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Examining the Effects of Light Pollution on Health and Diseases

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Examining the Effects of Light Pollution on Health and Diseases

A thesis submitted in partial satisfaction

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in Physiological Science

by

Sophia Anne Marie Barnachea Villanueva

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ABSTRACT OF THE DISSERTATION

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by

Sophia Anne Marie Barnachea Villanueva

Master of Science in Physiological Science

University of California, Los Angeles, 2024

Professor Cristopher S. Colwell, Co-Chair

Professor Gene D. Block, Co-Chair

A circadian rhythm is an endogenous oscillator with a period close to 24 hours that drives and regulates processes in the body. As light is known to be the strongest entrainment cue for circadian rhythms, the increasing use of artificial lighting in modern society raises concern over the negative impact of nighttime light pollution for both animal and human health. In the first study, the impact of artificial light intensity and temperature on nocturnal rodents was explored. We found that spectral control may mitigate some of the negative effects of nighttime light pollution poses on wildlife and we illustrate a need to account for light spectrum in circadian behavioral studies. In the second study, we utilized an ultradian LD cycle to determine the direct influence of aberrant light on autistic behavior in the *Cntnap2* mouse model of autism spectrum disorder. Our findings show that light exposure during the night without a circadian disruption does not elicit a worsening of the autistic phenotype.

The thesis of Sophia Anne Marie Barnachea Villanueva is approved.

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Acknowledgements

Chapter 2 is a version of Longcore, T., Villanueva, SAM., Nguyen-Ngo, K., Ghiani, C., Harrison, B., & Colwell, C. (2024). Relative Importance of Intensity and Spectrum of Artificial Light at Night in Disrupting Behavior of a Nocturnal Rodent. This work has been accepted and is currently in-press.

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Chapter 1: General Introduction

Chapter 1

General Introduction

The circadian clock is a temporal program that generates a 24-hr structure on biological processes from gene expression to behaviour. Circadian clocks are autonomous, producing rhythms even in the absence of daily environmental signals. The mammalian circadian timing system includes a central clock in the suprachiasmatic nucleus (SCN), which receives light information through a specialized pathway, as well as information about other relevant environmental cues, like the timing of food. The SCN communicates with the rest of the brain through several pathways, including arousal centers, the autonomic nervous system, as well as the hypothalamic-pituitary-adrenal (HPA) axis. Given this potent mix of output pathways, the central clock has the ability to regulate rhythms throughout the body. The result is a broad temporal structure that influences all biological systems. One of the central tenets of the circadian field is that this temporal structure is essential for our health (Cederroth et al., 2019; Roenneberg & Merrow, 2016) and its disruption has costs felt throughout our bodies.

Classically, the circadian timing system consists of a light input pathway, a central clock located in the SCN, and multiple output pathways. Molecular circadian clocks are widely distributed and control the temporal pattern of transcription locally. The daily light-dark (LD) cycle is a powerful regulator of behavior both through the direct effect of light, as well as synchronization of the endogenous circadian clock (Roenneberg et al., 2013). Light is detected by intrinsically photoreceptive retinal ganglion cells (ipRGCs) that make use of melanopsin as a photopigment (Rollag et a., 2003; Schmidt et al., 2011). The ipRGCs can also respond to rod- and cone-driven signals (Van Diepen et al., 2021). The ipRGCs integrate this light information (Lucas, 2013) and send a direct projection to the central circadian clock where this signal synchronizes

circadian oscillations. The ipRGCs also underlie, in part, the direct impact of light on mood and cognition (LeGates et al., 2014; An et al., 2020).

The SCN of the hypothalamus contains the “master” oscillatory network necessary for coordinating circadian rhythms throughout the body. A characteristic property of SCN neurons is that they generate a circadian rhythm of neural activity with higher spontaneous activity during the day than the night. Such neural activity rhythm is critical for the synchrony of cells within the SCN circuit, as well as the ability of this nucleus to drive outputs throughout the body. These rhythms in SCN electrical activity are driven by cell-autonomous molecular feedback loops and seem impacted in mouse models of neurodevelopmental disorders (Hogenesch and Ueda, 2011; Cox and Takashashi, 2019). A growing body of work suggest that the molecular clockwork is disrupted in neuropsychiatric disorders with a developmental origin or not, including mood and anxiety disorders as well as schizophrenia (Dollish et al., 2024; Seney et al., 2019; Vadnie et al., 2022).

The molecular clockwork driving circadian oscillations is found throughout the body. Neurons and glial cells express daily oscillations that provide a temporal structure to transcription, cell biology, and network properties of all major CNS systems that are impacted by neurodevelopmental disorders. Genome-wide analyses reveal global circadian control over processes involved in functionally important pathways, such as transcription, chromatin modification and remodelling, metabolism, inflammation, and cell signalling. Therefore, in trying to understand the mechanisms by which circadian disruption can exacerbate symptoms and pathology of neurodevelopmental disorders, several possible mediatory pathways have been proposed with the immune system looking as a particularly promising intersection between circadian rhythms and neurodevelopmental disorders.

The immune system is composed of a diverse network of cells, tissues, and organs (e.g. white blood cells, spleen, thymus, and bone marrow) that are linked by function and synchronized in time. The temporal patterning of immune function is a general feature, with the circadian system driving a more activated immune system when the organism is awake and damping it down during times of sleep (Man et al., 2016; Baxter and Ray, 2020). In peripheral organs, our understanding of the molecular interactions between the circadian clock and the immune system is fairly advanced. For example, studies have found clear evidence for the molecular circadian clock driving the expression or repression of immune genes (Hergenhan et al,. 2020). In addition, rhythmic acetylation or methylation of histones regulates transcription of inflammatory genes. There is even evidence that circadian clock proteins can engage in direct physical interactions with components of key inflammatory pathways, such as members of the NF-κB protein family. This intimate association between circadian rhythmicity and immune function likely serves to increase protection during times of day when we are out in the world, while limiting the cost of over-stimulation during times of sleep.

Neuroinflammation has been proposed as a contributor to neurodevelopmental disorders (Hughes et al., 2023; Hartmann et al., 2024), also supported by the presence of elevated levels of immunomediators in the CSF of individuals with ASD and their mothers (Carter et al., 2021; Wood, 2022). Glia cells, such as astrocytes and microglia cells, exhibit robust circadian rhythms (Prolo et al., 2005; Hayashi et al., 2013; Fonken et al., 2015; Colwell and Ghiani 2020), and so do some of the mediators they release in response to synaptic functions as well as injury or stress. Astrocytes are multifaceted cells involved in several functions, including responding to brain insults or stressors with striking changes in morphology and becoming “reactive”. Similarly, microglia, the immune resident cells of the brain, are continuously surveilling the CNS

environment and respond to minimal variations due to insults or stressors with morphological changes as well. Both cell types are in close contact with neurons and synapses, and importantly daily changes in their morphology have been shown to be rhythmic and likely under circadian control. A growing body of evidence implicates the circadian clock and its components in mediating inflammation along with some of the functions of astrocytes and microglia (Musiek et al., 2013; Nakazato et al,. 2017; Lananna et al., 2018; Griffin et al., 2019).

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Chapter 2: Exploring the impact of artificial light intensity and temperature on the activity of nocturnal rodents

Chapter 2

Exploring the impact of artificial light intensity and temperature on the activity of nocturnal rodents

Abstract

Roadway lighting has evolved into an integral component of modern society and the illumination of streets allows for safe transportation and work during hours of darkness. Light emitting diodes (LEDs) are becoming an increasingly popular mainstay of street lighting; in comparison to their incandescent counterparts, LEDs offer much brighter illumination, as well as more blue light production, which has introduced concerns over potentially harmful impacts on human and animal health. The California Department of Transportation is seeking to evaluate LED lighting with the goal of maximizing roadway visibility while minimizing negative effects on wildlife and biodiversity. We hypothesize that utilizing warmer temperatures of light would be less disruptive to the circadian rhythms and behavior of nocturnal rodents. To test this hypothesis, we subjected laboratory mice to various intensities and spectrums of light and determined the extent to which the photic stimulus suppressed activity levels. Adult mice of both sexes, held on a 12:12 hr light/dark cycle, were exposed to a one-hour pulse of 3 correlated-color temperatures (CCTs) (1750K, 1950K, and 3000K) at 4 different light intensities (0.01, 0.1, 5 and 50 lux) during their activity phase in the dark, at zeitgeber time (ZT) 14, two hours after lights off. Suppression of locomotor activity rhythm was strongly correlated with increasing CCT, as 1750K evoked the weakest activity disruptions and 3000K elicited the strongest effect in laboratory mice. Analysis of light-evoked cFos expression in the suprachiasmatic nucleus at 50lx demonstrated no significant difference between high and low (3000K and 1950K, respectively) CCT exposure. These findings not only revealed that spectral control may mitigate some of the detrimental effects that light

pollution at night poses on wildlife but importantly, highlight the importance of considering light spectrum in circadian studies of behavior.

Introduction

Circadian rhythms refer to an endogenous oscillation in every animal that is driven by the organism's own biological clock. These rhythms serve as a pacemaker by coordinating numerous physiological processes, such as sleep-wake cycles, hormone secretion, and gene expression, which promotes internal synchrony and carefully maintains the intricate interplay among fundamental mechanisms. Several key characteristics of a circadian rhythm include a period (time to complete one full cycle) close to 24 hours and entrainability to zeitgebers (Eckel-Mahan and Sassone-Corsi, 2015). Zeitgebers are external events that function to entrain the period of our circadian rhythms to the period of the environment, allowing organisms to predict changes in their environment, such as day/night changes. Light is the most potent zeitgeber, as its presence during the day signals activity onset to diurnal organisms and activity offset to nocturnal animals (Foster et al., 2020). Light is detected by intrinsically photosensitive retinal ganglion cells (ipRGCs) in the eye, which then communicate photic signals to a cluster of neurons in the hypothalamus known as the suprachiasmatic nucleus (SCN). The SCN operates as the body's central pacemaker by relaying the zeitgeber-driven timing signals throughout the body and regulating the circadian oscillations within each physiological system accordingly. Once detected by the SCN, darkness signals activity offset in diurnal organisms and activity onset in nocturnal organisms (Farnworth et al., 2016).

Artificial night lighting has become an instrumental and universal component of urban environments, allowing for safe driving and work during dark hours (Figure 2). The switch from incandescent to LED light sources has allowed for greater energy efficiency and overall illumination of roadways (Stone et al., 2012). However, LED lighting also emits greater concentrations of blue light than its predecessors (Longcore et al., 2017), which has introduced

concerns over wildlife health and overall biodiversity. Previous studies have demonstrated that one-hour pulses of blue light at night elicited sleep delays and increased arousal in mice. These observations were attributed to an increased ipRGC sensitivity to higher temperatures (longer wavelengths) of light, as the SCN experienced additional stimulation when exposed to blue-enriched light in comparison to white light. Blue light-induced neurological changes in the arcuate hypothalamic nucleus, cingulate cortex, and paraventricular thalamic nucleus were also observed (Wu et al., 2021). These findings offer insight into some of the circadian disruptions that inappropriate light exposure, particularly blue light, can induce in nocturnal rodents.

The California Department of Transportation aims to evaluate LED lighting in order to maximize visibility on roadways while minimizing detrimental effects on wildlife. Hence, we set out to examine the extent to which light intensity and spectrum can influence circadian rhythms in activity in nocturnal rodents. Our hypothesis is that higher CCTs and light intensities, similar to high-pressure sodium and incandescent lighting, will evoke stronger activity suppression in wild-type mice. Furthermore, we also hypothesize that higher CCTs will elicit greater cFos activation in the SCN.

