjects***

Male and female adult grass rats (*Arvicanthis niloticus*) were obtained from the breeding colony at Michigan State University [253]. Animals were housed under a 14:10 hr light-dark cycle (light phase beginning 6:00am) in Plexiglas cages (43 × 23 × 20 cm) with a metal tube provided for enrichment. Food (PMI Nutrition Prolab RMH 2000, Brentwood, MO, USA) and water were available *ad libitum*. Experimental animals were randomly assigned into two housing

conditions of either winter-like dim light (dimLD, ~50 lux at cage level as described in previous studies [9, 55]) or summer-like bright light (brLD, ~1000 lux at cage level) during the day for 4 weeks. The animals in dimLD (n=14/sex/condition) received ICV infusion of either orexin-A (OXA) or aCSF during week 4 throughout the beginning of the light phase, ZT 0-6 (Fig. 14). The brLD (n = 6/sex/condition) controls also received infusion of aCSF throughout week 4. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) and have been approved by the Institutional Animal Care and Use Committee of Michigan State University.

***Surgeries and OXA infusion***

Following two weeks of dimLD or brLD habituation, animals underwent a surgical procedure to have a cannula and minipump implanted (Fig. 14). Animals were anesthetized throughout the surgical process using a continuous intranasal flow of 2.5% isoflurane while fixed on a stereotaxic apparatus. A stainless steel 28-gauge cannula (Alzet, cat#0008663) was implanted into the lateral ventricle (anterior -0.6mm, lateral ± 1.0mm, ventral -3.0mm; from bregma). This cannula was attached with polyethylene tubing to a programmable minipump (iPRECIO SMP-310R, Alzet) placed subcutaneously in the lower thoracic region of the animal's back. The minipumps were placed with at least 4cm of tubing to allow the animal full range of movement. Following cannula and minipump implantation the animals were given a subcutaneous injection of meloxicam (2mg/kg body weight) and had access to oral meloxicam (1.5mg/ml) for 4 days in their drinking water for pain management. After a recovery period of 7 days, the minipumps were programmed to release either 3 µl of OXA (5nmol; California Peptide Research, cat#471-99) or a vehicle solution (aCSF) continuously at a rate of 0.5µl/hr for a 6-hour period (ZT0-ZT6) from days 22-28.

This dose has been used previously in our group for intranasal administration, and was re-verified during pilot experimentation for these dissertation projects [11]. Higher dosages, such as 10 nmol, have been reported to result in anxiety-like behavior [360]. A lower dose of 2.5 nmol was tested, though this dose did not yield changes in sleep or arousal compared to control animals and thus was not utilized. Faster flow rates, such as 1.0µl/hr and 0.5µl/min were also tested during pilot experimentation, yielding increased wakefulness during both the day and night or no significant changes in wake/sleep, respectively. OXA was infused from ZT0-ZT6 during the animals' natural peak of orexin secretion [149], as this experiment aims to enhance orexin and mimic natural release as though the animals were housed in brighter conditions.

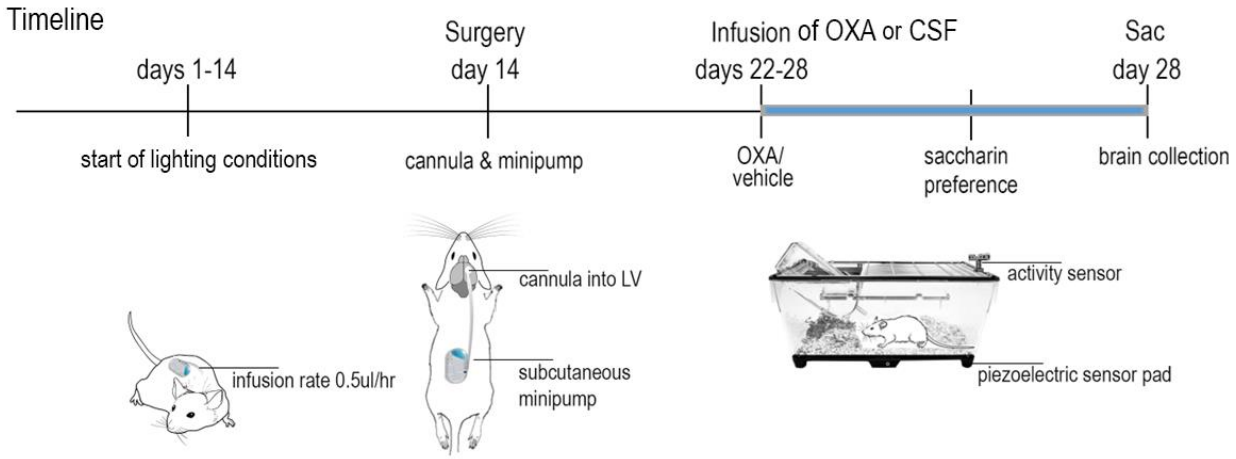


**Figure 14:** Surgical and behavioral timeline. Animals were habituated to lighting conditions for 14 days. Subjects underwent minipump and cannulation surgery on day 14 and were given one week to recover. Infusion of OXA or aCSF began on day 22 and lasted 7 days. Behavioral testing took place during the last 4 days of infusion. Animals were euthanized on day 28.

***Monitoring sleep/wakefulness***

Sleep/wakefulness was monitored continuously during the week of OXA/vehicle infusion using a piezoelectric system (Signal Solutions, Lexington, KY) in the same manner as previously described in Chapter 2 [259].

***Monitoring in-cage locomotor activities***

In-cage locomotor activities were monitored in animals throughout the week of treatment using an Actimeter system, an internet of things (IoT) sensor platform (Geocene Inc., Vallejo, CA), in the same manner as previously described in Chapter 2 [259].

***Saccharin solution preference testing***

Anhedonia can be reliably measured in animal models of depression using sweet-solution preference [341, 342, 361]. Grass rats (n = 14/sex/condition) underwent saccharin-preference testing on days 25-28, during the last 4 days of OXA/vehicle administration (Figure 15). Subjects were provided with two water bottles on day 25, to allow habituation to the presence of the novel bottle. Following the 48-hour habituation one of the bottles was replaced with a solution containing 0.1% saccharin (Sigma, MI) dissolved in water on day 27. The placement of the bottles containing the saccharin solution was counterbalanced. The amount of saccharin and water intake was measured after 24 hours on day 28. The sweet-solution preference (SSP) was calculated as the ratio of saccharin out of total liquid consumed each day. Animals had access to food ad-libitum throughout testing.

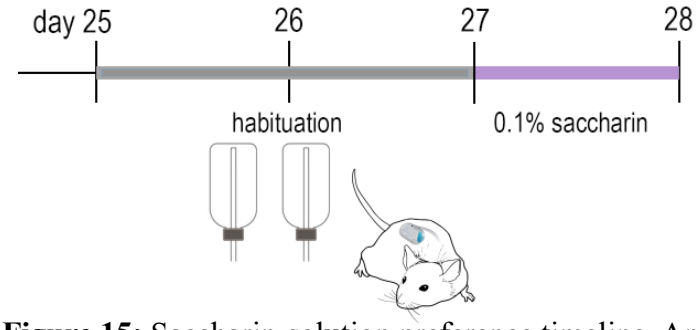


**Figure 15:** Saccharin-solution preference timeline. Animals were habituated to two bottles on days 25 and 26. On day 27, one of the bottles was replaced with 0.1% saccharin solution. Animals were euthanized on day 28, after a full 24 hours with the saccharin solution.

***Immunohistochemistry***

Animals (n = 6/sex/condition) in OXA/aCSF dimLD were euthanized during the last hour of orexin or vehicle infusion with sodium pentobarbital (150 mg/kg) and transcardially perfused with physiological saline followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde and then cryoprotected in 20% sucrose. Three alternate sets of 40-µm coronal sections were collected using a cryostat, with one set used for detecting the microglia marker Iba-1 using a rabbit primary antiserum raised against Iba-1 (1:4000; Wako Chemicals, Japan, RRID: AB_839506) and astrocyte marker anti-glial fibrillary acidic protein (GFAP) (1:1000, Santa Cruz Biotechnology, cat#sc-33673). The specificity of this Iba-1 antibody has been validated in a previous study [362]. Sections were incubated first in the Iba-1 and GFAP primary antiserum at 4 °C for 72 hrs and then in a fluorophore-conjugated secondary antibody (cy3 Donkey anti-rabbit, 1:1000, Jackson ImmunoResearch, AB_2338000; cy2 Donkey anti-mouse, 1:1000, Jackson ImmunoResearch, AB_2340820) at 4 °C for 24 hrs. Sections were mounted onto gelatin-coated glass slides, and incubated in DAPI (1:1000, NucBlue, ThermoFisher Scientific, cat # R37606) for 5 minutes, and cover slipped using Fluoromount-G Mounting Medium (ThermoFisher Scientific, Waltham, MA).

***Microglia and astrocyte number***

Iba-1 and GFAP immunoreactivity was visualized using a fluorescent microscope (Nikon Eclipse Ni-U, model 942498, Nikon Instruments Inc.; NY, USA). Photomicrographs captured at 20x from 4 to 6 sections for each brain region per animal were analyzed for Iba-1-immunoreactive (ir) and GFAP cells by an experimenter naïve to the subjects' experimental conditions. ImageJ (Version 1.8.0, RRID: SCR_003070) was used to count cells in the mPFC regions CG1 and CG2, CA1, and BLA corresponding to the images shown on plates 11-14, 29–

35, and 28–33, respectively, of a laboratory rat brain atlas [363]. A standardized square template (600x600 μm) was fit over each brain region, and cells were counted within the representative template. Total area analyzed ($\mu m^2$) was determined for each brain site and converted into cells per $mm^2$, for a more comparable analysis among the three sites.

***Microglia morphology***

For each brain region, 15 microglia identified by Iba-1-ir were selected at random from three micrographs per region taken by a 40× objective lens, for a total of 45 microglia per region. A six-image z-series was collected for each image to ensure visualization of glial processes. Microglia soma perimeter and area were traced and calculated using NIS-Elements 5.02 software. Microglia visible on the raw image were skeletonized and analyzed for branch length and endpoints, using ImageJ as described in previous studies [12, 364]. Branch length and number of endpoints on the skeletonized branches were analyzed to demonstrate the extent of microglia ramification and complexity [107, 365, 366]. Briefly, the image file was first converted into an 8-bit greyscale image and then color inverted to better visualize the positive Iba-1 staining. The image was further processed using an FFT bandpass filter, an unsharp mask filter, and a despeckle filter to remove noise and to sharpen the image while ensuring the cell maintains its original pixel radius. The image was then converted into binary form and a final despeckle step was applied. The picture was overlaid with the original to confirm dimensions. The image was then skeletonized and analyzed with the AnalyzeSkeleton 2D/3D plugin software package for branch length and endpoint measures[364]. Artifacts and skeleton fragments resulting from the IHC and the image acquisition process were removed from the analyses [364].

***RT-qPCR***

Animals (n=8/sex/condition) were euthanized with CO2, followed by rapid decapitation during the last hour of OXA or vehicle administration. Whole brains were flash frozen and stored at -80 °C. Brains were then sectioned at 200 µm via cryostat and thaw mounted onto slides. The mPFC, CA1, and BLA were punched from the slices using a 1-mm micropuncher (Harris Micropunch, Hatfield, PA). RNA was extracted with Qiagen RNeasy Plus Mini kits (Qiagen, Valencia, CA, Cat# 74134), and quantified using a Qubit Flex Fluorometer (Thermo Fisher Scientific). The RNAs were converted to cDNA using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA, Cat# 4368814).

The RT-qPCR was conducted using a SYBR green Master mix consisting of 5 ng cDNA and 0.25 µM of each primer set. Reactions were performed in triplicate. Primers for pro-inflammatory markers, anti-inflammatory markers, and neuroplasticity markers were designed based on corresponding sequences in Nile grass rats. Forward and reverse primer sequences used for pro-inflammatory markers CD11b, TNFa, IL-6, and the "housekeeping" gene HPRT1, anti-inflammatory markers IL-10 and IL-4, and neuroplasticity markers BDNF and TrkB are listed in Table 2. CT values were automated by the QuantStudio-5 Real-Time PCR System analysis software (Applied Biosystems, 272511214). The ΔΔCT method was used to calculate the level of gene expression in each condition relative to the aCSF controls.

| Primer | Forward Sequence | Reverse Sequence |
|---|---|---|
| CD11b | GCA GAC TTG CAA GGG TTC AG | GGA GGT ATC TTA CTC TTC GCT |
| TNF- α | GGT TTT CTC CAC CAA GGA AGT TTT C | TCT GCT TGC TGC CTG TGC |
| IL-6 | TCT ACT AGA GCC TAG TGA GCT CTG C | ACA TGA GTC AGA TAC CCG ACG A |
| HPRT1 | CTC ATG GAC TGA TTA TGG ACA GGA | GCA GGT CAG CAA AGA ACT TAT AGC C |
| IL-10 | AAA CTG AAA CCC GAG GGG TG | TTC CAA GGA GTT GCT ACC GT |
| IL-4 | CTG CGC CAT GAA TGA GTC CA | TAA AGC ACG GAG GTT CGG TTC |
| BDNF | GTC CCG CTA TCA AAA CCG CA | GCC TTC CTT CGT GTA ACC CCA |
| TrkB | TGC ACA TCG CTC AGC AAA TCG | ATC GGA TGG GCA ACA TTG TGT G |

**Table 2**: Forward and reverse primer sequences for CD11b, TNF- α, IL-6, HPRT1, BDNF, TrkB, IL-10, and IL-4.

### *Statistical analysis*

Data sets were examined for outliers (Grubb's Test for a Single Outlier), normality (Shapiro–Wilk's tests), and homogeneity of variance (Levene's tests). A mixed-model analysis with an autoregressive lag1 structure was used to analyze the behavioral time-series data within conditions and sex. Tukey's HSD post-hoc tests were used to further analyze differences between groups at individual time points of interest. Two-way between-subjects ANOVAs (light condition × sex) were performed for activity onsets/offsets and saccharin solution preference. Two-way between-subjects ANOVAs (light condition × sex) were performed for cell counts, microglia morphology, and mRNA expression of markers of interest. Significant main effects and interactions were followed by Tukey's HSD post-hoc tests to further analyze the effects of treatment within each sex. Statistical significance for all tests is indicated by $p < 0.05$.

## Results

### *Effects of OXA on wakefulness*

During the six-hour infusion, females in the dimLD OXA and brLD aCSF groups had significantly higher wakefulness compared to the dimLD aCSF group ($F_{2, 19} = 7.375$, $p = 0.004$).