Materials and Methods

Light Treatments

To calibrate the dimmest exposure, field data were taken in the Coachella Valley, California along State Route 62 as an example of open desert habitat with many native rodent species, located > 3 km from the nearest urban development. Data were collected using a Sky Quality Camera (Euromix Ltd., Llubljana, Slovenia) on nights with a new moon and after astronomical twilight. Cloud cover, which reflects light and increases light pollution, was variable. Both cosine-adjusted illuminance and hemispherical illuminance were extracted from the imagery. We plotted the relationship between hemispherical illuminance and cos-adjusted illuminance because hemispherical illuminance is important to exposure (light from all directions) while light meters used to measure light in laboratory conditions measure cos-adjusted illuminance. The average scalar illuminance at 15 locations from 10–400 m from a highway was 0.020 lx. This corresponded to a cos-adjusted illuminance of 0.007 lx. These compare with the illumination produced by a quarter (crescent) moon at its brightest of 0.008 lx (Krisciunas and Schaefer, 1991). The lower limit of our light meter is 0.01 lx, so we set this as the lowest exposure for the experiment so that we could measure it accurately. At the upper end, we chose both 5 lx, which is known to affect rodent circadian rhythms and could be experienced by rodents near roadway lighting and 50 lx, well above known impact thresholds to see the maximum influence of spectral differences. Between these extremes we selected 0.5 lx, which is somewhat greater than the light of a full moon (Kyba et al., 2017).

Custom lighting systems using LEDs were obtained from Korrus, Inc. (Los Angeles, California). The systems could be adjusted to spectral output to achieve different color temperatures. Because any particular CCT can be achieved in different ways, we compared possible outputs against

known lamp types in terms of their predicted melanopic effect, using methods described in Longcore et al. (2018). We selected three configurations to represent a range of melanopic effects when compared with daylight (D65) and that fall within the range of commercially available outdoor lighting. Ordered from highest melanopic effect to lowest compared with D65, they were 3000 K (54%), 1950 K (30%), and 1750 K (13%). For comparison, the melanopic effect as a percent of D65 for typical lamps is as follows: 4200 K LED streetlight (56%), 3000 K LED streetlight (45%), high pressure sodium streetlight (18%), phosphor-coated amber LED streetlight (10%).

Study Organism

All the animal procedures were reviewed and permitted by the institutional animal care and use committee at UCLA animal care committee's regulations. Sixteen WT mice (C57Bl/6J https://www.jax.org/strain/000664; 8 males, 8 females) were obtained at 3 months of age from Jackson Laboratory and placed into environmental control chambers. Although this strain of mouse does not produce melatonin, it has melatonin receptors (Ebihara et al., 1986) and its circadian system (light detection, physiological, and molecular mechanisms) is extremely well understood. The experiments were ended when the mice were 6 months old. Each mouse was individually housed to obtain rhythms in activity based on wheel running activity. The environmental chambers control sound, maintain temperature, humidity and allow the light to be varied in intensity and spectral properties using the custom LED illumination system.

Light Treatments and Photic Suppression of Nocturnal Activity (Negative Light Masking)

Mice were entrained to a light/dark (LD) cycle consisting of 12 hrs of light (350 lx; 3000 K) and 12 hrs of dark for at least two weeks, and then exposed to four levels of light intensity (0.01, 0.5, 5, and 50 lx), each at three different CCTs (1750, 1950 and 3000 K) for 1 hour at Zeitgeber Time

(ZT) 14, e.g. 2 hours after lights off (ZT0, lights on; ZT12, lights off). Mice were tested once per week with at least 6 days of recovery in 12h:12h LD conditions between exposures. Each mouse was subjected to all 12 light intensities in the following order: 5, 0.5, 0.01, 50 lx. For each intensity, the order of CCT exposure was 3000, 1950, 1750 K.

Home cage activity based on wheel running behavior was reported to a VitalView data recording system (Mini Mitter, Bend, OR). Wheel rotations were recorded in 3 min bins, and analysis was carried out using the El Temps chronobiology program (A. Diez-Noguera, Barcelona, Spain; http://www.el-temps.com/principal.html). Locomotor activity level during the light exposure was compared to the activity at the same phase (ZT 14–15) in the prior day. The percent change in activity was then calculated.

Photic induction of cFos in the SCN

A separate cohort of male and female WT mice (3-4 months old; n=3 per sex) was housed in normal LD conditions and exposed to either 1950 K or 3000 K light (50 lx) for 1 hour at ZT 14. The animals were euthanized with isoflurane (30%–32%) at ZT 15 and transcardially perfused with phosphate-buffered saline (PBS, 0.1 M, pH 7.4) containing 4% (w/v) paraformaldehyde (Sigma). The brains were rapidly dissected out, post-fixed overnight in 4% PFA at 4 °C, and cryoprotected in 15% sucrose. Coronal sections (50 μm), containing the middle SCN were obtained using a cryostat (Leica, Buffalo Grove, IL), collected sequentially, and paired along the anterior–posterior axis before further processing. Immunofluorescence was performed as previously described (Lee et al., 2018; Wang et al., 2023). Briefly, free-floating coronal sections containing the mid-SCN were blocked for 1 hr at room temperature (1% BSA, 0.3% Triton X-100, 10% normal donkey serum in 1xPBS) and then incubated overnight at 4ºC with a rabbit polyclonal antiserum against cFos (1:1000, Cell Signaling) followed by a Cy3-conjugated donkey-anti-rabbit

secondary antibody (1:300, Jackson ImmunoResearch Laboratories, Bar Harbor, ME). Sections were mounted and coverslips applied with Vectashield mounting medium containing the nuclear staining DAPI (4'-6-diamidino-2-phenylinodole; Vector Laboratories, Burlingame, CA), and visualized on a Zeiss AxioImager M2 microscope (Zeiss, Thornwood NY) equipped with an AxioCam MRm and a motorized stage.

cFos positive Cell Counting

Z-Stack Images (7um interval, 15 images) of both the left and right middle SCN were acquired with a 20X objective using the Zeiss Zen digital imaging software. Three observers masked to the experimental groups performed the cell counting. The SCN was visualized using the DAPI nuclear staining, traced, and the cells immuno-positive for cFos counted with the aid of the Zen software tool 'marker' in three to five consecutive sections. The values obtained in the left and right SCN of each slice were averaged. The means of the three-five slices were then averaged to obtain one value per animal and are presented as the mean ± standard deviation (SD) of 6 animals/light treatment. Data analysis was performed using Prism (Version 9.5.0; GraphPad Software, La Jolla, CA). The data passed the normality tests Shapiro-Wilk test and Kolmogorov-Smirnov test; hence, a two-tailed unpaired t test was employed to identify significant differences between groups.

Statistical Analysis

Data were collected and analyzed by observers masked to the experimental groups, and are presented as the mean ± SD. Statistical tests for significant differences between groups were performed using Prism 10.2.0 (GraphPad Software, La Jolla, CA). Normality was determined with the Shapiro-Wilk test and Kolmogorov-Smirnov test. A Student's t-test was employed to analyze statistically significant differences between two groups or one-way ANOVA followed by Bonferroni's multiple comparisons if more than two groups were present in the experimental

design. Two-way ANOVA followed by Bonferroni's multiple comparisons test with sex and CCTs as variables was used to analyze the percentage of suppression in activity and cFos induction in the SCN.

Results

Nocturnal activity was suppressed for all the CCTs at the higher light intensities (0.5lx & 50lx, Fig. 4&5, Table 1). Interestingly, the extent of activity rhythm suppression parallelled the increase in light intensities, with the light pulses at 50lx evoking the strongest effects (Fig. 4). At the lowest light intensity (0.01lx), the locomotor activity was not affected or differed among the different CCTs, however, at the lowest CCT 1750K there was an activity increase of 12.3% (Fig. 4). The 3000K at 5 lx and 50lx induced a significant activity suppression as compared to the other 2 CCTs (Fig. 5). The strongest suppression of activity was observed with a pulse given with an intensity of 50lx and a CCT of 3000K (Fig. 4&5; Table 1), while a pulse with similar intensity (50lx) but a CCT of 1750K elicited about half of the suppression in activity rhythms. Hence, the animals' behavior appears to be less disrupted by the lower CCT (1750K), since their normal activity behavior was unchanged or if so, a bit increased.

To better understand the response of the animals to the different CCTs, we investigated whether sex may play a role and differentially affect the response to the CCT light pulses. A significant effect of sex was found for the groups exposed at 50lx intensity, while the interaction between CCT and sex significantly affected the responses to 5lx (Table 1). At the lower intensity (5lx), a greater suppression of locomotor activity was observed between female and male wild-type mice only with the 3000K light pulse (Fig. 6, left panel). Furthermore, female mice exposed to a pulse with 1950K CCTs exhibited significantly less activity suppression than those exposed to 3000K (Figure 6, right panel). For light pulses at 50lx, the female wild-type mice seemed, in general, more sensitive than the males (Figure 6, left panel). Interestingly, when male mice were exposed to 1750K light pulses the activity suppression was significantly less than in females exposed to the same CCTs or to the same animals exposed to a pulse with the 3000K CCT. A

significant difference in activity suppression was also observed between female mice exposed to 1950K light pulses and 3000K light pulses (Fig. 6).

The effect of two CCTs (1950K or 3000K) was investigated on the light-evoked cFos response in the suprachiasmatic nucleus (SCN), a classic test of the intrinsically photosensitive retinal ganglion cells (ipRGCs) input to the circadian system in mammals. These cells express the photopigment melanopsin, which is maximally sensitive to colder lights (Hattar et al., 2002). As shown in Figure 7, no differences were observed in the number of cFos immunopositive cells in the SCN of wild-type mice exposed to 1950 or 3000K CCTs as well as to bright white light (about 5000K, generally used in indoor environment) at 50lx.

Finally, even though this experiment was not powered to assess sex differences, we observed that the two CCTs exerted a divergent effect on the number of cFos-positive cells in the SCN in male and female mice; in particular, the males seemed more sensitive to 3000K and displayed a higher number of cFos positive cells. Two-way ANOVA revealed a significant effect of the interaction between sex and CCT (Table 1 and Fig. 8).

Discussion

This study investigated the effects of different light intensities and temperatures on activity rhythms in nocturnal rodents. We studied CCTs of 1750K, 1950K, and 3000K, which are the most common commercially-available lights that the California Department of Transportation would be able to order and implement. Light intensities ranged from 0.01 to 50lx, with light pulses being delivered for one hour at ZT14 during the rodents' subjective night. Activity data, as recorded through wheel running, demonstrated that increasing CCTs elicited greater activity disruptions in laboratory mice. Furthermore, increasing light intensity at each CCT induced greater suppression of activity. These findings suggest that light of higher intensities and higher CCTs (i.e. more blue-enriched light) elicits greater circadian disruption in mice, as demonstrated by greater suppression of activity during the dark period. Our results coincide with the notion that mice are typically prey in the wilderness and bright, blue-enriched photic stimuli would be expected to reduce their locomotor activity in order to avoid detection by predators.

Light induction of cFos in the SCN did not show any differences between equal intensities of 3000K and 1950K light. The neural circuits governing circadian control of nocturnal behavior have previously been demonstrated to travel through the SCN and onto motor pathways. As such, the difference in behavior can likely be attributed to differences at the motor circuit level, as opposed to differences in the mouse's detection of the light stimulus. The lack of significant difference in cFos cell counts could be that the spectral distributions of the two CCTs are too similar (i.e. contain too much overlap) to elicit a significantly different response in cFos immunoreactivity. Another conclusion that these findings may suggest is that the behavioral response is more sensitive to CCT differences than the light-evoked cFos response.

The behavioral findings from this study align with prior literature outlining the increased photosensitivity to blue light within ipRGCs and, thus, the SCN. This results in greater activation in response to blue light, which can elicit potentially deleterious effects if organisms are exposed to light at the wrong time of day. In diurnal organisms, light exposure signals activity onset, while light exposure signals activity offset in nocturnal organisms. This pathway is mediated by the retinal photopigment known as melanopsin, which contributes to the non-image-forming component of our vision (Fernandez *et al.,* 2018). Melanopsin has been shown to possess increased sensitivity to blue temperatures of light and relays external photic signals to ipRGCs. Based on the concentrations of light that are detected by melanopsin, ipRGCs are able to activate neurons of the SCN to not only impact peripheral clocks but also initiate physiological processes and behaviors that correlate with the perceived time of day (LeGates et al., 2012).

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Figures and Tables

Locomotor Activity	CCT	% of suppression	Kruskal-Wallis Test	
50lx	1750	44.4 ± 40	***H* = 13.16; *P* = 0.0014**	Total # of values = 48 # of groups = 3
	1950	40.5 ± 31		
	3000	78.5 ± 29		

Two-Way ANOVA

		Females	Males	CCT	Sex	Interaction (CCT x Sex)
5lx	1750	19.6 ± 11	4.02 ± 14	***F*(2,28)=6.96; *P*=0.004**	$F_{(1,14)}$=1.61; *P*=0.225	***F*(2,28)=4.54; *P*=0.019**
	1950	6.20 ± 10	13.9 ± 22			
	3000	34.3 ± 15	17.2 ± 25			
50lx	1750	67.3 ± 25	21.6 ± 41	***F*(2,21)=7.98; *P*=0.003**	***F*(1,21)=9.78; *P*=0.005**	$F_{(2,21)}$=0.886; *P*=0.427
	1950	49.9 ± 28	31.1 ± 32			
	3000	89.1 ± 11	67.9 ± 38			

cFos	CCT	# of positive cells	One-Way ANOVA	Unpaired t test (two-tailed)
	1950	123.7 ± 18	$F_{(3,12)}$=23.99; *P*<0.0001	$t_{(10)}$= 0.9409; P<0.3689
	3000	113.4 ± 20		
	WL	153.3 ± 23		
	DD	10.35 ± 1		

Two-Way ANOVA

		Females	Males	CCT	Sex	Interaction (CCT x Sex)
	1950	134 ± 13	114 ± 18	$F_{(1,8)}$=0,199; *P*=0.667	$F_{(1,8)}$=1.37; *P*=0.276	***F*(1,8)=7.25; *P*=0.027**
	3000	100 ± 15	127± 14			

Table 1. Statistical analyses were performed using Prism 10.5 (GraphPad). One-way ANOVA or Kruskal-Wallis (one-way ANOVA on ranks) Tests were used to determine statistical differences among three or more groups; Statistically significant values are in bold. Asterisk indicated statistically significant differences between different Color Temperature (CCT) groups within same sex and crosshatch indicate sex differences within the same CCT group. Values are shown as the Mean ± SD. DD= constant darkness. Degrees of freedom are reported within parentheses. WL: white light

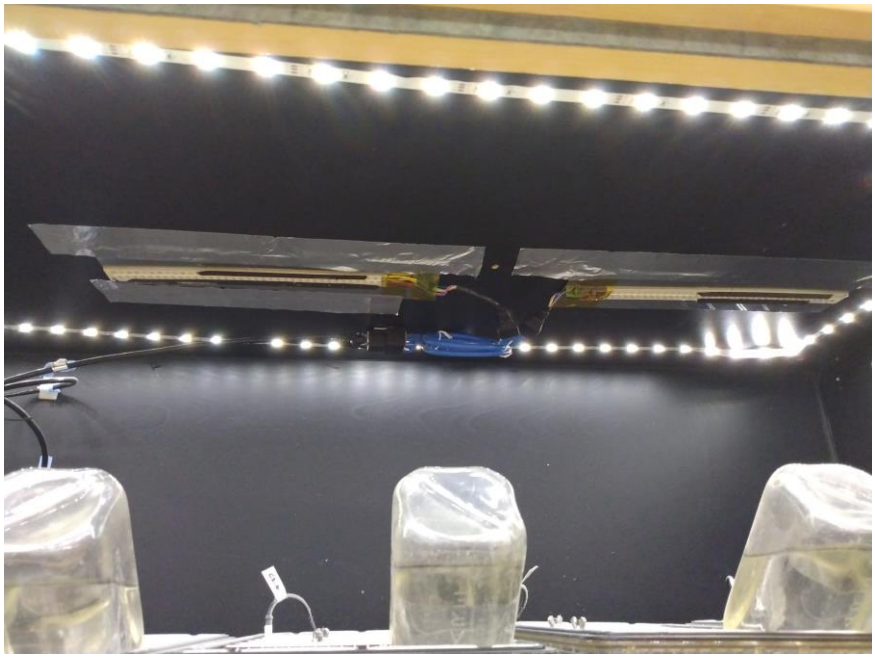

Figure 1. LED light strips for generating Light/Dark (LD) cycles in light-tight chambers. The LD cycle was generated via LED light strips that were placed along the perimeter of the environmental control chambers and were controlled by an external timer. The LD cycle consisted of 12 hours of light (250lx; 3000K) and 12 hours of darkness. K=Kelvin; lx=Lux

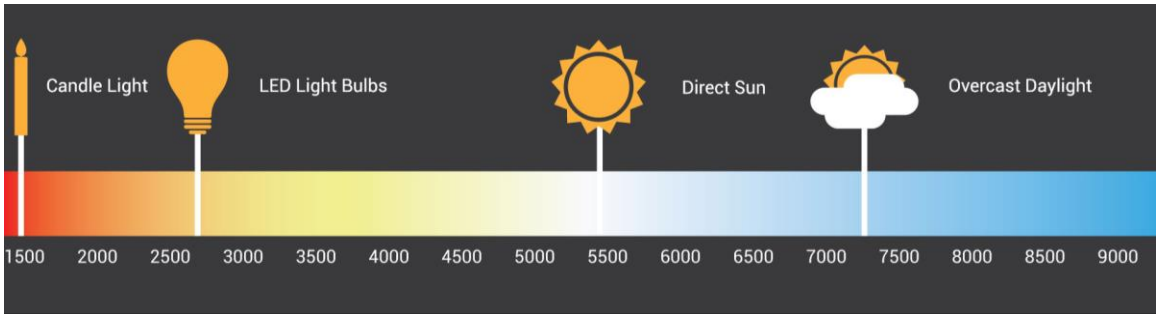

Figure 2. Color temperatures in the Kelvin scale. The correlated-color temperatures (CCTs) studied in this experiment were 1750K, 1950K, and 3000K. 1750K light represented the most red (highest wavelength) of light and the 3000K light represented the most blue (lowest wavelength) of light. These CCTs are the most common commercially-available LED lights that the California Department of Transportation may seek to implement. *(Cocoweb, Corona, California)*

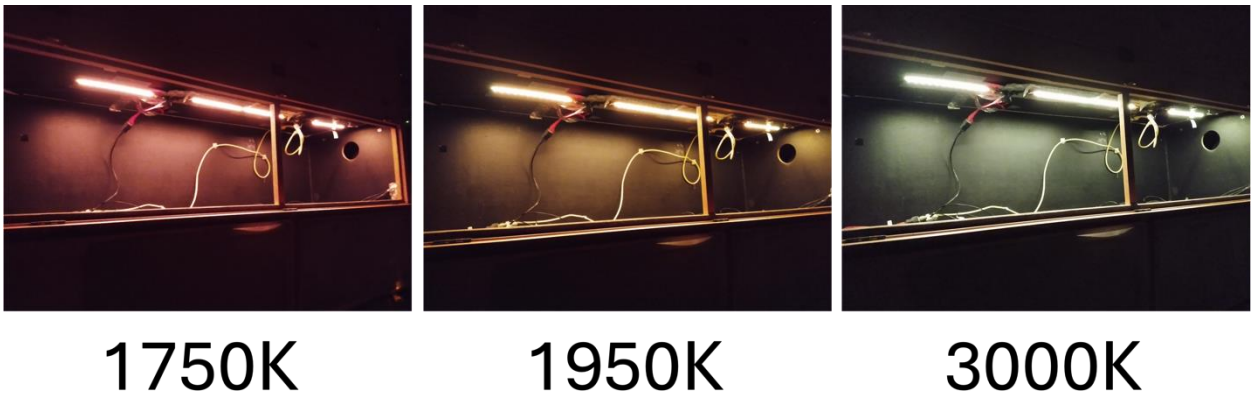

Figure 3. Custom LED light strips for delivering light pulse in light-tight chambers. Custom LED light strips were positioned along the ceiling of the light-tight chambers to deliver one-hour light pulses at ZT14 (two hours after lights off). K=Kelvin; ZT=Zeitgeber Time.

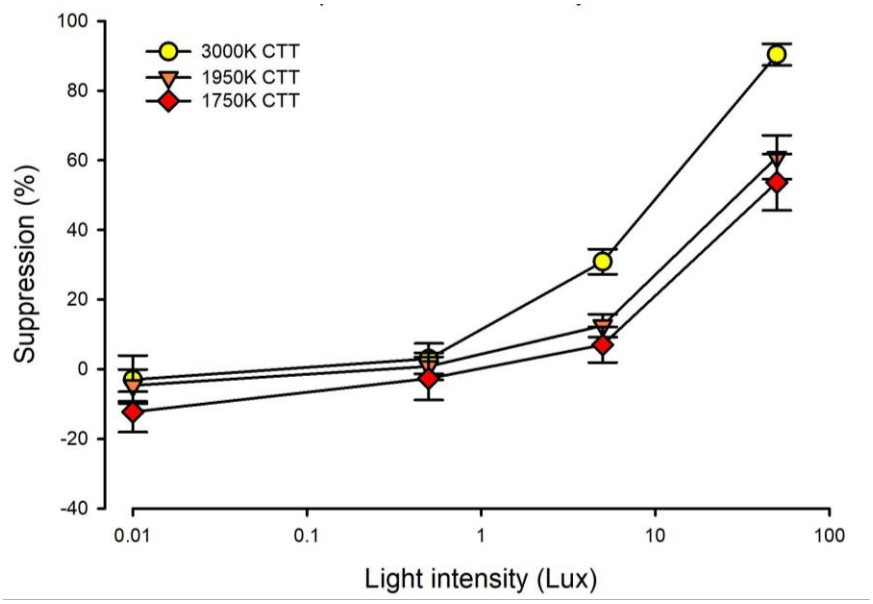

Figure 4. Effect of one-hour pulses with different CCTs and light intensity on mice locomotor activity. Light pulses at 1750K, 1950K, and 3000K with varying intensity levels from 0.01 to 50lx were delivered to wild-type mice (n=8 mice/CCT with an equal number of male and females) for 1 hour at ZT14. Activity was recorded with running wheels using the VitalView program. Percentage of suppression in activity was calculated by comparing the activity of each mouse on the day before the light pulse to the activity during the one hour light pulse. The 3000K light pulses show the strongest suppression at each light intensity setting. K=Kelvin; ZT=Zeitgeber Time; lx=Lux; CCT=correlated-color temperature.