This was not observed in males (ps > 0.05) (Fig. 16A and A'). There was no significant difference in wakefulness between the dimLD treatment groups in either sex when wakefulness was averaged for the six-hours post-infusion, though the brLD aCSF males had a higher wake percentage compared to the dim light groups ($F_{2, 19} = 4.557$, $p = 0.02$) (Fig. 16A and A'). The temporal dynamic of OXA's wakefulness-promoting effects during the six-hour treatment was further analyzed at 30-minute intervals (Fig. 16B, B'). There was evidence of anticipatory wakefulness in the dimLD OXA ($p = 0.002$) and brLD aCSF ($p = 0.003$) females prior to lights on and the beginning of infusion compared to dimLD aCSF (5:30, $F_{2, 18} = 10.49$, $p = 0.001$), which was not observed in males in the dimLD OXA or aCSF groups (ps > 0.05). There were numerous group differences throughout and post infusion (6:30, $F_{2, 18} = 3.67$, $p = 0.46$; 9, $F_{2, 18} = 5.84$, $p = 0.01$; 10, $F_{2, 18} = 4.51$, $p = 0.02$; 11, $F_{2, 18} = 4.02$, $p = 0.03$; 11:30, $F_{2, 18} = 5.27$, $p = 0.01$; 12, $F_{2, 18} = 3.92$, $p = 0.03$; 12:30 $F_{2, 18} = 10.46$, $p = 0.001$; 13, $F_{2, 18} = 3.68$, $p = 0.04$). Females that received orexin had significantly higher wakefulness than their dimLD aCSF counterparts at 5:30 ($p = 0.002$), 10 ($p = 0.03$), 11:30 ($p = 0.01$), 12 ($p = 0.03$), 12:30 ($p = 0.01$), and 13 ($p = 0.03$), as revealed through Tukey's post hoc testing. In males, there were no time points in which the OXA treated animals had significantly higher wakefulness compared to the dimLD aCSF group (ps > 0.05). At the end of infusion, dimLD aCSF males actually had significantly higher wakefulness compared to the OXA males ($p = 0.007$). In-cage locomotor activity was also assessed throughout treatment (Fig. A1). Females in the OXA-treated group had significantly earlier onsets compared to the dim-light vehicle group ($F_{1, 10} = 5.51$, $p = 0.04$) and significantly later offsets ($F_{1, 10} = 5.36$, $p = 0.04$) (Fig 16C). Similarly, OXA-treated females had a significantly longer active duration ($F_{1, 10} = 7.39$, $p = 0.02$) compared to the dim-light vehicle group (Fig. A1). There were no significant differences between groups in males (ps > 0.05).

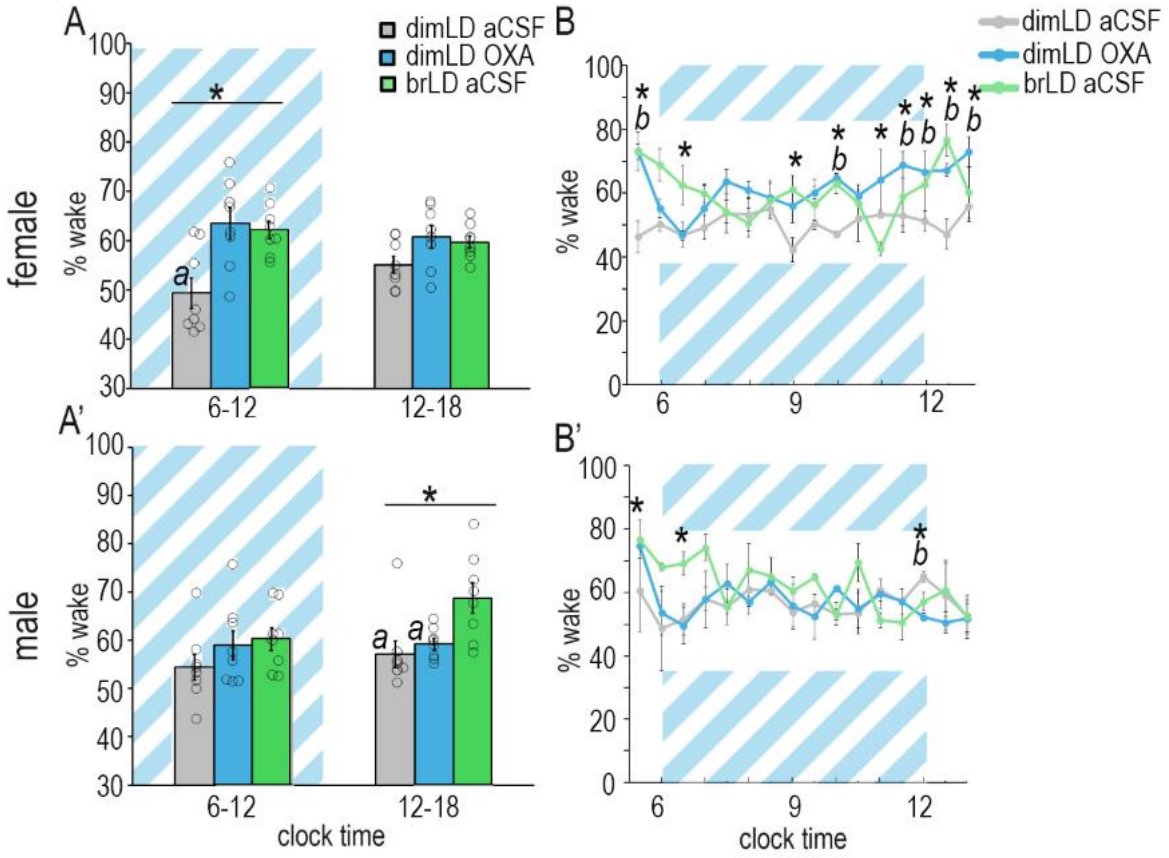


**Figure 16**: OXA administration promotes wakefulness in females. Higher percent of wakefulness was observed in dimLD OXA and brLD aCSF groups compared to the dimLD aCSF control group when averaged during treatment in females (A) but not in males (A'). The percentage of time in wakefulness was further compared in 30-minute intervals immediately prior to, during, and after infusion in females (B) and males (B'). The shaded area represents the infusion window. Data are shown as Mean ± SEM, n = 8/dimLD aCSF/OXA, n = 6/brLD aCSF, with "*a*" indicating significant difference from the brLD aCSF control condition in the bar charts, and "*b*" indicating significant differences between the dimLD aCSF and dimLD OXA groups in the line graphs. * $p < 0.05$.

***Effects of OXA on sleep***

A clear day/night difference in percentage of time spent sleeping was observed with higher sleep at night in both females (Fig. 17A, $F_{1, 42} = 544.71$, $p < 0.001$) and males (Fig. 17A', $F_{1, 42} = 459.16$, $p < 0.001$), which was expected given the diurnal nature of Nile grass rats. There was no significant effect of light condition or interaction between light and time in the percentage of sleep during the day or night, nor were there sex differences (ps > 0.05).

Consistent with the percentage of time spent sleeping, sleep bout length also showed a day/night difference with longer sleep bouts at night in both females (Fig. 17B, $F_{1, 40} = 114.97$, $p < 0.001$) and males (Fig. 17B', $F_{1, 40} = 193.47$, $p < 0.001$). There was a nearly significant effect of condition during the day in females, with dimLD aCSF groups having longer sleep bouts compared to the dimLD OXA and brLD aCSF animals ($F_{2, 19} = 3.48$, $p = 0.053$) (Fig. 17B). In males, the dimLD aCSF group had significantly shorter sleep bouts at night ($F_{2, 19} = 6.12$, $p = 0.01$).

Further analysis at 1-hour intervals revealed treatment group differences in sleep bout length at specific times of the night (Fig. 17C, C'). In females, there were no points in time over the course of the night where the orexin-treated animals had significantly longer sleep bouts than the dimLD aCSF group (ps > 0.05). Instead, towards the end of the night dimLD aCSF females had significantly longer sleep bouts (4:00, $F_{1, 21} = 4.67$, $p = 0.02$) (Fig. 17C). Males, however, in the dimLD OXA and brLD aCSF groups had significantly longer sleep bout lengths throughout the night compared to the dimLD aCSF controls (20:00, $F_{1, 21} = 5.8$, $p = 0.01$; 21:00, $F_{1, 21} = 5.73$, $p = 0.01$; 22:00, $F_{1, 21} = 13.64$, $p < 0.001$; 23:00, $F_{1, 21} = 15.51$, $p < 0.001$; 1:00, $F_{1, 21} = 6.01$, $p = 0.01$; 2:00, $F_{1, 21} = 18.59$, $p < 0.001$; 3:00, $F_{1, 21} = 5.705$, $p = 0.01$; 4:00, $F_{1, 21} = 5.44$, $p = 0.01$) (Fig. 17C').

The distribution of bout lengths also presented differently in females and males (Fig. 17D, D'). In females (Fig. 17D), there was a clear peak shift towards longer sleep bouts from day to night in the dimLD OXA and brLD aCSF groups that was not observed in the dimLD aCSF group. The orexin treated and bright light housed females had a peak in daytime sleep bout lengths at 240-480 seconds, while their peak in nighttime sleep bout lengths was 480-960 seconds (Fig 17D). The peak in sleep bout length for dimLD aCSF females was 480-960 seconds

during both the day and night (Fig 17D). During the day, dimLD OXA and brLD aCSF females had significantly fewer and shorter bouts (60-120 s, $F_{1, 11} = 4.97$, $p = 0.02$; 120-240 s, $F_{1, 11} = 5.6$, $p = 0.01$), while dimLD aCSF females had longer bouts (480-960 s, $F_{1, 11} = 5.14$, $p = 0.01$; 960-1920 s $F_{1, 11} = 8.16$, $p = 0.003$). In males, all groups had a clear peak shift from shorter sleep bouts during the day to longer sleep bouts during the night (Fig 17D'). There were no significant differences in sleep bout frequency during the day between conditions, however at night the dimLD OXA and brLD aCSF males had significantly longer bout lengths compared to the dimLD aCSF controls (1920-3840 s, $F_{1, 11} = 4.4$, $p = 0.03$) (Fig 17D').

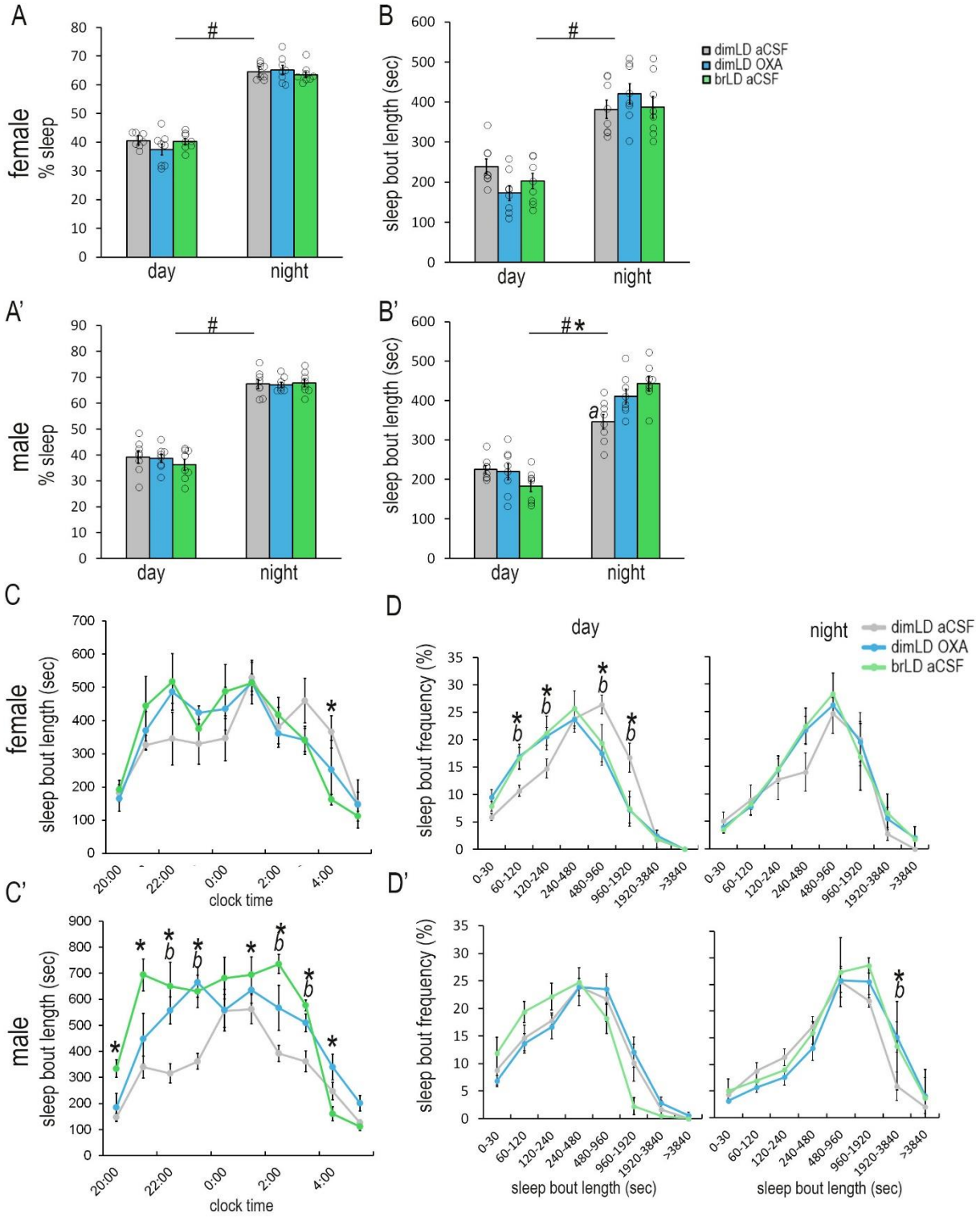


**Figure 17:** Quantity and quality of sleep. Overall sleep percentages during the day and night were not significantly different between conditions in either females (A) or males (A'). Overall sleep bout length during the day also did not differ between conditions in females (B) or males (B'), although dimLD OXA and brLD aCSF males had significantly higher nighttime sleep bout

**Figure 17 (cont'd)**
length compared to the dimLD aCSF group (B'). Analysis of sleep bout length in 1-hour intervals during the animals' dark phase revealed longer sleep bouts in the dimLD OXA and brLD aCSF groups compared to the dimLD aCSF group in males (C'), but not in females (C). Significant differences in sleep bout frequency were observed during the day in females (D) and the night in males (D'). Data are shown as Mean ± SEM, n = 8/dimLD OXA/aCSF, n = 6/brLD aCSF, with "*a*" in the bar charts indicating a significant difference from the brLD aCSF control condition in the bar charts, and "*b*" indicating a significant difference between the dimLD aCSF and dimLD OXA conditions. * indicates significant effect of condition, # indicates significant effect of time. $p < 0.05$.

***Effects of OXA on anhedonia***

Both females and males in the dimLD OXA and brLD aCSF groups had a significantly higher saccharin-solution preference compared to their dimLD aCSF counterparts (effect of condition, $F_{2, 42} = 8.67$, $p = 0.001$; effect of sex $F_{1, 42} = 0.006$, $p = 0.93$; interaction $F_{2, 42} = 0.57$, $p = 0.57$) (Fig. 18). There were no significant differences in SSP between the dimLD OXA and brLD aCSF groups (ps > 0.05). All subject groups, including the dimLD aCSF females ($M = 62.31$) and males ($M = 56.45$), were above the 50% chance level for saccharin preference when analyzed both collectively ($t_{63} = 9.03$, $p < 0.001$) and by condition (dimLD aCSF, $t_{25} = 4.23$, $p = 0.003$; dimLD OXA, $t_{25} = 7.18$, $p < 0.001$; brLD aCSF, $t_{11} = 5.62$, $p < 0.001$). This suggests that the animals preferred saccharin at a baseline, and anhedonia in this instance is measured as which group preferred it less.

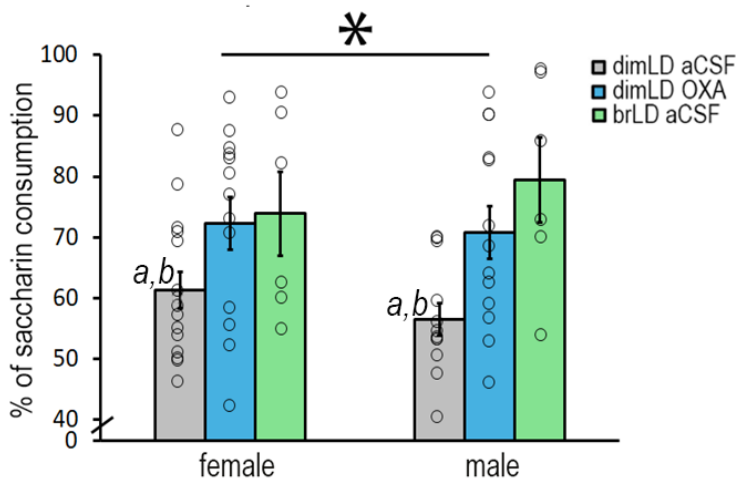


**Figure 18:** Saccharin solution preference. Both females and males in the dimLD OXA and brLD aCSF had a significantly higher SSP compared to the dimLD aCSF groups. Data are shown as Mean ± SEM, n = 14/dimLD OXA/aCSF, 6/brLD aCSF, with "*a*" indicating a significant difference from the brLD aCSF control condition, and "*b*" indicating a significant difference between the dimLD aCSF and dimLD OXA conditions. *indicates significant effect of condition, $p < 0.05$.

***Effects of OXA on synaptic plasticity markers***

There were no significant effects of treatment, sex, or an interaction for expression of BDNF in the mPFC (effect of condition: $F_{1, 37} = 0.3$, $p = 0.74$; effect of sex: $F_{1, 37} = 0.5$, $p = 0.49$; interaction: $F_{1, 37} = 0.35$, $p = 0.71$), BLA (effect of condition: $F_{1, 37} = 0.11$, $p = 0.89$; effect of sex: $F_{1, 37} = 0.005$, $p = 0.94$; interaction: $F_{1, 37} = 0.01$, $p = 0.99$), or CA1 (effect of condition: $F_{1, 37} = 2.12$, $p = 0.13$; effect of sex: $F_{1, 37} = 1.16$, $p = 0.29$; interaction: $F_{1, 37} = 0.04$, $p = 0.95$) (Fig. 19). Similarly, there were no significant effects of treatment, sex, or condition in the expression of TrkB in the mPFC (effect of condition: $F_{1, 36} = 1.34$, $p = 0.27$; effect of sex: $F_{1, 36} = 0.84$, $p = 0.35$; interaction: $F_{1, 36} = 0.5$, $p = 0.6$) or CA1 (effect of condition: $F_{1, 37} = 0.69$, $p = 0.5$; effect of sex: $F_{1, 37} = 1.57$, $p = 0.21$; interaction: $F_{1, 37} = 0.61$, $p = 0.54$) (Fig. 19). There was however a significant effect of both condition and sex in the BLA (effect of condition: $F_{1, 36} = 4.84$, $p = 0.01$; effect of sex: $F_{1, 36} = 9.56$, $p = 0.004$; interaction: $F_{1, 36} = 3.15$, $p = 0.055$), with TrkB

expression being upregulated in the female brLD aCSF group compared to dimLD OXA ($p <$ 0.001) and dimLD aCSF ($p = 0.02$) groups, and males having higher overall expression (Fig. 19).

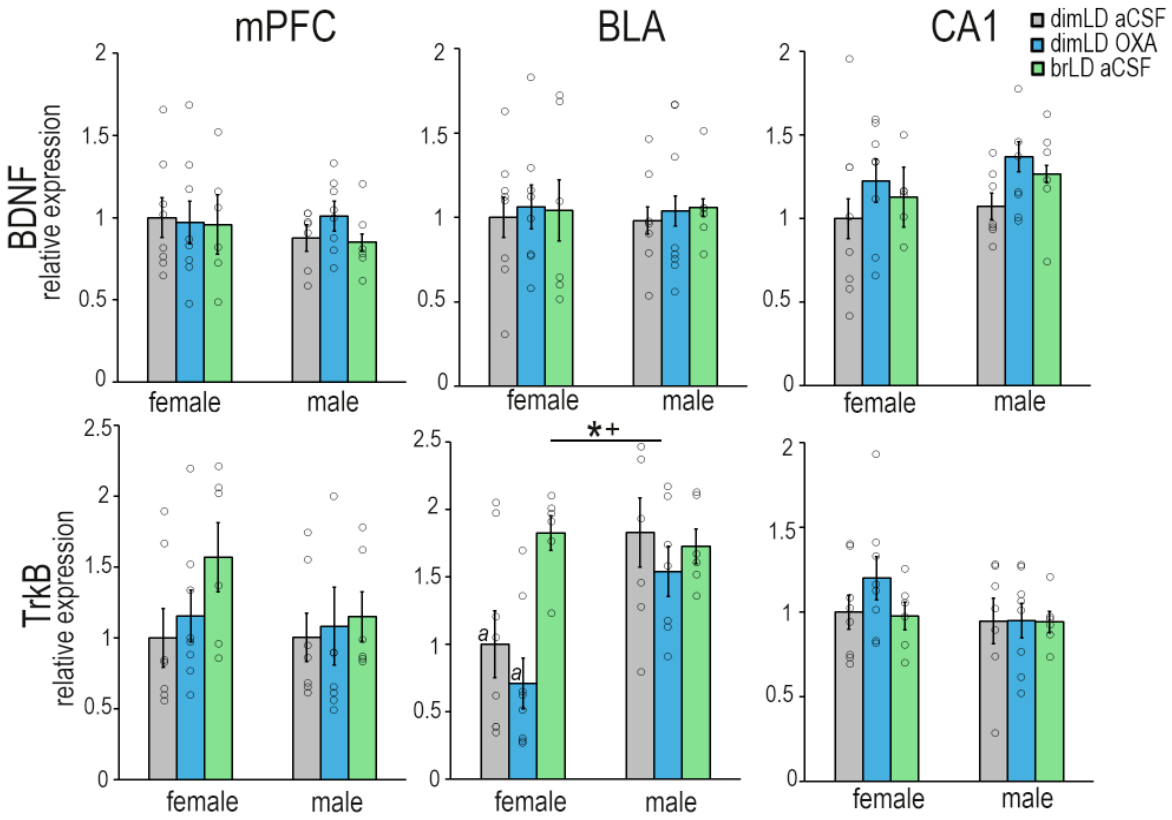


**Figure 19:** Levels of mRNAs for neuroplasticity markers BDNF and TrkB in the mPFC, BLA, and CA1 of females and males. Infusion of OXA did not significantly affect neuroplasticity markers in any of the regions analyzed. Females in the brLD aCSF group had higher TrkB expression than those in the other conditions in the BLA. Data are shown as Means ± SEMs, n = 8/dimLD OXA/aCSF, n = 6/brLD aCSF, with "*a*" indicating a significant difference from the brLD aCSF control condition. *indicates significant effect of condition, + indicates significant effect of sex. $p < 0.05$.

***Effects of OXA on anti-inflammatory markers***

In the mPFC, there was a significant effect of sex and a significant interaction between sex and condition in the expression of IL-4 (effect of sex: $F_{1, 37} = 7.14$, $p = 0.01$; effect of condition: $F_{1, 37} = 0.88$, $p = 0.42$; interaction: $F_{1, 37} = 3.94$, $p = 0.02$) (Fig. 20). Females had higher expression overall of this anti-inflammatory cytokine compared to males, and expression was highest in females who received OXA infusion ($p = 0.02$) (Fig. 20). Similarly, there was a

significant interaction between sex and condition in the mPFC expression of IL-10 (effect of sex: $F_{1, 36} = 3.59$, $p = 0.06$; effect of condition: $F_{1, 36} = 2.32$, $p = 0.11$; interaction: $F_{1, 36} = 3.13$, $p = 0.05$). Tukey's post-hoc analysis revealed that this was driven by upregulation in the female dimLD OXA group ($p = 0.002$), which had significantly higher expression compared to the dimLD aCSF ($p = 0.03$) and the brLD aCSF ($p = 0.001$) groups (Fig. 20).

In the BLA, there was a significant effect of sex and a significant interaction between sex and condition in the upregulation of IL-4 (effect of sex: $F_{1, 35} = 7.41$, $p = 0.01$; effect of condition: $F_{1, 37} = 3.15$, $p = 0.055$; interaction: $F_{1, 36} = 3.54$, $p = 0.04$), with females in the dimLD aCSF group having significantly higher expression than the dimLD OXA ($p = 0.03$) and brLD aCSF groups ($p = 0.01$) (Fig. 20). There was no significance in sex, condition, or an interaction between the two in BLA expression of IL-10 (effect of sex: $F_{1, 36} = 3.32$, $p = 0.07$; effect of condition: $F_{1, 36} = 1.16$, $p = 0.32$; interaction: $F_{1, 36} = 1.71$, $p = 0.19$) (Fig. 20).

In the CA1, there was a significant effect of condition in the expression of IL-4 (effect of sex: $F_{1, 36} = 0.87$, $p = 0.35$; effect of condition: $F_{1, 37} = 4.56$, $p = 0.02$; interaction: $F_{1, 37} = 2.08$, $p = 0.13$) (Fig. 20). There was collectively higher expression of IL-4 in the dimLD OXA groups compared to the dimLD aCSF ($p = 0.04$) and brLD aCSF ($p = 0.009$), though this effect was driven by the males (Fig. 20). When analyzed by sex, IL-4 expression in the OXA treated males was increased compared to their dimLD aCSF counterparts ($p = 0.01$), but not brLD aCSF ($p = 0.07$). The expression of IL-10 followed a similar pattern. There was a significant effect of condition (effect of sex: $F_{1, 36} = 1.562$, $p = 0.22$; effect of condition: $F_{1, 37} = 13.53$, $p < 0.001$; interaction: $F_{1, 37} = 2.93$, $p = 0.06$). The OXA treated animals had significantly higher expression overall compared to the dimLD aCSF animals ($p = 0.004$), and this upregulation was male led.

Males that received OXA had significantly higher IL-10 expression than those that received aCSF in dimLD ($p = 0.005$) and brLD ($p = 0.01$).

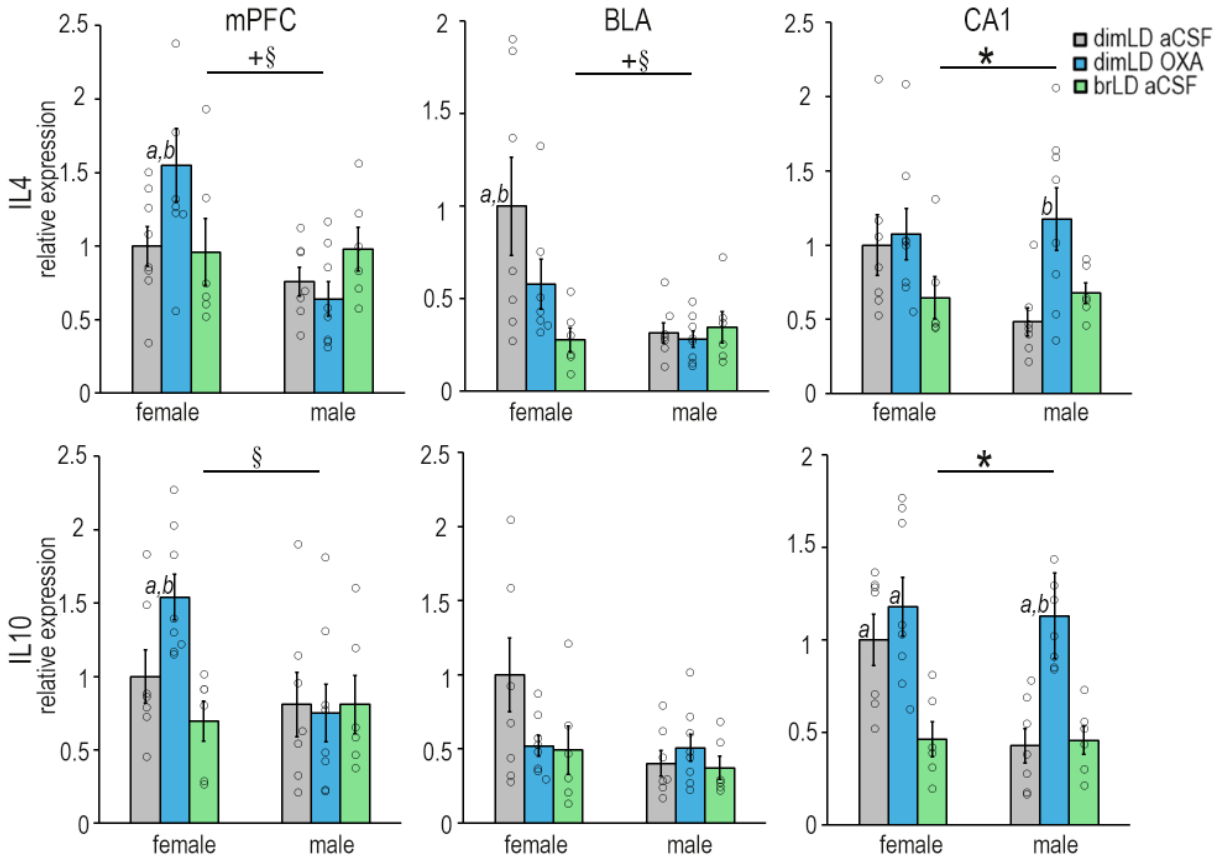


**Figure 20:** Levels of mRNAs for anti-inflammatory markers IL4 and IL10 in the mPFC, BLA, and CA1 of females and males. IL-4 and IL-10 were significantly upregulated in OXA-treated females in the mPFC and CA1, and in OXA-treated males in the CA1. Females had significantly higher IL-4 expression in the BLA compared to males. Data are shown as Means ± SEMs, n = 8/dimLD OXA/aCSF, 6/brLD aCSF, with "*a*" indicating a significant difference from the brLD aCSF control condition, and "*b*" indicating a significant difference between the dimLD aCSF and dimLD OXA conditions. *indicates significant effect of condition, + indicates significant effect of sex, § indicates significant interaction. $p < 0.05$.