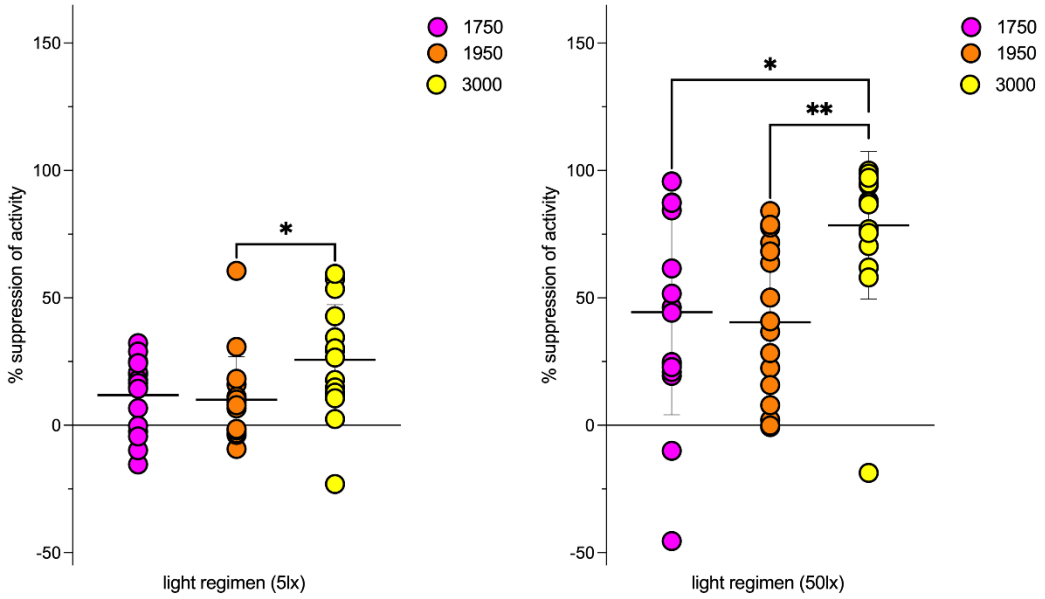

Figure 5. Effect of one-hour pulses with different CCTs on mice locomotor activity. One-hour pulses from ZT14 to ZT15 with three different CCTs (1750K, 1950K, and 3000K) of constant intensity (either 5lx, left or 50lx, right) were delivered to wild-type mice (n=16 mice/CCT with an equal number of males and females). Percent of activity suppression was calculated as described in Figure 4 and is depicted for each CCT. The 3000K CCT evoked the strongest inhibitory effect on locomotor activity regardless of the intensity. Data were analyzed using Kruskal-Wallis Test followed by Dunn's Multiple comparison Test (*P<0.0177; **P<0.0019). K=Kelvin; ZT=Zeitgeber Time; lx=Lux; CCT=correlated-color temperature.

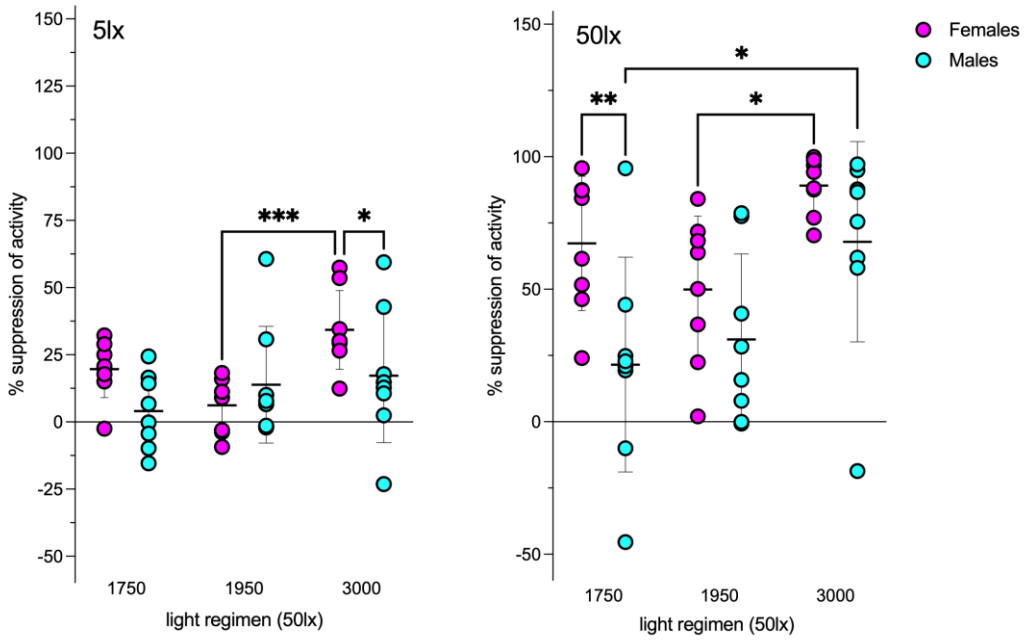

Figure 6. Sex-dependent effect of one-hour pulses with different CCTs on locomotor activity. Wild-type male and female mice (n=8/sex/CCT) were held in LD, and exposed to light pulses at three different CCT (1750K, 1950K or 3000K; either 5lx (**left panel)** or 50lx (**right panel**) at ZT 14 for 1 hour. Locomotor activity rhythms were recorded in cages equipped with wheels and activity suppression was calculated as reported in Figure 4. **Left Panel.** Female mice displayed the highest suppression in activity when exposed to 3000K light pulses as compared to both male exposed to the same CCT and females exposed to 1950K. **Right Panel.** Locomotor activity is significantly suppressed in female mice by all CCTs at 50lx when compared to their male counterparts. Percentage of activity suppression in male mice was positively correlated with increasing CCT at 50lx. Data are shown as the mean ± SD (n=8/sex/CCT) and were analyzed by two-way repeated measures ANOVA followed by Bonferroni's multiple comparisons (*P<0.05; **P<0.01; ***P<0.0005) (Table 1). K=Kelvin; ZT=Zeitgeber Time; lx=Lux; CCT=correlated-color temperature

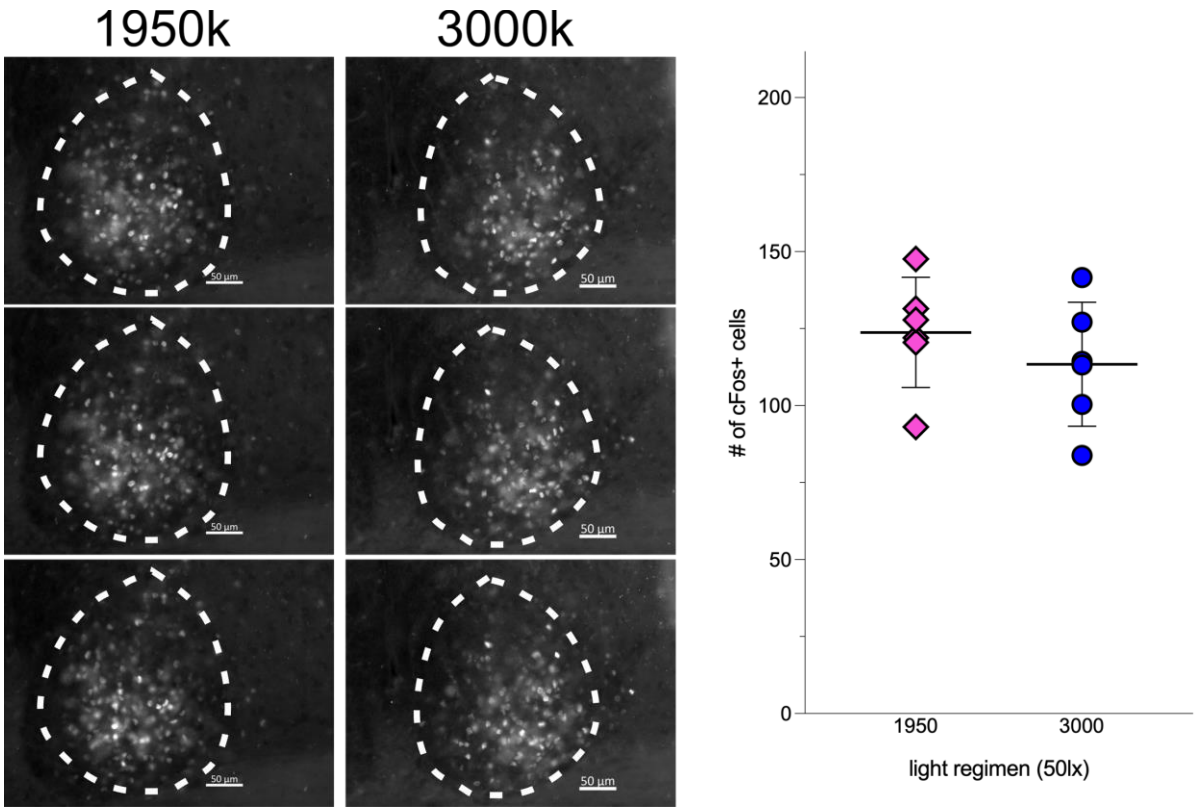

Figure 7. No differential cFos induction in the suprachiasmatic nucleus (SCN) of wild-type mice exposed to two different CCTs. Wild-type mice, held in LD, were exposed to either a 1950K or 3000K light pulse (50lx) for 1 hour at ZT 14. Mice were subsequently euthanized and perfused at ZT 15. An equal number of male and female wild-type mice were used. **Left Panels.** Representative serial images of light-evoked cFos expression in the SCN of wild-type mice. **Right Panel.** The number of cFos-positive cells was counted in both the left and right SCN in 3 to 5 consecutive coronal sections per animal and then averaged to obtain one cell count for each animal per pulse. Data are shown as the mean ± SD (n=6 mice/CCT) and were analyzed by Student's t-test (Table 1)**.** K=Kelvin; ZT=Zeitgeber Time; lx=Lux; CCT=correlated-color temperature.

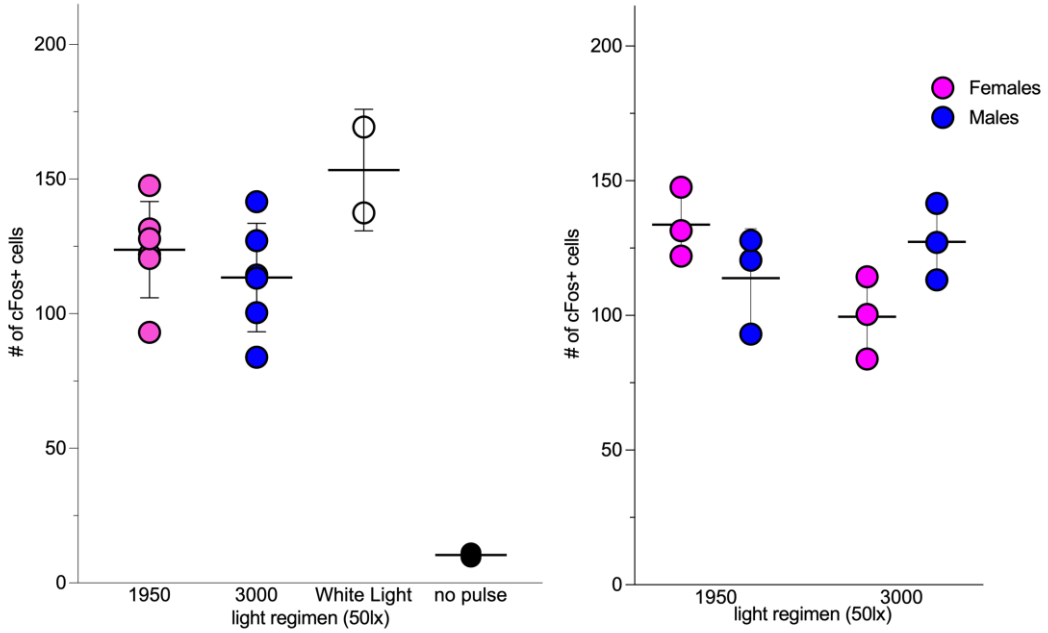

Figure 8. cFos expression in the suprachiasmatic nucleus (SCN) of wild-type mice is not differentially affected by CCT or sex. Wild-type mice, held in LD, were exposed to a 1950K(n=6; 50lx), 3000K light (n=6; 50lx), or white light pulse (about 5500K; n=3) for 1 hour at ZT 14 . Control mice did not receive the pulse (no pulse, n=2) Mice were subsequently euthanized and perfused at ZT 15. **Left Panel:** No changes in cFos-induction were observed in the SCN among mice to the different CCTs. Animals not exposed to light pulse exhibited no cFos activation. Data are shown as the mean ± SD (n=6mice/CCT) and were analyzed by one-way ANOVA followed by Bonferroni's multiple comparisons (Table 1). **Right Panel**: Evidence of divergent effects of CCT on cFos induction in male and female mice (n=3/sex/CCT). Two-way ANOVA followed by Bonferroni's multiple comparisons displayed a significant effect of the interaction between the two variables sex and light pulse/CCT (Table 1). K=Kelvin; ZT=Zeitgeber Time; lx=Lux; CCT=correlated-color temperature.