***Effects of OXA on pro-inflammatory markers***

In the mPFC, there was a significant difference in TNF-α expression between sexes, but not groups (effect of sex: $F_{1, 33} = 6.2$, $p = 0.018$; effect of condition: $F_{1, 33} = 2.01$, $p = 0.15$; interaction: $F_{1, 33} = 1.07$, $p = 0.35$) (Fig. 21). There was overall higher upregulation of IL-6 in the

dimLD aCSF animals, though there were no effects of sex or an interaction (effect of sex: $F_{1, 36}$ = 0.73, $p$ = 0.39; effect of condition: $F_{1, 36}$ = 7.03, $p$ = 0.003; interaction: $F_{1, 36}$ = 1.33, $p$ = 0.27). However, there was significant upregulation of microglia marker CD11b (effect of sex: $F_{1, 37}$ = 1.45, $p$ = 0.23; effect of condition: $F_{1, 37}$ = 4.21, $p$ = 0.02; interaction: $F_{1, 37}$ = 0.71, $p$ = 0.49). This effect was driven by females, with the dimLD aCSF group having higher expression than both the dimLD OXA and brLD aCSF groups ($p$ = 0.03).

In the BLA, females had significantly higher expression of both TNF-α (effect of sex: $F_{1, 37}$ = 13.84, $p$ = 0.001; effect of condition: $F_{1, 37}$ = 2.45, $p$ = 0.1; interaction: $F_{1, 37}$ = 0.21, $p$ = 0.81) and IL-6 (effect of sex: $F_{1, 36}$ = 10.52, $p$ = 0.003; effect of condition: $F_{1, 36}$ = 1.95, $p$ = 0.16; interaction: $F_{1, 36}$ = 2.11, $p$ = 0.13) than males, regardless of condition (Fig. 20). There were no significant differences in CD11b expression in sex or condition (effect of sex: $F_{1, 36}$ = 2.93, $p$ = 0.09; effect of condition: $F_{1, 36}$ = 1.39, $p$ = 0.26; interaction: $F_{1, 36}$ = 0.52, $p$ = 0.59). Similarly, in the CA1 there was an effect of sex but not of condition were no significant differences in expression of TNF-α (effect of sex: $F_{1, 36}$ = 0.51, $p$ = 0.48; effect of condition: $F_{1, 37}$ = 0.62, $p$ = 0.52; interaction: $F_{1, 37}$ = 1.05, $p$ = 0.35) or CD11b (effect of sex: $F_{1, 37}$ = 2.26, $p$ = 0.14; effect of condition: $F_{1, 37}$ = 2.22, $p$ = 0.12; interaction: $F_{1, 37}$ = 0.15, $p$ = 0.85). IL-6 had higher expression in females, though there were no effects of treatment (effect of sex: $F_{1, 35}$ = 5.08, $p$ = 0.03; effect of condition: $F_{1, 35}$ = 2.54, $p$ = 0.09; interaction: $F_{1, 35}$ = 0.81, $p$ = 0.45).

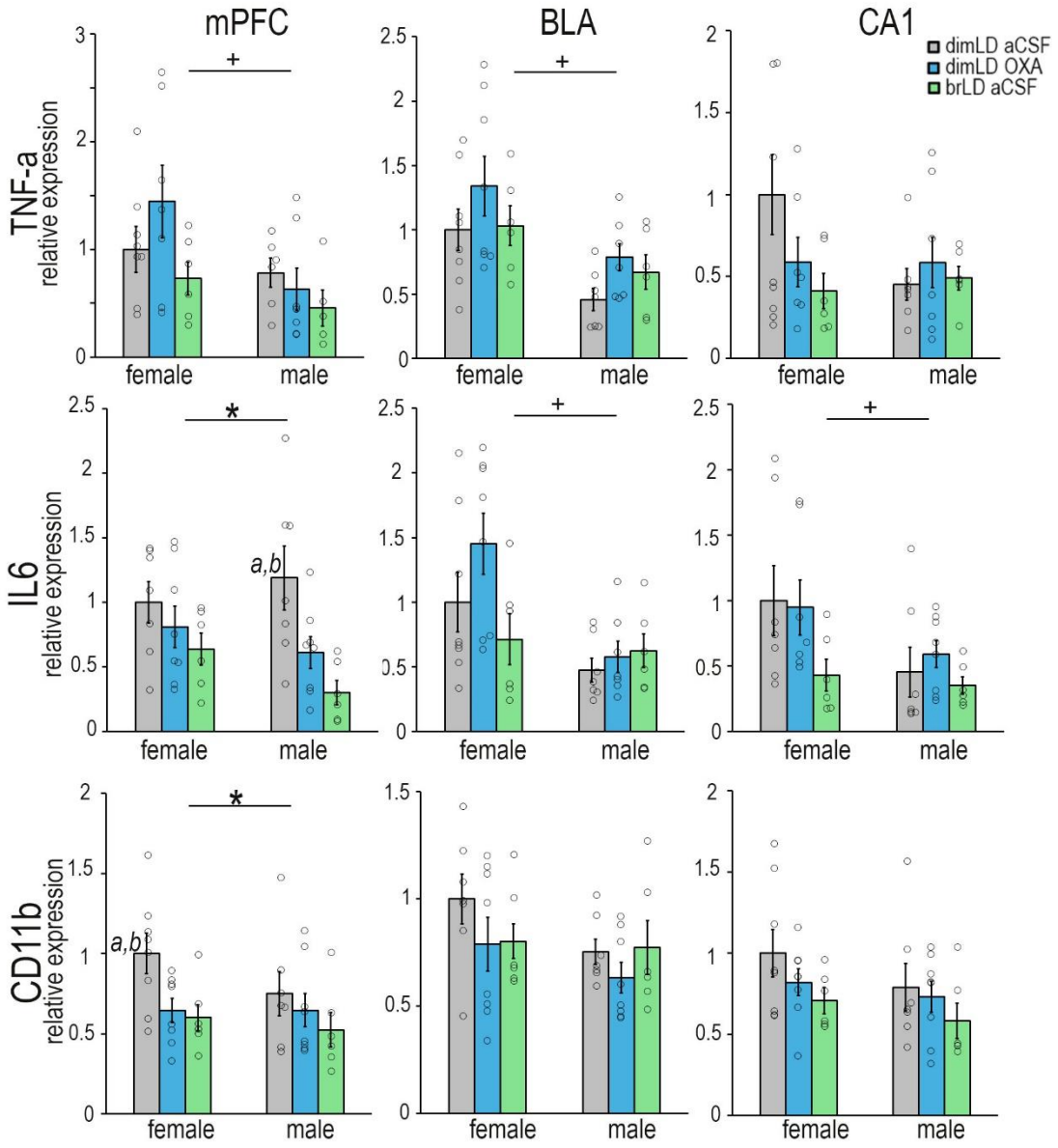


**Figure 21:** Levels of mRNAs for pro-inflammatory markers TNF-a, IL-6, and CD11b in the mPFC, BLA, and CA1 of females and males. Data are shown as Means ± SEMs, n = 8/dimLD OXA/aCSF, 6/brLD aCSF, with "*a*" indicating a significant difference from the brLD aCSF control condition, and "*b*" indicating a significant difference between the dimLD aCSF and dimLD OXA conditions. *indicates significant effect of condition, + indicates significant effect of sex. $p < 0.05$.

***Effects of OXA on glial proliferation and morphology***

In the mPFC there was a significant decrease in the number of Iba-1 labelled microglia (effect of sex: $F_{1, 20} = 0.23$, $p = 0.63$; effect of condition: $F_{1, 20} = 5.13$, $p = 0.03$; interaction: $F_{1, 20}$

= 0.2, $p = 0.65$) in the OXA-treated groups compared to the dimLD aCSF groups regardless of sex (Fig. 22A). This was also observed in the number of GFAP labelled astrocytes, particularly in the males (effect of sex: $F_{1, 20} = 7.63$, $p = 0.01$; effect of condition: $F_{1, 20} = 8.19$, $p = 0.01$; interaction: $F_{1, 20} = 0.69$, $p = 0.41$) (Fig. 23). There were no significant differences in microglia soma area (effect of sex: $F_{1, 20} = 0.41$, $p = 0.52$; effect of condition: $F_{1, 20} = 1$, $p = 0.32$; interaction: $F_{1, 20} = 0.31$, $p = 0.86$) or branch length (effect of sex: $F_{1, 20} = 3.57$, $p = 0.07$; effect of condition: $F_{1, 20} = 0.06$, $p = 0.79$; interaction: $F_{1, 20} = 2.15$, $p = 0.15$), though both OXA-treated females and males had significantly more endpoints than the dimLD aCSF groups (effect of sex: $F_{1, 20} = 1.12$, $p = 0.3$; effect of condition: $F_{1, 20} = 6.68$, $p = 0.01$; interaction: $F_{1, 20} = 0.89$, $p = 0.35$) (Fig. 22 B, C, D).

In the BLA, there was also a significant decrease in the number of microglia (effect of sex: $F_{1, 20} = 0.78$, $p = 0.38$; effect of condition: $F_{1, 20} = 3.99$, $p = 0.05$; interaction: $F_{1, 20} = 0.01$, $p = 0.89$) and astrocytes (effect of sex: $F_{1, 20} = 2.27$, $p = 0.14$; effect of condition: $F_{1, 20} = 6.09$, $p = 0.02$; interaction: $F_{1, 20} = 0.09$, $p = 0.76$) in the dimLD OXA groups compared to the dimLD aCSF groups (Fig. 22A, 23). Microglia soma area was significantly decreased in the OXA-treated animals (effect of sex: $F_{1, 20} = 0.02$, $p = 0.86$; effect of condition: $F_{1, 20} = 4.93$, $p = 0.03$; interaction: $F_{1, 20} = 0.04$, $p = 0.83$) (Fig. 22B). There were overall treatment differences in branch length (effect of sex: $F_{1, 20} = 1.86$, $p = 0.18$; effect of condition: $F_{1, 20} = 4.9$, $p = 0.03$; interaction: $F_{1, 20} = 0.55$, $p = 0.46$), though OXA administration decreased summed branch length in the females and increased summed branch length in the males (Fig. 22C). There were no effects of sex or condition on number of endpoints (effect of sex: $F_{1, 20} = 1.07$, $p = 0.31$; effect of condition: $F_{1, 20} = 2.81$, $p = 0.1$; interaction: $F_{1, 20} = 0.18$, $p = 0.67$) (Fig. 22D).

In the CA1, OXA treatment significantly lowered the number of Iba-1 microglia in females (effect of sex: $F_{1, 20} = 6.20$, $p = 0.02$; effect of condition: $F_{1, 20} = 5.02$, $p = 0.03$; interaction: $F_{1, 20} = 2.02$, $p = 0.15$) (Fig. 22A, Fig. A2). Orexin administration marginally decreased GFAP astrocyte number in males (effect of sex: $F_{1, 20} = 0.06$, $p = 0.81$; effect of condition: $F_{1, 20} = 3.79$, $p = 0.06$; interaction: $F_{1, 20} = 1.78$, $p = 0.19$) (Fig. 23). Each measure of microglia morphology was significantly decreased by OXA administration in both sexes, including soma area (effect of sex: $F_{1, 20} = 0.01$, $p = 0.93$; effect of condition: $F_{1, 20} = 5.92$, $p = 0.02$; interaction: $F_{1, 20} = 0.001$, $p = 0.98$), branch length (effect of sex: $F_{1, 20} = 0.12$, $p = 0.73$; effect of condition: $F_{1, 20} = 5.11$, $p = 0.03$; interaction: $F_{1, 20} = 0.42$, $p = 0.52$), and number of endpoints (effect of sex: $F_{1, 20} = 1.73$, $p = 0.84$; effect of condition: $F_{1, 20} = 5.14$, $p = 0.03$; interaction: $F_{1, 20} = 0.48$, $p = 0.49$) (Fig. 22 B, C, D).

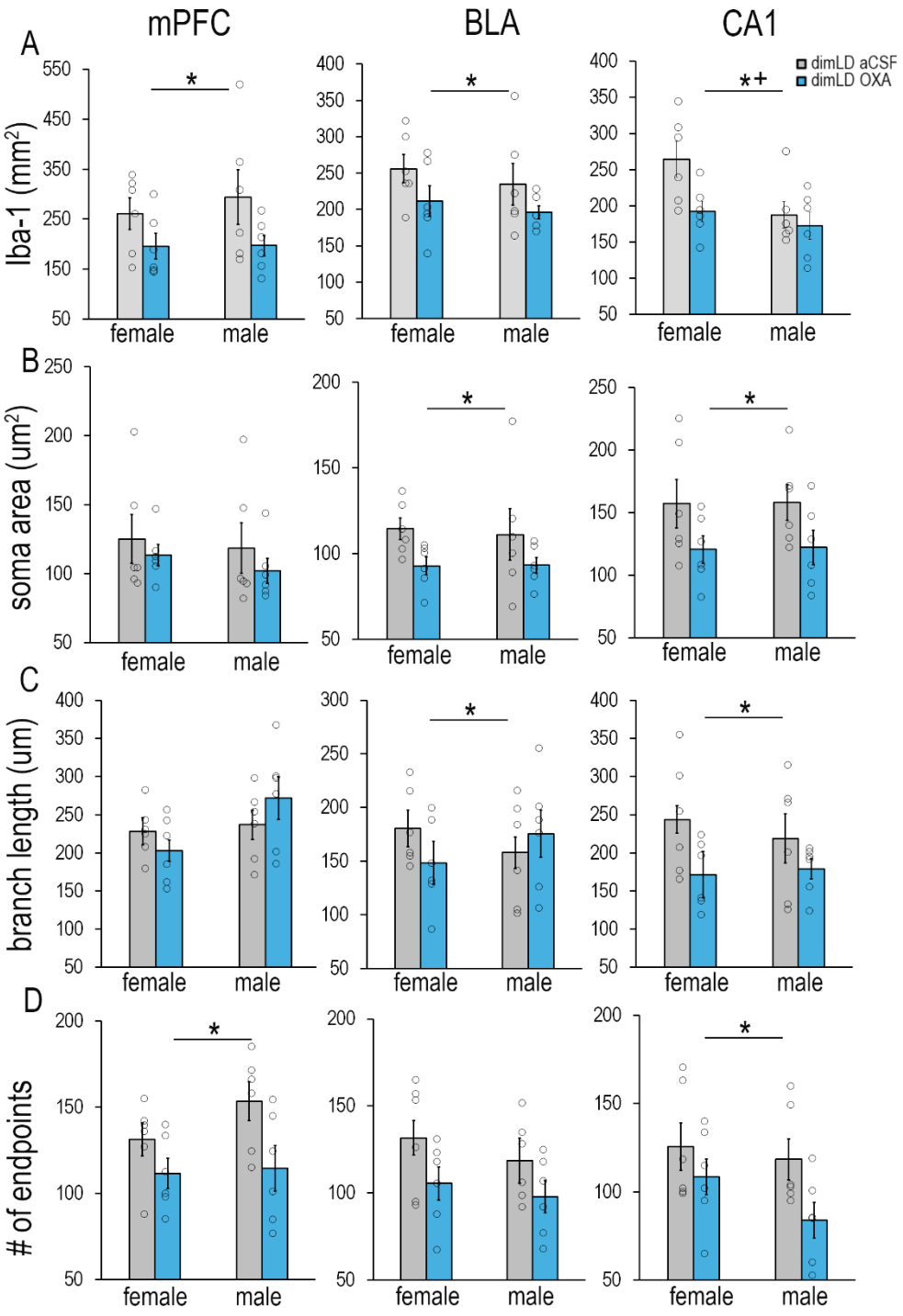


**Figure 22:** Effects of OXA administration on microglia proliferation and morphology in the mPFC. BLA, and CA1. Quantitative analysis of Iba-1-ir (A) cell density (per $mm^2$). Microglia soma area ($\mu m^2$) (B), summed branch length ($\mu m$) (C), and mean number of endpoints per cell (D). Data are shown as Means ± SEMs, n = 6/group. *indicates significant effect of condition, + indicates significant effect of sex. $p < 0.05$.

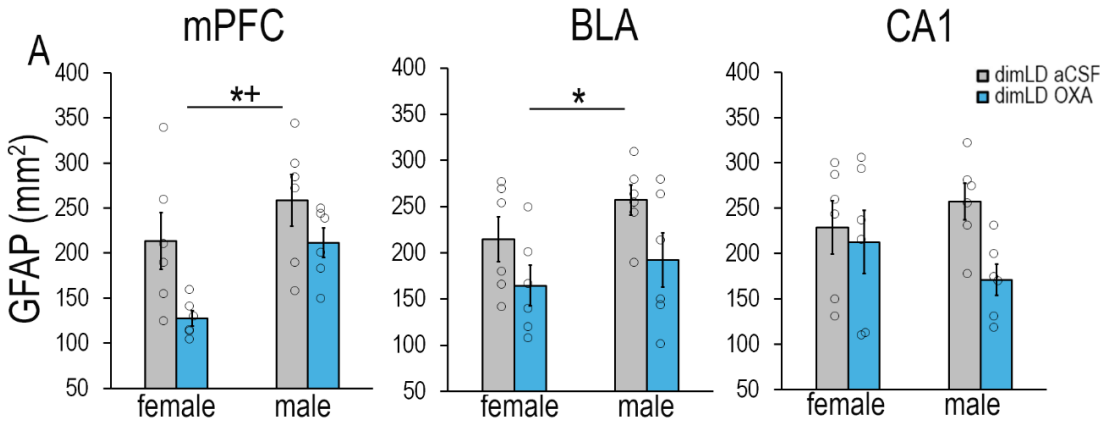


**Figure 23:** Effects of OXA administration on astroglia proliferation. Quantitative analysis of GFAP cell density (per $mm^2$). Data are shown as Means ± SEMs, n = 6/group. *indicates significant effect of condition, + indicates significant effect of sex. $p < 0.05$.

**Discussion**

This study tested whether orexin administration alone could modulate wakefulness, sleep, anhedonia, neuroplasticity, and neuroinflammatory markers, building upon prior findings of the effects of BLT on the orexinergic system. Orexin infusion had sex-specific effects: it increased wakefulness in females and improved sleep quality in males, with each group mimicking behaviors seen under bright light conditions. Both sexes, however, showed reduced anhedonia with OXA treatment, with saccharin preference similar to bright light control animals. In the brain, OXA upregulated anti-inflammatory markers IL-4 and IL-10 in a sex- and region-specific manner, decreased markers of microglia and astrocyte activation, and did not significantly affect neuroplasticity markers or pro-inflammatory cytokines. These findings indicate that orexin's protective effects may be due in part through increasing anti-inflammatory actions and interactions with microglia and astrocytes, rather than targeting pro-inflammatory cytokines or neuroplasticity markers directly. These results suggest that orexin modulates SAD-relevant behavioral and neural responses in a sex- and region-dependent manner.

***Effects of OXA on wakefulness and sleep***

During the six-hour infusion, the dimLD OXA females showed more wakefulness when compared to that of the dimLD aCSF females (~65% dimLD OXA vs 50% dimLD aCSF) (Fig. 16A). They were not significantly different in their percentage of time spent awake than females that were housed in brLD (Fig. 15A). This is similar to females during the hour of bright light therapy (~75% BLT vs 50% dimLD) (Fig. 3). However, unlike the BLT animals, wakefulness and arousal was only affected in the females. Males that were treated with orexin had no differences in wakefulness before, during, or after infusion, while males that received BLT had a higher percentage of wake during the light exposure (~80% BLT vs 50% dimLD). When analyzed in 30-minute bouts, these wakefulness effects in dimLD OXA females were more apparent during the last half of the six-hour infusion and persisted afterwards (Fig. 16B).

The orexin treated and brLD aCSF females also demonstrated anticipatory wakefulness before the beginning of the light phase and infusion, that the dimLD aCSF females did not (Fig. 16B). The presence of OXA alone acted as a salient wakefulness cue, similar to that of bright light. This is reflected in arousal and locomotor activity. Females in the dimLD OXA group had an advance in activity onset compared to the dimLD aCSF group, as well as almost an hour longer active duration (Fig. A1). While these findings are not consistent with the phase-delay and shorter active duration observed in the BLT animals, these findings are consistent with orexin's promotion of wakefulness [135, 136, 146]. Differences in activity could be due to the nature of the photic vs nonphotic cue, orexin delivery via ventricle rather than direct infusion into a particular brain region, as well as duration of light vs duration of orexin infusion. In mice that were given chemotherapy drugs, ICV infusion of OXA improved active-phase fatigue and lethargy in voluntary locomotor behavior, as well as increased active duration [367]. Similar to

our findings, the largest increases as well as anticipatory wakefulness were observed during the beginning of the mice's active phase [367]. ICV infusion of OXA also increased motor function during active phase in a Parkinson's rat model [368]. Fragmentation of wakefulness in the early active period was found to progress with severity of orexin neuronal loss in a mouse model of orexin neurodegeneration, suggesting that orexin's endogenous arousal effects may be strongest at the beginning of the active phase [369].

There was a clear day/night difference in the percentage of time spent asleep and in sleep bout length in both sexes of grass rats, regardless of treatment condition (Fig. 17). This is similar to the BLT cohorts, as well as previous research in diurnal rodents [272]. Males in the dimLD OXA group had significantly longer average sleep bouts at night compared to dimLD aCSF males. Sleep bout length was increased throughout the night in orexin treated males, while there was not a time point at night in which dimLD OXA females had a significantly longer sleep bout length than the dimLD controls. While there appears to be a trend towards longer sleep bouts in the beginning of the night, there was wide individual variability in the females that prevented significance (Fig. 17 C). Males also had a higher frequency of longer sleep bouts at night, indicating better sleep consolidation (Fig. 17 D'). This was not observed in females, further suggesting a sex difference in the arousal effects of orexin in sleep maintenance. Females instead that were treated with orexin or in the brLD aCSF groups had higher frequencies of shorter sleep bouts during the day compared to the dimLD aCSF group, consistent with increased daytime wakefulness. The dimLD OXA and brLD aCSF females had a peak in daytime bout length frequency of 240-480 seconds, which shifted to a peak of 480-960 seconds at night (Fig. 17 D). The dimLD aCSF females did not exhibit a shift in peak of sleep bout frequency between day and night, suggesting that they were sleeping in longer 480-960 second bouts regardless of active

phase. In orexin-ablated mice, sleep decreased during both the inactive and active phase; this was reversed with orexin treatment [369]. Orexin receptor agonists, developed for the treatment of narcolepsy, reduce sleep fragmentation, and increase sleep latency in both humans and rodents [192, 370, 371]. Orexin treatment, similar to BLT, leads to more consolidated sleep and better sleep quality.

OXA administration in humans and non-human primates is associated with promotion of wakefulness and reduction of nighttime sleep loss [192, 319, 320]. These effects could also be dependent on the duration of infusion. When the treatment week in the current study was analyzed by individual day during pilot testing, wakefulness and sleep bout length showed a clear change over time. Mid-day and pre-dark phase wakefulness was apparent after the first day of infusion, though the anticipatory pre-infusion wakefulness developed between days 3-6. Sleep bout length dramatically changed throughout the week of infusion, with the nighttime bout length increasing in the OXA animals and actively decreasing in the control animals as the days progressed. These findings justify the OXA infusion length; many OXA ICV infusion paradigms are short term, lasting between 1-3 days [372-374]. There is a need to study long-term orexin administration effects, as therapeutic effects in sleep and wakefulness would not have been observed in the current study if infusion were to have only lasted 1-3 days.

***Effects of OXA on anhedonia***

Anhedonia in the current study served as a behavioral analysis of depressive-like symptoms. Sweet-solution preference operates under the premise that a reduction in saccharine intake is indicative of lack of pleasure from enjoyable stimuli [340]. SSP is thought to involve activation of nucleus accumbens neurons that project to the ventral pallidum, emphasizing reward [375, 376]. There's great variety in SSP paradigms, with varying time schedules and

sugar/saccharin concentrations, which could all possibly affect sweet solution intake values. There are also strain and species differences in SSP [340]. The current study used a saccharin preference test that has been optimized to the grass rats and constraints of the experimental paradigm. We were limited, in that testing needed to be at the end of the 7-day infusion to reflect full treatment. We also could not remove the bottles before the time of sacking in case the removal of the saccharin could be perceived as a loss of reward [192-194]. The paradigm utilized was two days of habituation, followed by availability of saccharin on the third day during the early active phase. Saccharin was available for 24 hours, until sacking in the morning the next day.

The habituation period in sweet solution preference paradigms is necessary to avoid environmental novelty in test results, possibly affecting behavior and stress response [377, 378]. A meta-analysis showed 80.7% of 335 studies included habituation periods to the presence of two water bottles, though there was wide variation in duration of this habituation [379]. Habituation periods ranged from 4 hours to 10 days, depending on experimental constraints [379]. Most procedures recommend at least 12 hours of the actual sweet solution exposure, comprising at least a whole phase of the animal's active phase to minimize confounds[376]. Several days of exposure presents a different challenge, in that sweet-solution intake tends to decrease while individual variability in drinking patterns emerge [380-382]. Data from early experimentation of various SSP paradigms in the grass rats suggests that saccharin preference over 24 hours is not an effect of novelty, as saccharin preference did not significantly change across several days of saccharin access.

In the present study, the percentage of saccharin solution preference was significantly increased in the dimLD OXA and brLD aCSF groups compared to dimLD aCSF groups in both

sexes (Fig. 18). This suggests that the presence of orexin buffered anhedonia to the extent of bright light housing [6, 55]. These findings are consistent with other studies of orexin and anhedonia. Optogenetic activation of orexin terminals in the ventral tegmental area (VTA) increased sucrose consumption in a mouse model of depressive-like state [383]. Increased orexinergic signaling in the mPFC, VTA, and LH are associated with decreased anhedonia, despite variability in SSP methodology [384, 385] [383]. Sex differences have been reported, with female rats exhibiting lower SSP compared to males [386]. A sex difference in SSP was not observed in the current study, though there was a large amount of individual variation in each group. This could be due to the longer duration of saccharin exposure and emergence of individual drinking patterns.

***Effects of OXA on neuroplasticity***

We had previously found in the BLT cohorts that the expression of BDNF was upregulated in the BLA and CA1 of males that received light therapy, while BDNF expression was downregulated in the CA1 of females that received light therapy [95]. TrkB expression followed a similar pattern in females, with downregulation in the light therapy groups across brain regions. In the current study however, there were no significant differences in OXA treatment or sex for neuroplasticity markers (Fig. 19). The only neurotrophic factor in the current study that had significant upregulation was TrkB in the BLA of brLD aCSF females, which is in direct contrast the downregulation of BLA TrkB in females that underwent light therapy in the Chapter 2 experiments [95]. The lack of change in mPFC and CA1 neurotrophic factor expression was unexpected, as orexin is known to upregulate BDNF and promote synaptic plasticity [140, 183]. This is also in contrast with previous findings from our group, where there was higher levels of BDNF immunoreactivity in males housed in brLD [9, 10]. These differences

may be due to the method of quantification; when we've previously measured BDNF and TrkB protein levels, rather than mRNA between dim and bright conditions, the differences did not reach statistical significance [10].

In previous experiments, our group found that intranasal administration of OXA increased spatial memory and increased phosphorylation of $Ca^{2+}$ /calmodulin-dependent protein kinase II α and glutamate receptor 1 within the CA1 [11]. The concentration of the intranasal OXA was the same as the current experiment, yet the intranasal method resulted in an increase of molecules associated with synaptic plasticity. These discrepancies could be due to the differences in methods. The intranasal procedure involved 7 days of handling and 5 days of actual administration. The OXA solution was dropped onto the animal's nose for absorption, and during this process the animals were wrapped in a towel and scruffed in the palm of the experimenter's hand ventral-side up. This repeated handling, as well as possible restraint stress from being securely wrapped in the towel, could have affected responses in neuroplasticity [387]. In mice, acute restraint stress increased plasticity and facilitated long term potentiation in BLA to PFC microcircuits [388]. In rats, studies have shown that restraint stress increases dendritic spine density in the nucleus accumbens but decreases synaptic connectivity with astrocytes, with these changes enduring up to 3 weeks post-stress [389]. These alterations in plasticity following restraint stress may be specific to the plasticity marker and brain region, as other studies have shown that restraint stress reduces apical dendrites of pyramidal cells in the mPFC and the CA3 of the hippocampus, but not in the orbital PFC [390-392]. In the current study, there was no handling or involvement of restraint stress. However, the animals had undergone surgery, and were possibly still recovering from and adjusting to the cannula, tubing, and minipump in their

bodies. The ICV administration also lasted 7 days, rather than 5, which may have contributed to differences in neuroplasticity marker expression.

The mechanism of action between ICV and intranasal administration for OXA to reach the brain differs. The mechanisms of intranasal orexin delivery into the brain are not completely understood, though it is thought that the transportation of the peptide relies heavily on chemosensory and somatosensory innervation, beginning at the olfactory and trigeminal nerve perineural regions of the nasal epithelium and traveling to the olfactory bulb and sensory/spinal trigeminal region of the pons [393-395]. Once inside the brain, the peptide can then diffuse to different regions. Given the molecular size of OXA, as well as its ability to bypass the blood-brain barrier, the time course of its extracellular transport to the brain is thought to be within an hour [393, 396-398]. However, PET imaging of intranasal radiolabeled OXA in rodents and non-human primates suggests that the OXA is localized to the olfactory bulb and does not diffuse well to other brain regions [399]. Despite this finding, intranasal administration of OXA repeatedly shows behavioral outcomes associated with higher corticolimbic brain regions, such as improvements in short-term memory and alleviation of effects from sleep deprivation [319].