Chapter 3: Determining the direct influence of aberrant light on autistic behaviors in the *Cntnap2* mouse model of autism spectrum disorder

Chapter 3

Determining the direct influence of aberrant light on autistic behaviors in the *Cntnap2* mouse model of autism spectrum disorder

Abstract

Previous studies have shown how the circadian rhythms in humans and animals are negatively impacted by light exposure at the wrong times of the day, leading to disrupted sleep and cognitive, motor, and metabolic dysfunction. Individuals with neurodevelopmental disorders such as autism spectrum disorders (ASD) are known to experience sleep/wake cycle alterations, which can increase their exposure to light at night. We have previously shown that a mouse model of ASD, called *Cntnap2* KO, is more vulnerable to the effects of nighttime light exposure: WT and *Cntnap2* KO mice both exhibited disrupted locomotor activity, exacerbated excessive grooming behavior and diminished social preference. In order to determine whether the effects elicited by nighttime light exposure on the *Cntnap2* KO mice are due to exposure to the light itself or to a circadian disruption and malfunctional suprachiasmatic nucleus (SCN), we exposed WT and *Cntnap2* KO mice to a 3.5h light, 3.5h dark (T7) ultradian cycle. Importantly, this lighting cycle has been reported to expose mice to light without major disruptions of the circadian cycle without disrupting gene expression rhythms measured in the SCN. Thus, the T7 cycle allows us to test the hypothesis that light at night, as well as circadian disruption, are required to increase repetitive behavior and reduce social interactions in the *Cntnap2* KO model. To test this hypothesis, adult WT and *Cntnap2* KO mice were held on a 12:12 hr light/dark cycle for two weeks before entering either constant darkness (DD) or the T7 cycle. Locomotor activity was recorded using infrared motion detectors and quantified by measuring circadian period, fragmentation, and total activity. We found that WT mice under T7 exhibited a lengthened free-

running period, a significant reduction in power (%V), total activity, relative amplitude and intraday stability. The *Cntnap2* KO mice under T7 exhibited a lengthening of period along with a reduction in amplitude and intradaily stability. Critically, the DLaN-evoked increase in grooming behavior as well as the reduction in social interactions seen in the *Cntnap2* KO mice was not seen when the mice were held under T7. These findings are consistent with the hypothesis that light exposure during the night without a disruption of the circadian system does not exacerbate the autistic phenotype.

Introduction

With the advancement of technology and the implementation of artificial lighting in modern architecture and transport networks, there is a growing concern over the negative impacts of nighttime light pollution on human health. Dim light at night (DLaN) is a common environmental perturbation of the circadian timing system and has been linked to a range of deleterious consequences (Stevenson et al., 2015; Lunn et al., 2017). Prior work in pre-clinical models has demonstrated that light at night negatively impacts the metabolism (Fonken et al., 2013a; Plano et al., 2017), immune function (Bedrosian et al., 2011; Fonken et al., 2013b; Lucassen et al., 2016), mood and cognition (Lazzerini Ospri et al., 2017; An et al., 2020; Walker et al., 2020).

Individuals with neurodevelopmental disorders such as autism spectrum disorders (ASD) are known to experience sleep/wake cycles alterations. ASD is characterized by impaired social interaction and communication, alongside repetitive behavior patterns. It is considered as a lifelong disorder with consequences on the individual's social, academic, working performances and quality of life (Li et al., 2016). 1 in 68 children are diagnosed with ASD and parents of children with ASD have reported sleep problems with more frequent daily stress and worsened daytime behaviors (Baio, 2014). Due to disrupted sleep, ASD patients are more likely to be exposed to light via electronic screens or home lighting systems at night and may be particularly vulnerable to their disruptive effects (Devnani & Hegde, 2015; Elrod & Hood, 2015; Mazurek & Sohl, 2016).

Variants of the contactin-associated protein-like 2 (*Cntnap2*) gene are associated with ASD in both patient and mouse models. We have previously shown that *Cntnap2* KO mice models are more vulnerable to the effects of nighttime light exposure where dim light at night for two weeks worsened their sleep/wake cycle, locomotor activity rhythms, increased grooming behavior, and

reduced social interactions (Wang et al., 2020). In mouse models, excessive grooming and reduced social behaviors are commonly used measures of autistic symptoms. The deleterious effects of DLaN may be mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs) expressing the photopigment melanopsin, which is maximally sensitive to blue light (480nm). In an additional study (Wang et al., 2022), the usage of a light-emitting diode array enabled us to shift the spectral properties of the DLaN while keeping the intensity of the illumination. First, we confirmed that the short-wavelength enriched lighting produced strong acute suppression of locomotor activity (masking), robust light-induced phase shifts, and cFos expression in the suprachiasmatic nucleus in wild-type (WT) mice, while the long-wavelength enriched lighting evoked much weaker responses. *Opn4*^{DTA} mice, lacking the melanopsin expressing ipRGCs, were resistant to DLaN effects. Importantly, shifting the DLaN stimulus to longer wavelengths mitigated the negative impact on the activity rhythms and 'autistic' behaviors (i.e. reciprocal social interactions, repetitive grooming) in the *Cntnap2* KO as well as in WT mice. The short-, but not the long-wavelength enriched, DLaN triggered cFos expression in the basolateral amygdala (BLA) as well as in the peri-habenula region, raising that possibility that these cell populations may mediate the effects.

The DLaN used in the prior work, both causes disruption of the circadian system and exposes mice inappropriately to light at night. Both acute effects of light and circadian disruption are likely to contribute to the behavioral phenotypes observed in the *Cntnap2* KO mice. Prior work by the Hattar groups used an ultradian cycle consisting of 3.5-h light and 3.5-h dark (T7) to determine that contribution of light alone (LeGates et al., 2012; Duy and Hattar, 2017). They found that the T7 aberrant light cycle does not affect sleep architecture, rhythms in core body temperature or activity rhythms. This cycle did not cause circadian arrhythmicity in either output rhythm, although the circadian period was lengthened. Mice housed in the T7 cycle showed

rhythms in PER2 expression in the SCN similar to those of littermates housed in the T24 cycle, indicating no disruption of internal rhythmicity of the SCN pacemaker. Furthermore, Per2 levels in the liver of mice housed on the T7 light cycle were intact and in phase similar to mice under T24. Together, these data show that the T7 light cycle does not disrupt sleep or causes circadian arrhythmicity. Although the circadian timing system and sleep remain intact under the T7 light cycle, mice are exposed to light at all circadian phases during the experimental time course owing to the mismatch between the imposed light cycle and the period length. Thus, the T7 cycle will allow us to determine the direct influence of aberrant light exposure on autistic behaviors in the *Cntnap2* KO mice.

Materials and methods

Animals

All animal procedures were performed in accordance with the UCLA Animal Care Committee's regulations. As in previous studies, *Cntnap2*^{tm2Pele} mutant mice back-crossed to the C57BL/6J background strain were acquired (B6.129(Cg)- *Cntnap2*^{tm2Pele} /J, https://www.jax.org/strain/017482, (Poliak et al.,1999). *Cntnap2* null mutant (KO) and wild-type (WT) mice were obtained from heterozygous breeding pairs. Weaned mice were genotyped (TransnetYX, Cordova, TN) and group-housed prior to experimentation. In comparison to our previous studies, mice of both sexes were used in this study to examine any sex differences. 108 mice were used, 54 WT and 54 *Cntnap2* KO, with an equal number of males and females in each genotype at 3 months of age.

Cage conditions and activity

Mice were housed individually to monitor and collect locomotor activity rhythms using a top-mounted passive infrared (PIR) motion detector reporting to a VitalView data recording system (Mini Mitter, Bend, OR). The cages were placed in circadian control chambers where an environment with a temperature range of 65-75° and humidity levels of 30-40% was maintained. The mice had free access to food and water. The mice were entrained in a 12:12 LD cycle for two weeks before exposure to dim light at night (DLaN, 5 lux of light during lights off), 3.5h light: 3.5h dark (T7) ultradian cycle, or constant darkness. Each lighting condition was evaluated over two weeks.

Detected movements will be recorded in 3 min bins, and 14 days of data were averaged for analysis using the Clocklab program (Actimetrics, Wilmette, IL; http://actimetrics.com/products/clocklab/). This program will determine cage activity by averaging

the 14 days of PIR-detected motion (movements/h) and the relative distribution of activity during the day versus the night (day activity, %). The strength of the rhythm will be determined from the amplitude of the $\chi 2$ periodogram at 24 h, to produce the rhythm power (%V) normalized to the number of data points examined. Precision will be determined by first determining the daily onset of activity over 14 days, and then determining the deviation from the best-fit line drawn through the 24 onset times.

Immunohistochemistry

Following 14 days under either constant darkness or T7, the animals were euthanized with isoflurane (30%–32%) at CT18. After blood collection from the portal vein, mice were transcardially perfused with phosphate buffered saline (PBS, 0.1 M, pH 7.4) containing 4% (w/v) paraformaldehyde (Sigma). The brains were rapidly dissected out, post-fixed overnight in 4% PFA at 4°C, and cryoprotected in 15% sucrose. Coronal sections (50 μm) were obtained using a cryostat (Leica, Buffalo Grove, IL), collected sequentially, and paired along the anterior–posterior axis before further processing.

Immunohistochemistry was performed as previously described (Wang et al., 2023). Free-floating coronal sections containing the BLA were blocked for 1 h at room temperature in carrier solution (1% BSA, 0.3% Triton X-100 in 1xPBS) containing 10% normal donkey serum and then incubated overnight at 4◦C with a rabbit polyclonal antiserum against cFos (1:1000, Cell Signaling) followed by a Cy3-conjugated donkey-anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Bar Harbor, ME). Sections were mounted and coverslips applied with Vectashield mounting medium containing DAPI (4′ -6-diamidino-2-phenylinodole; Vector Laboratories, Burlingame, CA), and visualized on a Zeiss AxioImager M2 microscope (Zeiss, Thornwood NY) equipped with an AxioCam MRm and the ApoTome imaging system**.**

cFos positive Cell Counting

Z-Stack Images (7um interval, 40 images) of both the left and right BLA were acquired with a 20X objective using the Zeiss Zen digital imaging software. Three observers masked to the experimental groups performed the cell counting. The BLA margins were visualized using the DAPI nuclear staining, traced, and the cells immuno-positive for cFos counted with the aid of the Zen software tool 'marker' in three to five consecutive sections. Values obtained in the left and right BLA of each slice were averaged. The means of three-five slices were then averaged to obtain one value per animal and are presented as the mean ± standard deviation (SD) of 4 animals/light treatment. Data analysis was performed using Prism (Version 10.5.0; GraphPad Software, La Jolla, CA).

Behavioral tests

One cohort of mice (30 WT and 30 *Cntnap2* KO, equal number of males and females in each genotype) was used to test social and grooming behavior. The mice were entrained in a 12:12 LD cycle for two weeks before either continuing the LD cycle, exposure to dim light at night (DLaN, 5 lux of light during lights off), or the T7 cycle for another two weeks. On the 14th day, they were placed in a novel arena for 10 minutes to measure their grooming and exploration behavior during their subjective active phase. After habituation to the arena, they were tested using the three chamber sociability protocol (Yang et al., 2011). In this test, mice are free to roam an arena with three chambers. The central chamber remained empty while the flanking chambers contained an up-turned metal-grid pencil cup: one remained empty as the novel object (O), and an age- and sex-matched WT stranger mouse (S1) was placed in the second up-turned cup. The stranger mice had previously been habituated to the cups for 3 x 15 min sessions. To test for social preference, the mice were presented with the choice of O or S1. All three chamber tests were

performed under dim red light (<2 lx at arena level) during the animals' night. Video recordings of each arena were captured using surveillance cameras, supplemented with infra-red lighting, connected to a video-capture card (Adlink Technology Inc., Irvine, CA) on a Dell Optiplex computer system. Mice were automatically tracked using ANY-maze software (Stoelting, Wood Dale, IL).

Social preference was determined by comparing the dwell time of the tested mouse in the two chambers, and social preference index is calculated using the following: $(S1 - O)/(S1 + O)$, where a higher value indicates greater social preference. The time that the tested mouse spent investigating the object and the stranger mouse is also determined.

Grooming and investigating behaviors were manually scored and averaged post-hoc by two independent observers who were blind to the treatment and genotype conditions. ANY-maze software determined distance traveled and chamber dwell time during the behavioral tests.

Statistical Analyses

Data analysis was performed using Prism (Version 10.5.0; GraphPad Software, La Jolla, CA) or SigmaPlot (version 12.5, SYSTAT Software, San Jose, CA). Two-Way ANOVA with genotype and lighting/cycle condition (T7 and DD) or Three-way ANOVA with genotype, sex and lighting/cycle condition were used to analyze the parameters of the activity rhythms and other behavioral tests. Holm-Sidak test for multiple comparisons was used when appropriate. Data are shown as the mean as the mean ± standard deviation (SD). Alpha=0.05.

Results

In our first set of experiments, we set out to determine whether we could measure free-running rhythms in locomotor activity under an ultradian T7 rhythm. Mice were placed in T7 or in constant darkness (DD) for at least two weeks, and locomotor activity was measured. As shown by the representative actograms (Fig. 2), both genotypes exhibited free-running rhythms with periods longer than 24 hours. There were significant differences in the cycle lengths between the genotypes (Table 1). Next, we analyzed several locomotor activity parameters measured under the T7 treatment using a three-way ANOVA with genotype, sex and lighting treatment as factors. We found significant of the T7 treatment total activity, power, amplitude and cycle-to-cycle stability compared to those values under constant darkness (Table 1). This data set also allows us to compare the activity parameters of *Cntnap2* KO to WT mice. We found significant differences in period, total activity and stability (Table 1). Surprisingly, power was not different between the WT and *Cntnap2* KO. Finally, sex differences were a striking feature of this data set. We found significant differences in period, total activity, amplitude, and fragmentation (Table 1).

When the three-chamber social approach test was administered to adult mice during the night (ZT 14-16), WT typically spent more time in the chamber with a stranger mouse than in the one containing the novel object (Fig. 5). This social preference was reduced when the mice were held under DLaN but not when the mice were held on the T7 cycle (Table 2). The *Cntnap2* KO mice exhibited a significantly lower social preference than WT mice ($t = 3.924$, $p < 0.001$). Similarly, the KO mice under DLaN exhibited reduced social interactions compared to the LD controls while the T7 group was not different from LD (Fig. 5). Therefore, exposure to DLaN, but not the T7 exposure, disrupted social behavior in both genotypes, and this negative impact was strongest in the *Cntnap*2 KO.

We examined grooming behavior in the night (ZT 14-16) for each of the six groups. The WT mice spent a limited amount of time grooming and that amount did not vary with DLaN or T7 conditions (Fig. 5; Table 2). The *Cntnap2* KO spent more time grooming that WT controls ($t=5.899$; $p < 0.001$). DLaN significantly increased the amount of grooming, while T7 group was no different the LD controls (Fig. 5; Table 2). Therefore, the DLaN, but not T7 exposure, drove large increases in repetitive behavior in the *Cntnap2* KO but not in WT mice.

Finally, we sought to test the hypothesis that 2 weeks exposure to DLaN would trigger cFos immunoreactivity in the Basolateral Amygdala (BLA). Compared to mice held on a standard LD cycle or constant darkness (DD), WT and *Cntnap2* KO mice exposed for 2 weeks to the short-λ enriched DLaN displayed an increased number of cFos positive cells in BLA (Fig. 6). Conversely, exposure to the T7 cycle did not affect cFos expression. Albeit preliminary, these data suggest that the magnitude of cFos induction appears to be stronger in the mutant mice.

Discussion

In previous work, we have found that exposing mice to DLaN disrupts diurnal rhythms in locomotor activity (Wang et al., 2020). For *Cntnap2* KO mice, this nightly light exposure drove increases in repetitive behaviors and reduced social interactions (Wang et al., 2020). We also found that shifting the spectrum of light away from blue/green and into the red wavelengths reduced the negative effects of the light exposure (Wang et al., 2022). Under these conditions, the DLaN exposure resulted in the mice being directly exposed to light as well as leading to a disrupted circadian system. Conceptually, it is very difficult to tease these two factors apart. Still, prior work done in the Hattar group provide a blue-print of one strategy to help distinguish the acute effects of light from those of light-evoked circadian disruption (LeGates et al., 2012; Duy and Hattar, 2017). In this earlier work, the authors applied a light cycle termed T7 (3.5 h of dark, 3.5 h of light) to their mice. The T7 cycle exposes mice to light at all circadian phases without causing sleep deprivation or circadian arrhythmicity. In this earlier work, the authors found that chronic exposure to the T7 cycle caused depression-like behaviors and learning deficits in adult mice (LeGates et al., 2012), highlighting the direct effects of light on mood and learning function. We sought to use the T7 to examine autistic-like behaviors in the *Cntnap2* KO mice.

We found that *Cntnap2* KO mice on a T7 did not exhibit the DLaN evoked increase in repetitive behaviors and reduction in social behaviors. In addition, the *Cntnap2* KO mice on a T7 did not exhibit an increase in cFos expression in the BLA as seen under DLaN. These data suggest that light alone does not drive the increase in autistic-like behaviors in the Cntnap2 mice. There is an interaction between light at night with the circadian disruption which drives behavioral outcome. The underlying mechanisms are not yet understood.

We also carried out a detailed analysis of the activity rhythms under the T7 cycle. As described by Hattar's work, both WT and the *Cntnap2* KO mice retained rhythmicity under the T7. The period or cycle length of both genotypes was lengthened but all mice retained rhythmicity. The T7 cycle is beyond the limits of entrainment so the mice are exhibiting free-running rhythms under these conditions. As previously described, the T7 exposed mice to light while maintaining rhythmicity and this ultradian cycle can be used to distinguish effects of light from the impact on circadian arrhythmicity.

Under the T7, the *Cntnap2* KO mice exhibited weaker rhythms than those seen in WT mice. The mutants exhibited daily rhythms with less overall activity, lower power, more fragmentation and increased cycle-to-cycle variability compared to WT mice under the same conditions. Most of our prior data with the *Cntnap2* KO was collected while the animals were under an LD cycle (Wang et al., 2000; 2022). This this case, the activity data was collected under essentially free-running conditions. The fact that the disruption in the rhythms continued to be observed under these conditions adds support to our conclusion that the *Cntnap2* mutation impacts the circadian system. Sex differences were a prominent feature of this data set with males apparently more vulnerable than females. Future work will need to explore these differences.

Finally, we carefully compared the circadian parameters measured under T7 with those measured under DD conditions. For both WT and Cntnap2 KO mice, the locomotor activity parameters are clearly impacted by the ultradian conditions. Specifically, we see that the rhythms under T7 exhibit reduced power with increased fragmentation and cycle-to-cycle variability. \While this does not invalidate the value of the T7 LD cycle, our findings do raise a cautionary note. Under exposure to this ultradian light schedule, the mice maintain rhythmicity but not with the same robustness as observed in constant dark conditions. Nevertheless, our findings suggest

that night-time light exposure alone is not sufficient to exacerbate the autistic-like behaviors in the *Cntnap*2 KO mice.