This mode of transport differs from ICV administration, in the current study orexin reached the brain through slow infusion into the lateral ventricle over 6 hours rather than through rapid extracellular transport in the perineural space around the olfactory and trigeminal nerves. This likely affected which brain regions the OXA was able to reach, and for how long it was available. The OXA in the CSF was intended to flow throughout and bathe the brain, rather than traveling mainly through diffusion. As the delivery methods were so different, the dosage of OXA may have also needed to be altered to mimic the exact effects of intranasal orexin on plasticity. In nonhuman primates, the dosage of intravenous administration of OXA needed to be

increased by 10-15 fold to mimic the effects of intranasal OXA on memory improvement [319]. Though not ICV, this highlights the effect that time, delivery method, and delivery route can have on the efficacy of OXA. The differences between previous findings in neuroplasticity are likely from both the method for orexinergic upregulation and the effects of handling or surgery.

OXA's lack of effects could also be attributed to measurement of these neurotrophic factors 14 days post-surgery and 7 days into treatment. Immediately following brain injury, BDNF mRNA expression increases in both the site of injury as well as the hippocampus in a reparative effort [137]. These levels decline after 24 hours, and are no longer significant after 36 hours [400]. TrkB receptor mRNA expression is also upregulated in the hippocampus and dentate gyrus following cortical injury, and also return to resting levels within 36 hours [401]. In human post-mortem tissue, low signaling BDNF genotypes were associated with better protection and healing in acute TBI-related mortality [402, 403]. BDNF is likely temporally dynamic, both in injury and treatment response [402]. BDNF and TrkB could have been upregulated in the current study immediately after surgery, acting as an endogenous neuroprotective response to the presence of the cannula, and then decreased prior to orexin treatment. Neurotrophic factors could have still been depressed following initial inflammatory upregulation from surgery, and orexin treatment would have needed to have been longer to see rebound and upregulation. The BLT paradigm, as well as our typical brLD paradigm, where we noted regional BDNF and TrkB changes lasted 4 weeks in duration. The current study only had one week of treatment. There is not a consensus in the literature on increases of BDNF due to pharmacological treatment alone, possibly due to these temporal or compensatory dynamics [404-406].

***Effects of OXA on anti-inflammatory factors***

To explore the role of orexin as a proponent of anti-inflammatory signaling, we examined the expression of anti-inflammatory cytokines IL-4 and IL-10. Both facilitate neuronal healing and repair, and IL-10 specifically is implicated in pain management following injury [343-345, 407]. We found that IL-4 and IL-10 were both significantly upregulated in orexin-treated females in the mPFC and in orexin-treated males in the CA1 (Fig. 20). This suggests that orexin could be exerting therapeutic effects via enhancement of anti-inflammatory cytokines, rather than enhancement of neurotrophic factors or suppression of pro-inflammatory cytokines. While both the dimLD aCSF and dimLD OXA groups experienced the same chronic daytime light deficiency, the presence of orexin promoted active healing and repair in the mPFC and CA1. The expression of IL-4 and IL-10 in the brLD aCSF groups was consistently low, regardless of sex. This makes sense, as the brLD animals were not undergoing chronic environmental stress from daytime light deficiency that has been shown to promote inflammatory response [305]. There was no need for active ongoing anti-inflammatory response, as there was a lack of an active ongoing threat to the neural environment.

OXA administration has been previously demonstrated to heighten anti-inflammatory response in rodents. In TBI and stroke, the infusion of OXA upregulates anti-inflammatory cytokines both in vitro and in vivo [172, 408, 409] [354] [174]. The effects of this increased neuroprotection and repair can be visualized, in that the area of injury or infarct size is significantly decreased within the same day following orexin treatment compared to controls [172, 408, 409]. In male mice, one dose of intranasal OXA post-intracerebral hemorrhage was enough to upregulate both IL-4 and IL-10 in the cortex [410].

There was no effect of orexin treatment on anti-inflammatory cytokines in the BLA in the current study, although there was a baseline sex difference of higher expression overall of IL-4 in females. This sex difference was also observed in the mPFC, with expression of IL-4 and IL-10 This sex-specific response was not surprising, as sex differences in these cytokines have been previously reported. At the protein level, female mice show a higher baseline of both IL-4 and IL-10 compared to males in the cortex and hippocampus [411, 412]. However, hippocampal mRNA expression of these cytokines have been shown to have a greater magnitude in response to viral threat in males compared to females [411]. This was observed in the current study; the orexin treated males had upregulation of CA1 IL-4 and IL-10 comparable to the dimLD female groups (Fig. 20).

The orexin-driven actions of anti-inflammatory cytokines suggest a potential strategy for mitigating the extent of neuronal damage or pain behaviors triggered by heightened pro-inflammatory response. The upregulation of IL-4 and IL10 could have contributed to the daytime wakefulness and arousal in females, through combatting pro-inflammation induced lethargy. Analyzing a variety of both anti- and pro-inflammatory markers is important for a more complete understanding of inflammatory response.

***Effects of OXA on pro-inflammatory factors***

*Pro-inflammatory cytokine expression*

Both sex- and brain-region specific responses in pro-inflammatory cytokine expression were found in a previous study comparing grass rats chronically housed in dimLD or brLD [305]. The findings of the current study, however, were more similar to the responses of animals given BLT, in that there was a lack of treatment effect (Fig. 21) [95]. There was a wide variety of individual differences in each group in the current study, preventing some of the clearer

expressional trends from reaching significance. For example, TNF-α expression in the CA1 of females and IL-6 expression in the mPFC of males appear to be higher in the dimLD groups compared to the brLD aCSF and orexin-treated group, though there was such a wide range in individual variation that no statistical conclusions can be drawn (Fig. 21). Significance was only found between sexes, in that females had higher expression of TNF-α and IL-6 in the mPFC and BLA regardless of condition compared to the males.

These findings were unexpected, as orexin has been demonstrated to suppress pro-inflammatory cytokine expression through both peripheral and neural administration [174, 348] [349]. The presence of orexin alone can reduce TNF-α and IL-6 expression [172, 174] [349]. In a mouse model of orexinergic neuron degeneration, lack of orexin significantly upregulated expression of TNF-α and IL-6 mRNA, while ICV infusion of OXA reduced this expression to the level of controls [172]. In vitro microglia cell lines suggest that orexin directly inhibits pro-inflammatory response via microglia OX1R and OX2R receptors, as OXA pretreatment decreases microglial production of TNF-α, IL-6, and other apoptotic chemokines [172, 174].

There could be a time-sensitive window for the effects of orexin on pro-inflammatory response. TNF-α and IL-6 may have only been affected during the first few days of treatment, which could have been negligible by the end of the week-long infusion. Many studies that report the effects of orexin on pro-inflammatory cytokines do so within the context of short-term or immediate OXA exposure, typically with an infusion window of 1-3 days or a single microinjection. Few studies examine inflammation following chronic orexin administration, and those that do vary in infusion rate and concentration. Orexin is thought to act quickly in its interactions with microglia. One group reported that OX1R expression on microglia increased immediately after an injection with LPS, peaking at 7 hours post inflammatory threat. This

expression returned to baseline within 24 hours [413]. This pattern is similar to cytokine reaction, responses are highest immediately following inflammatory threat. Cytokine expression has been shown to be strain and homeostatic-threat dependent, with some acute stress paradigms reporting a 48-hour discrepancy in return to baseline cytokine expression [354].

The prediction that pro-inflammatory cytokines would be downregulated in response to OXA was partially based in our previous findings with dimLD and brLD housing, however a major difference between the previous and current paradigm is that these animals in the dissertation experiment underwent surgery [305]. There is a greater inflammatory threat by having underwent surgery, despite the animals having a week of recovery before the infusion began. Following ablation of orexin neurons in mice, microglia were immediately activated but returned to normal phenotype, number, and reactivity after 4 weeks of recovery [369]. The initial shock and recovery from surgery, as well as the presence of the cannula in the brain, could have affected pro-inflammatory cytokine reactivity. There could have been initial upregulation and overactivation to the physical injury, causing a ceiling effect with reactivity and then a subsequent blunted response to the chronic stress of dimLD housing. Some studies have reported that peripheral IL-6 and TNF-α show attenuated responses after an initial event of intense prolonged activation, such as a major injury [414-416].

The lack of effects with pro-inflammatory cytokines could also be due to the method of detection. Cytokine secretion, particularly of interleukins, can be post-translationally regulated [417, 418]. Thus, mRNA measurements may not always reflect the level of protein, which could explain the couple of inconsistencies between cytokine expression and microglia expression observed in the current study. Many proteins optimize cellular function through post-translational modifications, which would not necessarily be comparable to mRNA expression [419-422].

Other methods to measure protein, such as a western blot or ELISA, could be used in conjunction with qPCR in future studies using this tissue, in order to better understand what happened with TNF-α and IL-6 expression in these animals. Additionally, there was no measure of cytokines in the periphery. Elevated plasma levels of pro-inflammatory cytokines have been found in patients during depressive episodes, with levels falling back to normal following antidepressant treatment [126, 423]. Though beyond the scope of the current experiment, this study could have been enhanced with the collection of trunk blood, so that plasma TNF-α and IL-6 could have been analyzed. It may also be that the presence of OXA alone did not act as a direct buffer to pro-inflammatory cytokines; rather, it could predominantly act on anti-inflammatory cytokines or other inflammatory mediators to exert its effects.

*Microglia expression, proliferation, and morphology*

Microglia activation, as measured by CD11b mRNA expression, was only significantly lowered in the orexin groups in the mPFC, largely driven by females (Fig. 21). The presence of OXA buffered this expression to the point that orexin-treated females resembled the brLD females (Fig. 21). Our previous work in nonsurgical and behaviorally naïve cohorts suggested that CD11b was also downregulated in the mPFC of females, but this trend did not reach significance [305]. This sex- and region-specific characteristic has been shown in stroke models; studies with mice have shown that females have higher cortex and PFC CD11b reactivity compared to males [424]. There were trends in the BLA of females and in both sexes in the CA1, though these failed to reach significance. In our previous findings, CD11b was only significantly downregulated in the CA1 of females [305].

The proliferation of microglia followed a similar trend to that of the astrocytes, with orexin buffering the total number of immunopositive cells in the mPFC and BLA of both sexes,

and in the CA1 of females (Fig. 22A). This female-specific CA1 finding is consistent with brLD results from our previous study [305]. All three brain areas showed decreased microglia proliferation in the OXA groups in the current study, unlike our previous findings. This set of experiments analyzed treatment and control groups within dimLD, rather than baseline dimLD vs brLD. These animals had undergone surgery and were housed in an environment meant to invoke SAD-type responses, and OXA administration alone was able to reduce pro-inflammatory microglia response in these animals facing chronic environmental stress.

The expression, number, and morphology of microglia are all indicators of what inflammatory actions are taking place. While proliferation may not increase, the microglia that are already in the area may have changed into an activated phenotype, suggesting that a functional inflammatory response is still taking place. Microglia are diverse in morphological characteristics, and will actively modify their soma shape and area, as well as the shape and length of their processes. The broad morphological spectrum of microglia responses is thought to be related to their diverse range of functions, and are indicative of homeostatic state. The classic phenotypes of microglia are 'ramified' resting and 'active' amoeboid, however genetic, molecular, and pharmacological interventions have revealed a wide variety of microglia phenotypes and physiological roles both in vivo and in vitro[425, 426]. There's an importance in understanding spatiotemporal dynamics of microglia, as it can lead to a better understanding of how microglia contribute to disease state and psychiatric disorders.

Ramified "resting" microglia can be characterized by their small soma and frequent extension and contraction of their processes, which is thought to aid in surveillance of the neural environment and play a neuroprotective role in initial excitotoxicity [108, 115, 427-429]. Amoeboid microglia are the classical activated state with an enlarged soma and retracted

branches, resembling an amoeba. This phenotype occurs in rapid response to injury, infection, or an acute stressor; their main role is apoptosis, and to keep the area phagocytically active in order to remove the perceived threat [430-434]. The endpoints of the processes can change independently of the rest of the cell, with ball-and-chain or bulbous structures on the ends of the branches. This is thought to be involved in phagocytosis and aid in the movement of microglia[429, 435-438]. Rod microglia, named for their rod-like elongated somas and polar processes, form in lines around injured neurons[110, 439, 440], while some "honeycomb" or "jellyfish" microglia are only formed in response to TBI and blood brain barrier damage [441]. Finally, and most relevant to the current study, there's hyper-ramified microglia. These cells are named for their exaggerated stretched branches and branch complexity, beyond that of resting state. They also have an enlarged soma compared to resting state, but not to the same degree as amoeboid. They are thought to be temporally dynamic, in that they form in response to chronic stress and may play a role in stress-related synaptic adaptations [97, 102, 442, 443].

In the current study, microglia in the aCSF dimLD groups had significantly larger soma area than the OXA treated groups in the BLA and CA1 (Fig. 22B). These microglia had longer summed branch lengths in the aCSF dimLD females in the BLA, and both sexes in the CA1 (Fig. 22C). Additionally, microglia in the mPFC and CA1 of the vehicle dimLD groups had an increased average number of branch endpoints compared to the orexin-treated groups (Fig. 22D). The enlarged soma area, increased branch length, and increased number of endpoints are all indicative of the hyper-ramified phenotype, suggesting chronic stress and chronic pro-inflammatory signaling. This morphology is consistent with what we've observed in animals that were housed in dimLD for 4 weeks [305]. These results suggest that orexin administration was neuroprotective, in that the OXA-treated animals had microglia soma areas, branch lengths, and

branch endpoints that were consistent with a resting phenotype observed in brLD [305]. Orexin in this paradigm may interact with microglia and anti-inflammatory cytokines, rather than through TNF-α and IL-6.

Orexin has been demonstrated to affect microglia morphology. In mice, amoeboid microglia in the cortex reduced soma size and increased the length of their retracted processes following an ICV injection of OXA [413]. Similarly, pretreatment with OXA prior to injection with lipopolysaccharide (LPS) prevented the formation of amoeboid morphology. Animals that did not receive orexin treatment had significantly larger soma sizes following LPS [444]. The number of OX1R on microglia have been shown to rapidly increase after LPS, regardless of OXA treatment, suggesting that orexin modulates the functional activity of microglia [413]. This holds clinical relevance, in that orexin-based therapeutic approaches could lessen inflammatory aspects of mood disorders or other health conditions.