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Figures and Tables

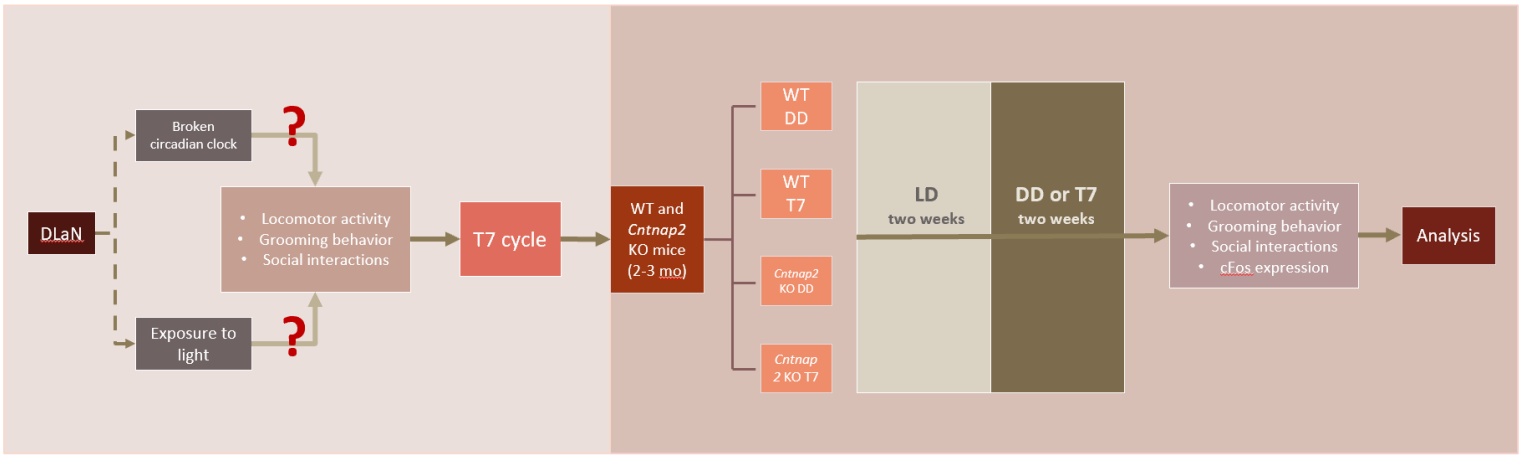

Figure 1: Experimental design

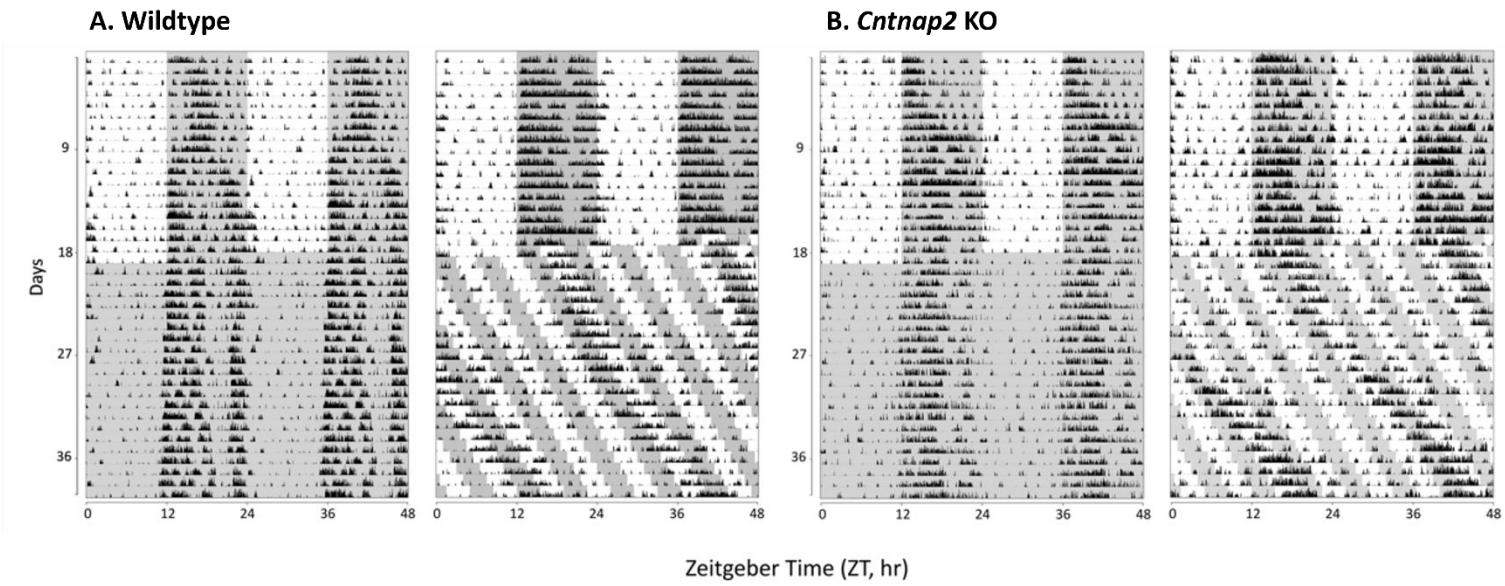

Figure 2: **Representative actograms of locomotor activity.** WT and *Cntnap2* KO animals under DD and T7 exhibit free-running rhythms with periods closer or longer than 24 hours, respectively. After two weeks of habituation, single housed mice were placed either in constant darkness (DD) or on a T7 cycle (3.5h:3.5h LD) for two weeks. Activity was recorded using infrared cameras. Activity levels in the actograms were normalized to the same scale (85% of the maximum of the most active individual. Each row represents two consecutive days, and the second day is repeated at the beginning of the next row.

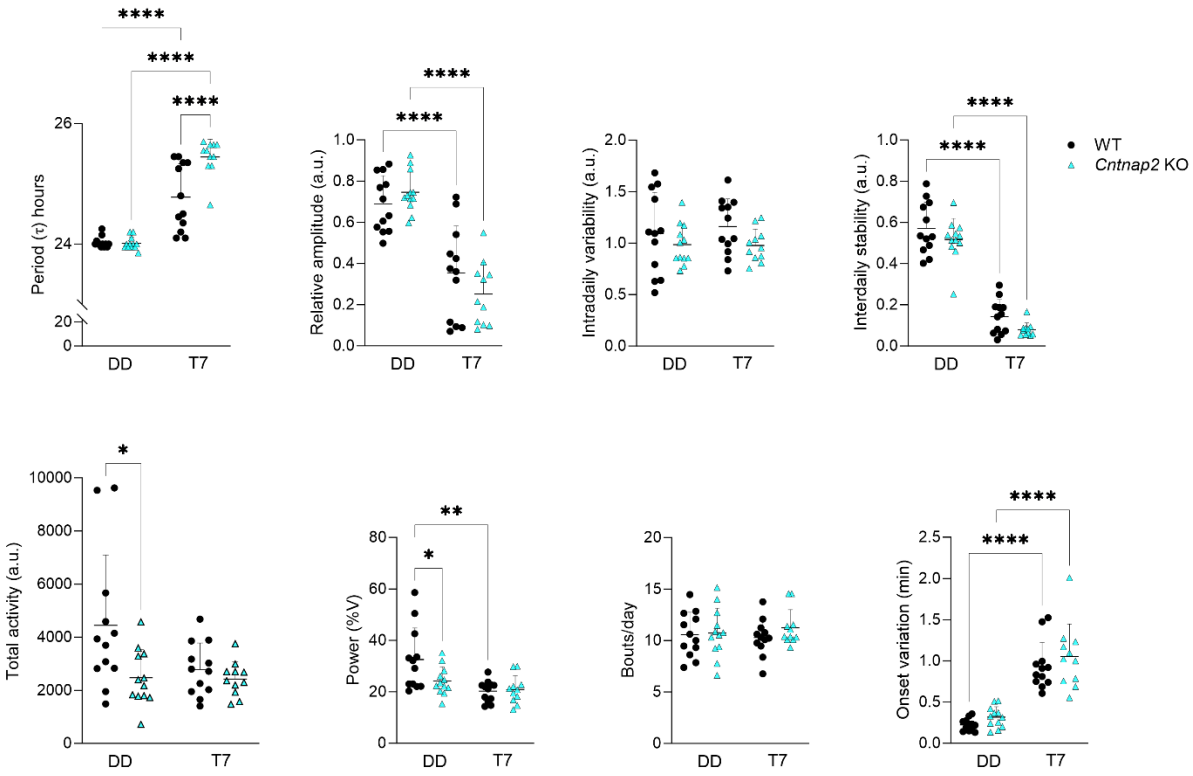

Figure 3: Altered daily rhythms of cage activity in WT and *Cntnap2* KO mice held on a T7 cycle for two weeks. Mice were held in constant darkness (DD) or on a T7 cycle (3.5h:3.5h LD) for two weeks. Several circadian parameters were influenced by genotypes and/or both environmental conditions. Period amd onset variations were significantly increased in both genotypes held in T7. Power and total activity were not affect in the mutants in T7. Data were analysed by two way ANOVA with genotype and light/cycle as variants followed by Holm-Sidak's multiple comparisons test. alpha = ($P < 0.05$).

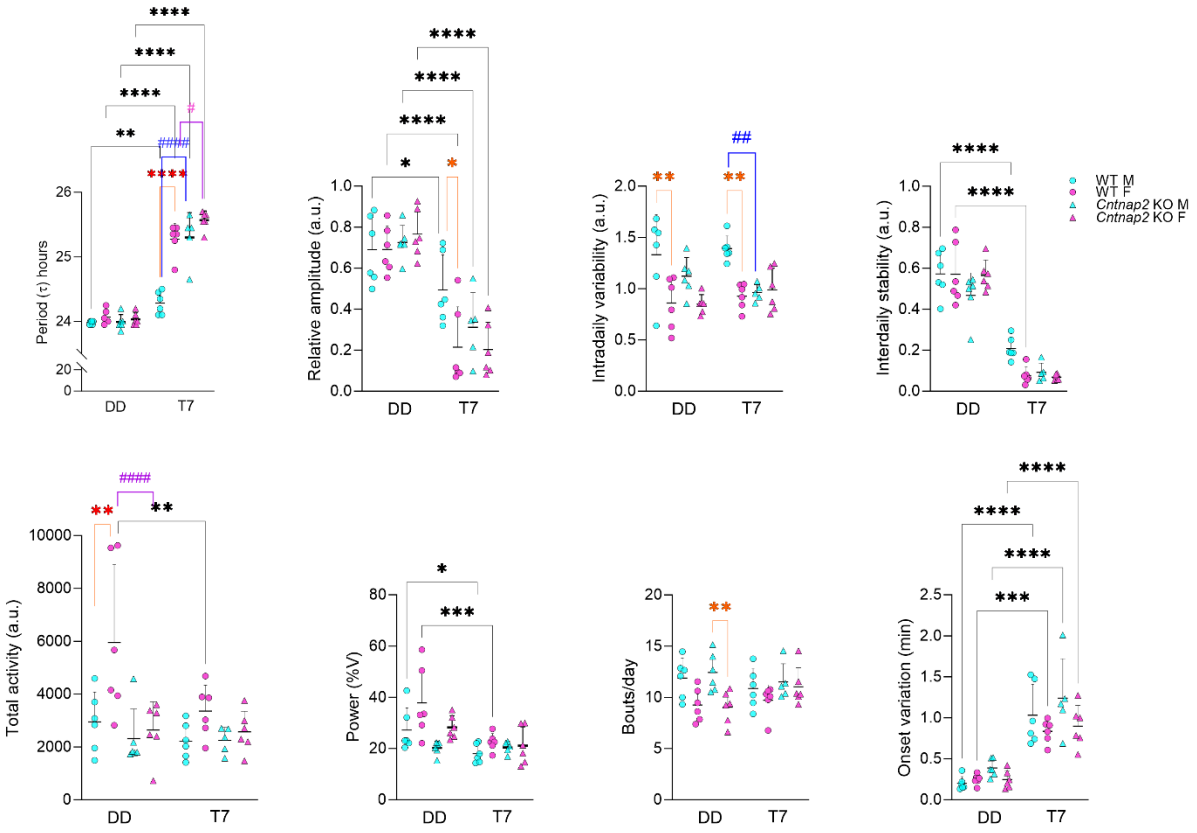

Figure 4: Sex-dependent effect of T7 on rhythms in activity in WT and *Cntnap2* mice. Mice were held in constant darkness (DD) or on a T7 cycle (3.5h:3.5h LD) for two week. Several circadian parameters were influenced by sex in both genotypes and by both environmental conditions. A significant effect of sex, genotype and light cycle was observed on both period and total activity. Period was significantly lengthened by T7 in WT females, as well as in mutant of both sexes. The effects of T7 on power and amplitude of the rhythms, as well as onset variability were more pronounced in WT mice, regardless of sex. Data were analysed by three-way ANOVA with genotype, sex and light/cycle as variables (table 1) followed by Hom-Sidak's multiple comparisons test. Significant ($P < 0.05$) sex differences within genotype are indicated with orange *, significant effect of cycle/light treatment is indicated by black *, purple and blue # indicate a genotypic difference but same sex and light cycle.

Parameter	Genotype	Sex	Cycle T7 or DD
Period	**$F_{(1, 46)}$=35.404, P < 0.001**	**$F_{(1, 46)}$=41.136, P < 0.001**	**$F_{(1, 46)}$=406.767, P < 0.001**
Total activity	**$F_{(1, 46)}$=9.768, P = 0.003**	**$F_{(1, 46)}$=10.158, P = 0.003**	**$F_{(1, 46)}$=4.457, P = 0.041**
Power	$F_{(1, 46)}$=3.713, P = 0.061	**$F_{(1, 46)}$=8.760, P = 0.005**	**$F_{(1, 46)}$=15.117, P < 0.001**
Amplitude	$F_{(1, 46)}$=0.338, P = 0.564	**$F_{(1, 46)}$=4.561, P = 0.039**	**$F_{(1, 46)}$=92.026, P < 0.001**
Stability	**$F_{(1, 46)}$=5.323, P = 0.026**	$F_{(1, 46)}$=0.342, P = 0.562	**$F_{(1, 46)}$=290.203, P < 0.001**
Fragmentation	$F_{(1, 46)}$=3.159, P = 0.083	**$F_{(1, 46)}$=9.742, P = 0.003**	$F_{(1, 46)}$=0.168, P = 0.684

Table 1: Analysis of key locomotor activity parameters by three-way ANOVA with genotype (WT, *Cntnap2* KO), sex (male, female), and treatment (DD, T7) as factors. Degrees of freedom are reported within parentheses, alpha=0.05. Bold type indicates statistical significance.

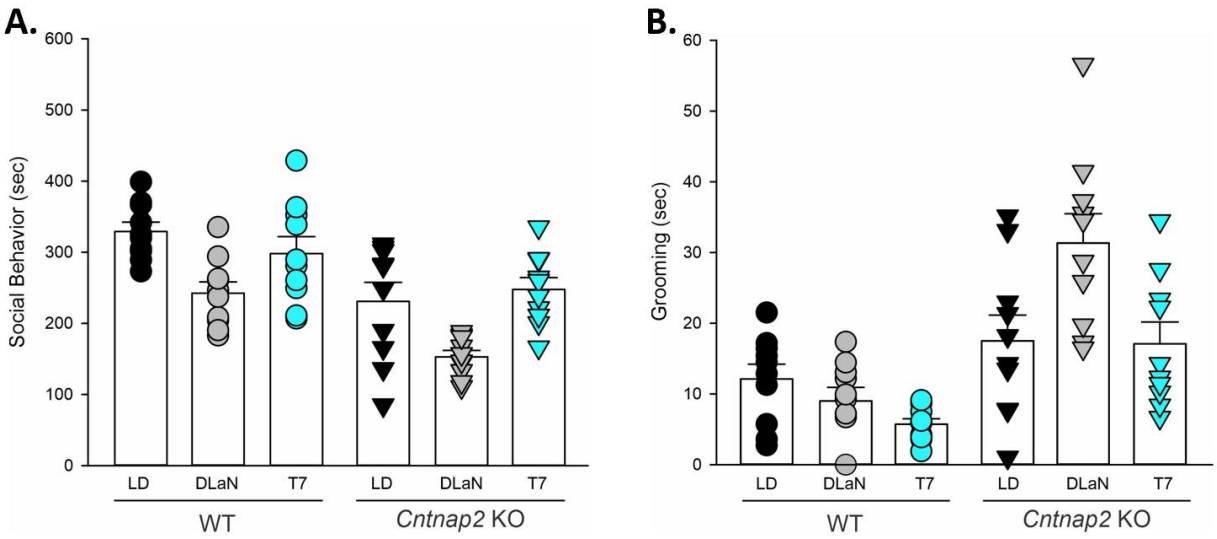

Figure 5: Social preference and grooming behavior are not impacted by T7. Mice were held on regular LD cycle, DLaN (10lx) or on a T7 (3.5h:3.5h LD) for two week and then assessed for social and grooming behavior at ZT 18 or CT 18 for the T7. In the histograms, values from individual animals in the LD control environment, under DLaN or T7 are shown. Data were analyzed using a 2-way ANOVA with genotype and treatment (Table 2) as variables. (A) Lack of effect of T7 on the social behavior in the WT and *Cntnap2* KO mice, whilst exposure to DLaN decreases social interaction in both genotypes. (B) Repetitive behaviors measured as grooming is worsened by DLaN but not by the T7 cycle in the mutants. WT mice did not show evoked grooming behavior under DLaN,

Parameter	Genotype	Treatment	Interaction
Social interactions	**$F_{(1, 59)}$=30.125, P < 0.001**	**$F_{(2, 59)}$=13.279, P < 0.001**	$F_{(2, 59)}$=1.029, P = 0.364
Grooming	**$F_{(1, 59)}$=34.799, P < 0.001**	**$F_{(2, 59)}$=5.361, P = 0.008**	**$F_{(2, 59)}$=5.057, P = 0.010**

Table 2: Analysis of Autistic-like behaviors by two-way ANOVA with genotype (WT, *Cntnap2* KO), and treatment (LD, DLaN, T7) as factors. Degrees of freedom are reported within parentheses, alpha=0.05. Bold type indicates statistical significance.

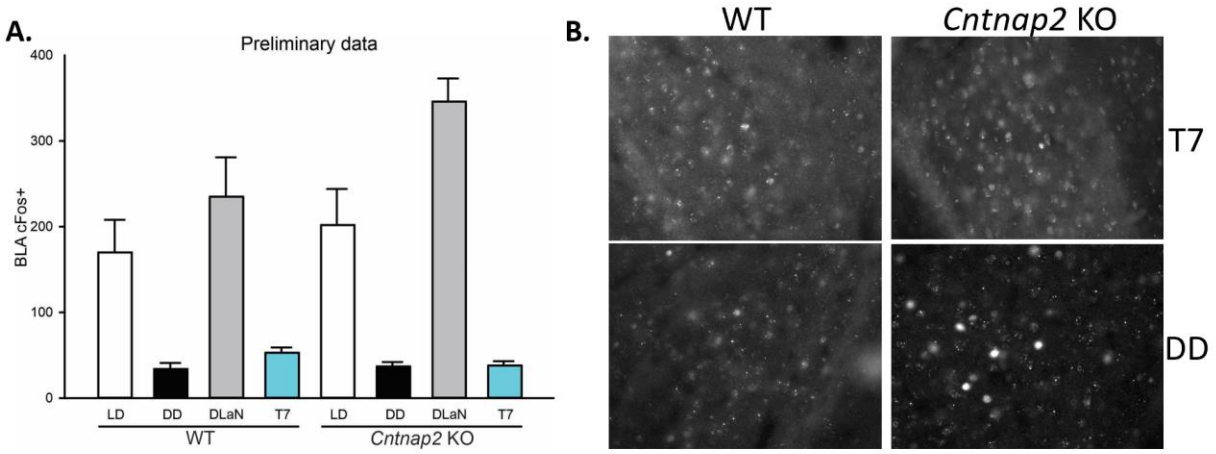

Figure 6: DLaN, but not T7, elicits a selective increase in cFos immunopositive cells in the basolateral amygdala (BLA). Such effect is more pronounced in the mutants. Brains were collected at ZT 18 from WT and *Cntnap2 KO* mice held in LD, or exposed to a short-λ DLaN (10 lx), and at CT18 from mice held in constant darkness (DD) or on a T7 (3.5h:3.5h LD) cycle for two week. (A) Quantification of the cFos positive cells in the basolateral amygdala (BLA) (B). Representative images of cFos staining in the BLA. The number of cFos positive cells was determined bilaterally in 3-4 coronal sections/animal/genotype/light condition. The values from the 3-4 consecutive slices/animal were averaged to obtain one value per animal (n=4-7), and are shown as the mean ± SEM.

Chapter 4: Discussion and future directions

Chapter 4

Discussion and future directions

During this thesis work, we carried out two sets of experiments separately described in Chapter 2 and 3. The influence of light spectral properties on circadian rhythms is of substantial interest to laboratory-based investigation of the circadian system and to field-based understanding of the effects of artificial light at night. The tradeoffs between intensity and spectrum regarding masking behaviors are largely unknown, even for well-studied organisms. In the first set of experiments, we used a custom LED illumination system to document the response of wild type house mice (Mus musculus) to 1-hr nocturnal exposure of all combinations of four intensity levels (0.01 lux, 0.5 lux, 5 lux, and 50 lux) and three correlated color temperatures (1750 K, 1950 K, and 3000 K). The highest activity suppression at 50lux was with 3000K light with higher component of blue wavelengths. An interesting aspect of this data is that female mice were more sensitive to light exposure and displayed larger suppression of activity. Assessment of light-evoked cFos expression in the suprachiasmatic nucleus at 50 lux showed no significant difference between high and low CCT exposure. The significant differences by spectral composition illustrate a need to account for light spectrum in circadian studies of behavior and confirm that spectral controls can mitigate some, but certainly not all, of the effects of light pollution on species in the wild.

In future experiments, we hope to further investigate how light at night impacts the molecular clock, including photic regulation of p-CREB and Period1 in the central pacemaker. In consideration of the negligible differences in photic induction of cFos activation between the 1950K and 3000K light pulses, we hope to repeat these experiments with CCTs that contain a greater spectral difference. By evaluating CCTs of a greater temperature disparity, we hope to further elucidate the relationship between various CCTs at night and the differential light-evoked

cFos responses in the SCN. We hypothesize that at the same light intensity, a higher, more blue-enriched CCT will stimulate the above-mentioned circadian mechanisms to a greater extent and thus, evoke greater cFos activation in the SCN. However, we should also consider that this lack of differences could be due having a low sample number, hence we were unable to properly detect sex-divergent effects.

In a second set of experiments, we sought to use an ultradian LD cycle to separate the effects of light from those of circadian disruption. We have previously shown that a mouse model of ASD, called *Cntnap2* KO, is more vulnerable to the effects of nighttime light exposure: WT and *Cntnap2* KO mice both exhibited disrupted locomotor activity, exacerbated excessive grooming behavior and diminished social preference (Wang et al., 2020; 2022). The DLaN used as an environmental stressor both disrupted the circadian system and exposed the mutant mice to light at night. In order to determine whether the effects elicited by nighttime light exposure on the *Cntnap2* KO mice are due to exposure to the light itself or to a circadian disruption, we exposed WT and *Cntnap2* KO mice to a 3.5h light, 3.5h dark (T7) ultradian cycle. Prior work by the Hattar groups used this T7 cycle to separate the impact of light at night from light-evoked circadian disruption (LeGates et al., 2012; Duy and Hattar, 2017). They found that mice under the T7 aberrant light cycle do not exhibit circadian disruption but do exhibit light evoked changes in mood-like behavior. Thus, they concluded the light at night alters mood but does not work through circadian disruption to cause these changes.

We sought to use the T7 cycle to i. disentangle the effect of light at night from circadian disruption on autistic-like behaviors; ii. determine the direct influence of aberrant light exposure on autistic behaviors in the *Cntnap2* KO mice; iii. test the hypothesis that light at night and circadian disruption are both required to increase repetitive behavior and reduce social interactions

in the *Cntnap2* KO model. Hence, adult WT and *Cntnap2* KO mice were held on a 12:12 hr light/dark cycle for two weeks before entering either constant darkness (DD) or the T7 cycle. Critically, the DLaN evoked increase in grooming behavior as well as the reduction in social interactions seen in the *Cntnap2* KO were not seen when the mutant mice were held under T7. These findings are consistent with the hypothesis that light exposure during the night without a disruption of the circadian system does not evoke a worsening of the autistic phenotype. Under the T7 the mice exhibited a longer cycle length but were clearly rhythmic. Compared to DD conditions, this lighting did reduce the amplitude of activity as well as increase cycle to cycle variability in both WT and *Cntnap2* KO mice. Sex differences in the circadian parameters were evident across both genotypes, with males showing the most sensitivity. Interestingly, DLaN drove a robust increase in cFos expression in basolateral amygdala while the T7 exposed mice did not show this sign of neural activity. Future work will need to explore these sex difference but also the cFos expression in the BLA.

Finally, we have preliminary evidence that DLaN elicits an inflammatory response that we believe may drive its negative effects on mouse behavior. We intend to test this hypothesis with measures of peripheral and central inflammation. As part of these studies, we will examine the impact of the T7 cycle which we expect will not elicit inflammation. Future work will then explore the mechanisms through which DLaN, but not T7, increases inflammation in the mouse models of ASD.

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