*Astrocyte proliferation*

In the current study, we observed a decrease in astrocyte proliferation in orexin-treated animals in both sexes in the mPFC and BLA (Fig. 23). In the mPFC, males had overall higher astrocyte proliferation than females. Astrocytes play a dynamic role in inflammatory response and serve both pro- and anti-inflammatory roles. They can be neuroprotective through metabolic support, antioxidant defense, and the release of neurotrophic factors. Similar to microglia, astrocytes can either aid or impair the survival of neurons, depending on if inflammatory conditions are normal or dysregulated [350, 351]. Following inflammatory threat, microglia will proliferate in the area of perceived injury and release pro-inflammatory cytokines. Cytokines such as IL-6 can cause activation and aggregation of astrocytes, which will prompt them to produce further pro-inflammatory factors, furthering a damaging cycle of sustained damage

[445, 446]. While not the initial responders to inflammatory threat, astrocytes are key in the amplification of inflammatory response and the cascade of signaling that is crucial for transitioning from pro- to anti-inflammatory response. Over-active astrocytes and microglia accelerate the process of neuronal death [350, 352]. Following traumatic threat, both astrocytes and microglia have been shown to express OX1R, suggesting their sensitivity to orexin and the crucial role that the orexinergic system could play in early inflammatory response [179, 180]. OXA infusion could mediate astrocytic inflammation, and has been shown to act specifically on astrocyte's different roles during pro-inflammatory reaction including attenuation of initial activation, astrocytic production of and signaling to pro-inflammatory cytokines, and astrocyte-mediated apoptosis [353] [178]. Our results were consistent with previous OXA infusion studies, in that the presence of OXA alone decreased astrocytic proliferation compared to controls, particularly in the mPFC [353].

Within the mPFC in the current study, males had overall higher astrocyte proliferation than females. Sex differences in astrocytes have been previously reported, though they seem to be brain-region dependent. In vitro, male astrocytes from PFC tissue show greater reactivity to inflammatory insult compared to female-derived cells [447, 448]. However, in the mouse CA1 and dentate gyrus, females have more resting and surveillant astrocytes compared to males [449]. There were no significant differences in sex or OXA treatment in the CA1 in the current study, although there was a trend of lowered astrocytic proliferation in the males. Hippocampal GFAP has been shown to be sensitive to estrous cycle in rats, and its immunoreactivity is highest during proestrus in CA1, CA3, and the dentate gyrus compared to females during diestrus and males [450]. Males had the lowest GFAP content in the dentate gyrus compared to females at any point in their estrous cycle[450]. In the hypothalamus however, male rats have significantly higher

GFAP protein and mRNA compared to females [451]. Males also have higher resting astrocytes in the medial amygdala, though this effect is lost in mutant males lacking androgen receptors [452]. In response to threat, there are noted sex differences in both proliferation and phagocytic capacity. For example, male mice have higher astrocytic release of phagocytic chemokines, and recruit more immune cells to the cortex, both in vivo and in vitro [424, 453]. There is an importance of studying both sexes with inflammatory factors, as sex steroids can have different effects and implications throughout the neuroinflammatory process.

**Conclusion**

This study directly tested if orexin modulates wakefulness, sleep, anhedonia, neuroplasticity, and neuroinflammatory markers, through the use of OXA infusion in the absence of bright light. Orexin infusion affected wakefulness and sleep in a sex-specific manner, with increased wakefulness in females and improved nighttime sleep in males, mimicking behaviors seen in control brLD groups. Both sexes in the OXA treatment group showed a higher SSP than the vehicle dimLD group, suggesting that orexin alone was able to decrease anhedonia. While no significant changes in neuroplasticity markers were found, orexin upregulated anti-inflammatory cytokines IL-4 and IL-10 in the mPFC and hippocampus, suggesting a protective role. Microglia and astrocyte proliferation was decreased across brain regions, and expression of microglia activation marker CD11b was reduced in the mPFC of females that received OXA. This buffering effect is further supported by the vehicle dimLD animals displaying a hyper-ramified and activated proinflammatory microglia phenotype, that was not observed in the OXA-treated animals. These results highlight sex- and brain region-specific effects of orexin on behavior and inflammation, supporting its role as a modulator of responses relevant to SAD. The findings of this study provide further understanding of the orexinergic system as a therapeutic target in

affective disorders, as well as the mechanisms through which light influences the brain and behavior.

# **CHAPTER 4:** Discussion and conclusion

## Effects of BLT on wakefulness/sleep, central orexin, neuroplasticity, and neuroinflammation

Using a diurnal rodent model of SAD, the present study examined the impacts of BLT on sleep/wakefulness, daily rhythms of in-cage locomotor activity, and the central wakefulness-promoting orexin/hypocretin system. The behavioral effects of BLT observed in the current experiment in promoting alertness/wakefulness, improving sleep quality, and entraining daily rhythms are consistent with those reported during BLT in humans [25, 199], supporting the validity of the diurnal grass rat as a model for elucidating the neural mechanisms underlying these therapeutic effects of BLT. The BLT group showed a higher level of wakefulness during light treatment, better sleep quality at night and improved entrainment of daily rhythms compared to the control group. Our findings on the central orexin system highlight sex-specific responses to BLT in hypothalamic orexin and orexin receptor expression in corticolimbic brain regions. The impact of BLT on the orexin system was sex- and brain region-specific, with males showing higher OX1R and OX2R in the CA1, while females showed higher PPO but lower OX1R and OX2R in the BLA, compared their the same-sex controls. Following 4 weeks of early morning BLT, numerous changes were found in the expression of neuroinflammatory or neuroplasticity markers in corticolimbic brain regions of diurnal grass rats when compared to animals in the red-light control group. For the neuroplasticity markers, BLT downregulated BDNF in the CA1 and TrkB in all three brain regions in females, but upregulated BDNF in the BLA and CA1 in males. For the neuroinflammatory markers, BLT reduced TNF-α in the BLA of females, and upregulated CD11b in the mPFC and IL-6 in the BLA in males. These results suggest that neuroplasticity and neuroinflammation could contribute to potential mechanisms

underlying the therapeutic effects of BLT. Together, these findings suggest that light affects the orexinergic system, as well as other behavioral and neural responses relevant to SAD.

**Effects of OXA on wakefulness/sleep, anhedonia, neuroplasticity, and neuroinflammation**

Based upon the previous findings establishing the relationship between BLT and orexin, the present study examined if orexin administration alone modulates wakefulness/sleep, anhedonia, neuroplasticity markers, and neuroinflammatory markers. The effects of OXA on arousal and sleep were sex-specific, with OXA improving wakefulness in females and improving sleep quality in males. The orexin-treated females showed higher levels of wakefulness prior to, during, and post orexin infusion compared to the dim light control females. Behaviorally, their wakefulness mimicked the females housed in bright light. Orexin-treated males showed increased sleep bout lengths throughout the night compared to their dim light controls. Behaviorally, their sleep mimicked the males that were housed in bright light. Both females and males in the orexin groups showed less anhedonia compared to the dim light controls. The behavioral effects of OXA administration observed in the current experiment in promoting alertness/wakefulness, improving sleep quality, and improving anhedonia are consistent with those reported with orexin treatment in humans [25, 192, 199, 320], supporting the validity of the diurnal grass rat as a model for elucidating the neural mechanisms underlying these therapeutic effects of orexin.

Our findings in the brain highlight sex- and region-specific responses to orexin. There were no significant effects of orexin treatment on neuroplasticity markers, however there were treatment effects in the expression of anti-inflammatory markers IL-4 and IL-10. Both IL-4 and IL-10 were upregulated in OXA females in the mPFC. In the CA1 IL-4 and IL-10 was upregulated in males. For pro-inflammatory markers, orexin only significantly downregulated

microglia marker CD11b in females in the mPFC. Orexin could exert its protective effects via upregulation of anti-inflammatory cytokines to facilitate healing and repair, rather than acting directly on pro-inflammatory cytokines or neurotrophic factors. Microglia and astrocyte proliferation were decreased by the presence of orexin in both sexes across brain regions. Microglia soma area, branch length, and number of endpoints are all indicators of an activated pro-inflammatory phenotype. Soma area was decreased in both sexes in the OXA groups in the BLA and CA1, branch length was decreased in the BLA and CA1, and mean number of endpoints was decreased in the mPFC and CA1. Both anti- and pro-inflammatory state was influenced by orexin, differently in males and females, and could play a role in sex differences in the behavioral changes occurring over seasons and in response to daytime light intensity. Together, these findings suggest that orexin modulates behavioral and neural responses relevant to SAD, in a sex- and brain-region dependent manner.

**Framework and limitations**

These dissertation experiments used a diurnal model to study therapeutic interventions within a SAD paradigm. It was hypothesized that the orexinergic system is a neural mechanism in the effects of daytime light deficiency, due to its wakefulness promoting, neuroplasticity promoting, and anti-inflammatory properties [135, 136, 146, 166, 181, 192, 315, 454] (Fig. 1). I first established how orexin responds to BLT, and then directly tested orexin's modulatory effects in the absence of a bright light photic cue. Both males and females that received BLT exhibited higher wakefulness during the light exposure and better sleep quality at night compared to controls. In the OXA-treated animals however, the females had increased daytime wakefulness and a longer active duration, while the males showed increased nighttime sleep quality compared to controls. It would have been valuable to know how sleep architecture changed throughout the

night in animals that received BLT or OXA treatment, particularly the OXA females. Future studies with grass rats could incorporate in vivo electrophysiological recordings to better understand the effects of orexin on REM and NREM sleep[455]. Anhedonia, however, in both sexes decreased with orexin treatment, consistent with controls housed in brLD.

Within the brain, it was expected that orexin would upregulate neurotrophic factors and buffer pro-inflammatory response, as orexin alone has been demonstrated to increase BDNF as well as suppress pro-inflammatory factors in rodent models of stress, injury, and sickness [137, 169-173]. In the BLT animals, BDNF and TrkB were downregulated in females but upregulated in males, following the direction of OX1R and OX2R expression (Fig. 9, 10, 11). When BDNF and TrkB protein levels were previously compared between grass rats housed in bright or dim daytime light conditions, the differences did not reach statistical significance despite mature BDNF and the phospho- over total-TrkB ratio in the CA1 being ~30% lower in the bright light group [10]. This pattern would suggest that orexin affects neurotrophic factors in a sex-specific pathway, however there were no effects of orexin treatment or sex on these factors in the absence of bright light (Fig. 19). Orexin may help modulate neuroplasticity, but it is likely not the only critical factor. There were also very few effects of BLT or OXA on pro-inflammatory cytokines, and not in the expected direction (Fig. 12, 13, 21). There was, however, a buffering effect of OXA treatment on microglia and astrocyte number as well as pro-inflammatory microglia phenotype (Fig. 22, 23). A limitation of this study is that there was not any available tissue for IHC analysis in the BLT/red-light control cohorts, and thus microglia activation is only defined through CD11b expression in these groups. Additionally, there was no available tissue from the Chapter 2 brLD control group for IHC, due to lack of programmable minipump battery life and availability of age- and sex-appropriate animals.

Instead of directly affecting pro-inflammatory cytokines, OXA treatment instead affected anti-inflammatory cytokines. IL-4 and IL-10 are considered to be neuroprotective, and were significantly upregulated in females in the mPFC and males in CA1 (Fig. 20). This suggests that although OXA-treated animals were undergoing chronic daytime light deficiency, there was active healing and repair occurring in the neuronal environment that was not present in vehicle dimLD animals. The actions of IL-4 and IL-10 could have had a feedback loop with the microglia, helping to suppress pro-inflammatory actions. Microglia can produce both IL-4 and IL-10, which in turn signal other microglia and recruit more anti-inflammatory factors [456, 457].

It is unclear if orexin acted directly or indirectly on the inflammatory factors. While microglia express orexin receptors, orexin has been implicated in nuclear factor kappa light-chain enhancer of activated B cells (NF-kB) and mitogen-activated protein-kinase (MAPK) pathways in an indirect mechanism for inflammation [353]. NF-kB is an inflammatory mediator that regulates the transcription of pro-inflammatory factors such as TNF-α for the chemical cascade needed for apoptosis [458]. Its major subunit and mechanism of action is through p65/p50, which is able to translocate to the nucleus and lead to expression of pro-inflammatory genes[459-461]. Similarly, MAPKs act through p38 and extracellular signal-regulated kinase (ERK) to induce microglia and astrocyte mediated inflammatory response [462, 463]. OXA through OX1R binding inhibits the nuclear translocation of NF-KB p64 [353]. OXA also inhibits phosphorylation of MAPK/ERK and MAPK/p38, preventing the protein AP-1 from reaching the nucleus and contributing to pro-inflammatory gene expression [353]. It would be valuable in future studies to study the molecular mechanisms of OXA, to better understand its direct and indirect effects in inflammation and other downstream neural responses.

The current experiments focused on whether orexin was *sufficient* to buffer behavioral and neural responses relevant to SAD. Future studies that would provide deeper insight into the mechanistic role of orexin could test if orexin is *necessary* to buffer these responses. This could be achieved by selectively knocking out orexin receptors with the use of a viral vector. Grass rats that were treated with a viral vector containing OX1R-shRNA to knockdown OX1R in the CA1 showed impaired spatial memory in the Morris Water Maze, as well as decreased apical dendritic spine density, highlighting the importance of orexin in hippocampal-related learning and plasticity [11] As orexin projections and receptors are so widely spread, there were concerns in the present study of which brain region to silence OX1R/OX2R (Fig. 1). We were not measuring a specific region-based neural or behavioral outcome, and instead were quantifying neuroplasticity and neuroinflammation, which have migratory characteristics and exert actions throughout the brain. Another option would be to silence the orexin neurons themselves, or their precursor PPO. Optogenetics have been successfully utilized in selectively and reversibly manipulating orexin neuron populations [464]. Chemogenetic approaches, such as designer receptors exclusively activated by designer drugs (DREADDS), have also demonstrated the ability to stimulate and suppress orexin neural activity [465]. In the current experiments, selectively deactivating orexin and its projections during BLT administration would confirm which of the behavioral and neural outcomes were solely due to orexin, and not additional modulatory factors.

Orexin could be exerting its therapeutic effects through interactions with other neurotransmitter systems, such as serotonin (5-HT). Orexin neurons directly project to the dorsal raphe nucleus (DR), which is home to a dense population of serotonergic neurons [466-469]. These orexinergic projections are excitatory and stimulative to DR 5-HT, while 5-HT is able to

inhibit orexin neurons through activation of 5-$HT_{1A}$ receptors[470-473] . Orexin-serotonin interactions are thought to play a role in mood regulation, as well as regulation of sleep/wakefulness states [474]. Mice lacking the 5-$HT_{1A}$ receptor gene in orexin neurons exhibit longer NREM sleep time and have decreased wakefulness during their active phase, and display more depressive-like responses following restraint stress [470].

Serotonin dysregulation is implicated in depressive disorders, and selective serotonin reuptake inhibitors are a popular and reliable antidepressant medication [475]. Increased pro-inflammatory cytokines and activated microglia have been shown to upregulate enzymatic degradation of the 5-HT precursor tryptophan, consequentially decreasing 5-HT binding ability [125, 476]. Attenuated 5-HT is implicated specifically in SAD patients [477, 478]. PET and MRI scans have suggested that patients with SAD have deficits in serotonin transporter protein function [25-27]. BLT has been demonstrated to increase the efficiency of synaptic serotonin transporters, comparable to that of healthy controls [479]. The role of 5-HT in SAD could be due in part to its seasonal fluctuations, with lower expression in winter months as evidenced by hypothalamic 5-HT in human post-mortem tissue [480].

5-HT demonstrated sensitivity to both lighting condition and orexin in the grass rats [6, 165, 481]. DimLD housing resulted in attenuated 5-HT in the DR, as well as lower density of 5-HT fibers in the anterior cingulate cortex (ACC) [6]. Functional connectivity between light, orexin in the LH, and the DR has been suggested by Fos [165]. BrLD housing increased Fos-ir in both orexin cell populations and the DR, and this activation was lost with the use of an OX1R antagonist [6]. Daily use of an OX1R antagonist in the ACC over 5 days decreased 5-HT-ir fibers in the ACC, DR, nucleus accumbens shell, oval bed nucleus of the stria terminalis, and periaqueductal grey [481]. This highlights an orexin-5-HT pathway in grass rats that could be

involved in the regulation of mood and affective behavior [6, 54, 138]. Orexin receptors play a functional role in the DR, with in-situ hybridization showing that OX1R is localized in the dorsal and lateral wings while OX2R is found in the ventral DR, involved in affective processes and sensory-motor function, respectively [481]. This orexin receptor distribution is sex-specific, with OX1R DR expression being higher in females regardless of lighting condition[145]. This baseline difference could play a role in the sex-differences observed in the current study as well as sex-differences in SAD and depressive disorders.

The findings of these experiments demonstrate that orexin improves wakefulness and sleep quality in a sex-specific manner, decreases anhedonia, upregulates certain anti-inflammatory factors in a sex- and region-specific manner, and downregulates pro-inflammatory microglia activation. Overall, these results suggest that orexin plays a modulatory role in the effects of daytime light deficiency, though further research is needed in orexin's molecular mechanisms as well as its interactions with other neurotransmitters to fully understand its actions in the behavioral and neural responses relevant to SAD.

**Sex differences in orexin**

The current study observed sex differences in the expression of PPO, orexin receptors, and in orexin-induced wakefulness/sleeping behaviors. Orexin has been demonstrated to be sexually dimorphic in both resting levels and reactivity in rodents and human models. It may be that orexin regulates the neural responses and behavioral symptoms of depressive disorders differently between the sexes.

Female rats have higher basal lateral hypothalamic PPO expression compared to males [482, 483]. This is also true of orexin neuronal activation, as measured through cFos, and circulating OXA in CSF [482, 484]. This pattern is conserved with hypothalamic OX1R

expression, although OX2R seems to be subregion specific with expression only increased in the paraventricular nucleus (PVN) of females compared to males [483, 485]. There is also increased orexin receptor expression in the pituitary and adrenal gland of females, though these differences are not as pronounced [486]. These findings suggest that there are possible sex-specific functions of orexin at several points in the hypothalamic-pituitary-adrenal (HPA) axis and neuroendocrine system.

There is a lack of consensus on the role that the estrous cycle plays in these sex differences. While some groups have reported fluctuations in orexin neuronal expression throughout the estrous cycle, these findings seem to be dependent upon the strain and age of the rats. Some have found increased LH orexin protein expression and mRNA expression, as well as increased OX1R expression, in proestrus compared to diestrus [487, 488], while others observe no fluctuations throughout the cycle [489]. These discrepancies suggest that gonadal hormones may not regulate orexin expression. Supporting this, gonadectomy in adult females and males and subsequent hormone replacement with testosterone or estrogen did not affect hypothalamic PPO or orexin receptor mRNA [490]. Orexin may instead have organizational effects. Neonatally demasculinized rats that are treated with estrogen and progesterone in adulthood show increased expression of orexin and orexin receptors, to the same level as females[491, 492]. Similarly, females that were neonatally androgenized do not show orexinergic responsivity to the same extent as their female control counterparts [491]. The exact mechanism through which these organizational effects occur has yet to be determined, as well as which time points are sensitive or critical periods.

Similar patterns in the sexually dimorphic expression of orexin have been reported in humans. Women have higher levels of central orexin, as measured by CSF and orexin

immunoreactivity in the LH, than men [493] in a healthy resting state, though these differences are more pronounced in women with MDD, Alzheimer's, and dementia [386, 493, 494]. Anterior cingulate cortex and dorsolateral prefrontal cortex OXA immunoreactivity has been shown to be increased in women with MDD compared to men with MDD in postmortem tissue [386]. These sex differences were not observed in cortical and prefrontal areas in patients without MDD, suggesting that these sex differences may be region-specific [386]. It is worth noting that these samples were from patients that were postmenopausal, and organizational effects of orexin may not be as robust. Diurnal fluctuation of OXA was also lost in both men and women with MDD, while it was intact in age-matched controls [386]. This absence of diurnal regulation could contribute to different sleep-wake disruptions in depressive disorders [495]. Insomnia, associated with hyperactivity of orexin, is more common in females, while narcolepsy, associated with hypofunction of orexin, is more common in males [496-498].

These differences in resting levels and reactivity may be functional for sex-specific stress responses, particularly those relevant to the pathophysiology of depressive and other psychiatric disorders. An inhibitory DREADDS study demonstrated that elevated orexin in female rats was responsible for heightened stress-induced impairments in cognition and HPA response to repeated stress [482]. Chromatin immunoprecipitation has shown that glucocorticoid receptors act directly on the orexin promotor to increase PPO in the LH of females [482]. Additionally, there is a significant positive correlation between corticotropin-releasing hormone to both LH PPO mRNA and PFC OX1R expression in female rats during chronic unpredictable mild stress [386].

Altered orexin signaling is also implicated in stress-induced abrupt waking in females, and this sleep disruption can last for up to 21 days after the stressor has ended [499].

Interestingly, this abrupt waking was observed both in females that had undergone the stress as well as in females that had witnessed their cage mates undergoing the stress event. These sex-specific orexin-mediated behaviors are clinically relevant, as chronically heightened arousal and startle response, sleep disturbances, and hypervigilance are key symptoms and diagnostic criteria for stress related disorders like PTSD [500, 501]. Beyond the HPA axis, sex differences in hypothalamic orexin could be associated with maternal care. OXA administration into the medial preoptic area has been shown to promote pup licking in lactating rats, and this effect was reversed with an OX1R antagonist [502].

In the current study, the downregulation of OX1R in females that received BLT may have actually been a therapeutic response. OX1R and hyperreactivity is implicated in female stress response, and downregulation of these receptors may be buffering a hyper-reactive stress response to dimLD. The increased PPO was consistent with the increased resting PPO in healthy female rats. This is further supported by the females having increased sleep quality at night, similar to males, while the red-light females with upregulated OX1R and OX2R expression had sleep disruptions. Increased daytime wakefulness was observed in the OXA treated females, but not males, consistent with the arousal effects of orexin in females. The males instead showed increased sleep quality at night, and both sexes displayed less anhedonia compared to dimLD controls. These changes are not due to fluctuations in estrous stage, as the grass rats are induced ovulators and will not cycle unless paired with a male. Orexin may have sex-mediated neural effects and affect SAD-related symptoms differently in males and females. As such, orexin-based therapies could potentially affect symptoms of stress-related illnesses in a sex specific manner. Higher orexinergic reactivity in women is worth considering when assigning dosage of drugs that affect the orexinergic system. Ambien, a common insomnia drug, is metabolized more

slowly in women and is one of the few FDA approved drugs with sex-specific dosing [496, 503]. Further research into sex-specific dosing of orexin-based medication needs to be done, so that treatments targeting the orexin system can be equally effective in both sexes.

**Orexin as a therapeutic target**

In a healthy individual, orexin exists in a balance. Loss of orexinergic signaling is associated with narcolepsy, while overabundance is associated with anxiety and insomnia[317]. In the last decade, orexin antagonists such as Suvorexant and Lemborexant have gained popularity in clinical populations[504]. These dual orexin receptor antagonists (DORAs) were approved by the FDA in 2014 for the treatment of insomnia, aiming to suppress orexin's wakefulness promoting properties[504, 505]. Suvorexant works through selective antagonism of ligands OXA and OXB to prevent receptor binding, while Lemborexant is a competitive antagonist that binds to both OX1R and OX2R to prevent ligand binding[506, 507]. DORAs are recommended to be taken at night, or before at least 7 consecutive hours of sleep, as there have been reports of residual pharmacologic effects; the binding and release of Suvorexant in particular is slow, and can be unpredictable in its pharmacokinetics [508].

DORAs hold promise, in that inhibiting orexin receptor actions result in increased total sleep without shifting sleep profile, unlike other hypnotic insomnia medications [509]. Suvorexant and Lemborexant have no reports of physical dependence or withdrawal symptoms, even after 1 year of daily use [505, 510]. Orexinergic drugs are beneficial in this manner, in that traditional drugs for insomnia act through benzodiazepine receptors and are highly addictive [511]. However, chronic inhibition of orexin can result in depressive symptoms, and Suvorexant and Lemborexant are not recommended for patients with existing depressive disorders [512-

514]. Depressed patients tend to have lower circulating CSF OXA, as well as reduced plasma OXA; DORAs would only exacerbate this decrease in orexinergic signaling [515, 516]

Side effects of these medications include sleep paralysis, hypnagogic hallucinations, dizziness, and abnormal thought pattern [512-514]. Women are more likely to have severe side effects, including somnolence, headache, nightmares, and continued episodes of insomnia[505, 517-520]. These differences in symptom severity could be due to sex-specific orexinergic effects, and more research is needed in dosage and timing of DORA medications. Sex differences were observed in the current study, with BLT downregulating OX1R and OX2R in females, and OXA infusion promoting daytime wakefulness and a longer active duration only in females. Sex differences exist not only in pathology of neurological and affective disorders, but also in physiological processes (reviewed in [521]). There is importance in understanding how the orexinergic system acts in different sexes in order to better understand the development of the pathologies that DORAs are aiming to treat.

**Conclusion**

Both BLT and orexin administration have demonstrated impacts on wakefulness, sleep, and neuroinflammation, in the context of SAD. BLT enhanced wakefulness during light treatment, improved sleep quality at night, and regulated daily rhythms. There were sex-specific impacts on the orexin system, and sex- and region-specific effects on neuroplasticity and pro-inflammatory markers. Similarly, OXA administration enhanced wakefulness during the day in females, and improved sleep quality at night in males. Anhedonia was lessened in orexin-treated animals. The presence of orexin modulated the expression of both anti-inflammatory cytokines and pro-inflammatory microglia and astrocytes, also in a sex- and region-specific manner. These findings highlight the importance of considering sex and brain region differences when

developing orexin-based therapeutics, as there may be specific functional mechanisms. These results provide novel insights into how seasonal light conditions and orexin influence a variety of neural functions including neuroinflammation and neuroplasticity, aligning with behavioral outcomes highly relevant to SAD and other depressive disorders in humans [522].

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# APPENDIX

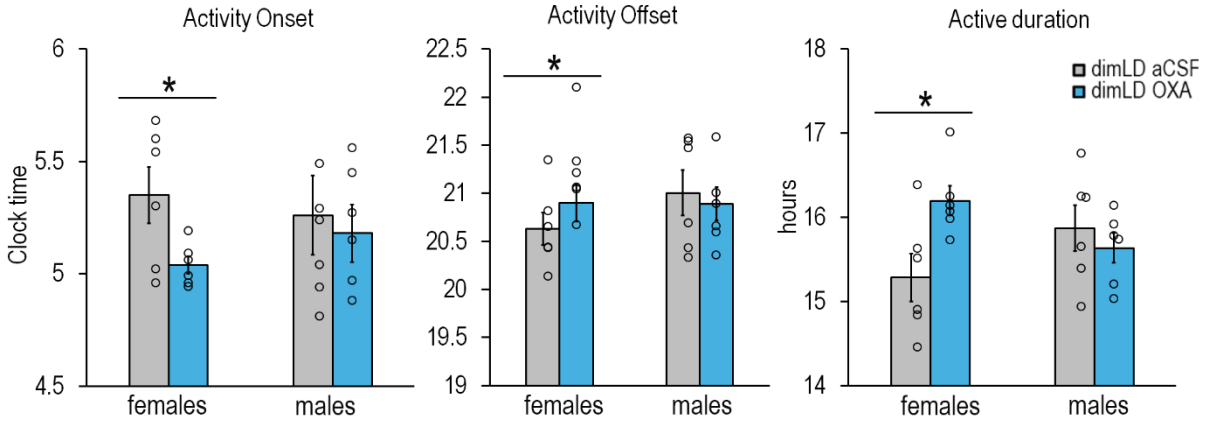


**Figure A1:** OXA promotes earlier onsets, later offsets, and a longer active duration in females. Data are shown as Mean ± SEM, n = 6/dimLD aCSF/OXA, * $p < 0.05$.

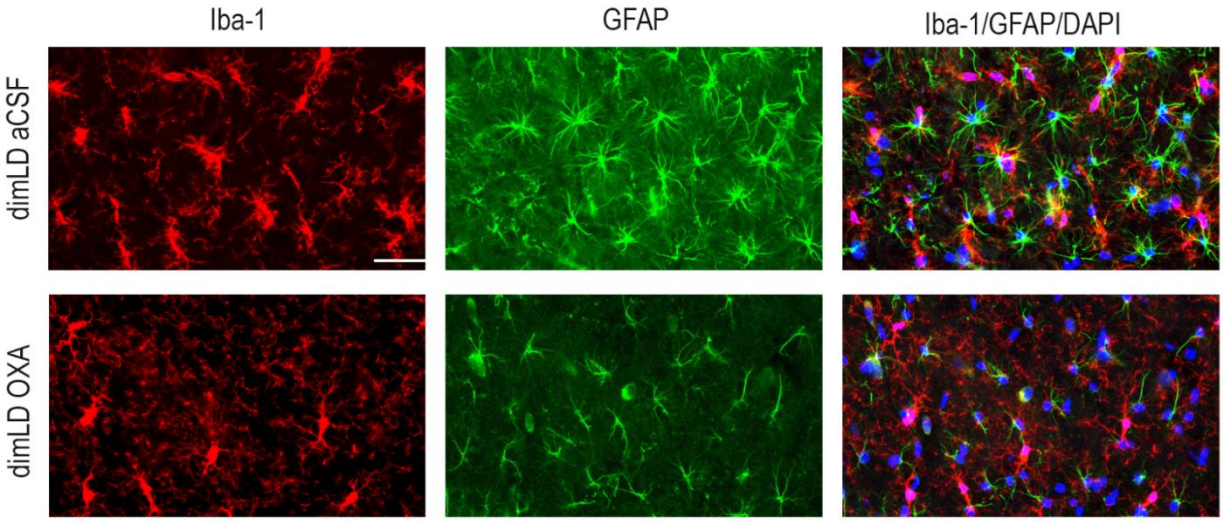


**Figure A2:** Effects of OXA administration on glial proliferation and morphology in the CA1. Representative Iba-1, GFAP, and combined Iba-1/GFAP/DAPI immunostaining in the CA1 of females that received aCSF or OXA infusion. Scale bar, 50 µm.