Perinatal Photoperiod Affects the Serotonergic System and Affective Behaviors

By

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For my family:

Mom, Dad, Seth, Sarah and Grace

I could not have done it without you.

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PREFACE

I took a deep breath as I stood over the shoulder of a 10 year old boy with ADHD who was looking through a 10 year old compound microscope at a drop of creek water. For the first time since I met the child a few months earlier he was completely still. He looked up at me with wide, fascinated eyes and said the four most gratifying words anyone teaching science will ever hear, “That is so COOL!” I was teaching a science and nature camp class at Oakland School summer camp between my second and third years of college. While I was showing the children the kinds of unseen life that flourish in regular creek water, I found myself reflecting on how I became interested in biology in much the same manner. My parents had given me a microscope similar to the one the children were using at camp when I was in early middle school. That microscope spawned a fascination with the complexities of life that has endured throughout my adult life.

During my junior year in high school I chose to do an independent study of microbiology. Based on this project I was subsequently awarded a summer internship in a microbiology lab at the University of Virginia. This experience taught me that I was absolutely fascinated with how life is maintained and that research is the basis for all medical treatments and procedures. It became clear to me that a single person can make an amazing impact in the biomedical field. As I continued my education I started to realize that we live in a very exciting time in the field of science. Not only are we developing new scientific techniques to answer longstanding biological questions but the general public is becoming more and more interested in applying the results the scientific community is generating to their daily lives.

In college, Dr. Chris Winter accepted me as his research assistant and introduced me to the field of circadian rhythms. He taught me the importance of our biological rhythms and how disruptions of these rhythms can lead to a variety of public health concerns. While my research in college was mainly at the population level, I became increasingly interested in the

mechanisms that drive our rhythms and how those mechanisms are altered to produce behavioral phenotypes. This curiosity led me to search for a field of study within biology that would fuel my craving for understanding many of these complex issues. I discovered the world of molecular, cellular and systems neuroscience.

These events, along with a myriad of happenings not related to science or education, brought me to graduate school at Vanderbilt University. During my first year while I was still rotating through labs in the department, I had a conversation with a senior graduate student, Jeff Jones, about electrophysiology and how it can be used to study the functions of neurons. I was enthralled with the idea of being able to watch neurological activity happen in real time and decided that I would like to pursue a specialty in electrophysiology. I was accepted into Dr. Douglas McMahon's laboratory. After discussing the research his lab was conducting, I decided that I would like to target my research toward understanding how environmental signals such as light can alter the circadian clock and therefore affect mood disorders. One of the main reasons I decided to study how environmental signals influences behavior and specifically mood disorders was because of the relative abundance of these disorders as well as the prevalence of undiagnosed mood disorders in the population.

This drive along with several studies out of northern Europe and the UK, correlating season of birth with the prevalence of mood disorders later in life, lead me to be increasingly interested in how the amount of light an organism receives during development could enduringly alter neurochemical pathways related to mood disorders. Thus I endeavored to investigate the effects of developmental light cycles on the serotonergic system, one of the main neuromodulatory systems implicated in the control of affective behaviors. In this dissertation I will illuminate and discuss my findings regarding how developmental light cycles can produce enduring changes in the serotonergic system and therefore influence affective behaviors during adulthood. While there are still numerous unanswered questions regarding this phenomenon, I hope that my work has increased our understanding of how environmental signals during

development can result in long lasting changes in neurological function. It is also my hope that this work provides a foundation on which future research can build to decrease the prevalence of mood disorders worldwide.

Chapter 1

Introduction

The study of the influence of photoperiod (the duration of light in a 24 hour period) on physiology and health dates back to the time of Hippocrates, who famously wrote, "Whoever wishes to investigate medicine properly should proceed thus: In the first place consider the seasons of the year…" Since that era, some scientists and physicians have wisely taken this theory to heart. Our seasons result from a 23.5 degree tilt to the earth's rotational axis[1-3]. This axial tilt was likely caused by the earth being impacted by a planetoid known as Thia[a] during the formation of our solar system[4]. This impact not only knocked Earth onto its current axial tilt, but also expelled enough of the earth's mantle and crust into the cold recesses of space to eventually form our moon.[4,5].

The fact that the seasons are caused by the axial tilt of the earth was first proposed by Hippocras around 150 BCE, predating the heliocentric model of Nicolaus Copernicus. The "Copernican revolution" eventually resulted in the discovery of Earth's elliptical orbit, with the Sun at one of the elliptical focal points theorized first by Johannes Kepler in the 17th century. This discovery lead to the idea that seasons may be caused by the distance from the sun as the earth traverses its orbital path around the sun. Counterintuitively this elliptical orbit actually plays a very small role in our seasons, with only about 7% of the light/dark seasonal variation due to our elliptical orbit [3]. Therefore, it took nearly 2,000 years from the time Hippocrates first proposed a tilt of the earth could result in seasons for the idea to be commonly accepted.

In this dissertation I will concentrate on the photoperiodic aspects of seasons rather than the variation in the temperature cycle over the seasons. Due to the earth's axial tilt, the farther from the equator the more extreme the changes in the light cycle are across seasons. At each pole the photoperiods are so extreme that during the winter solstice the sun never breaks the horizon resulting in a full 24 hours of darkness, while during the summer solstice the light period

takes up the entire 24 hour day. The earth's axis being tilted also means that the Northern and Southern Hemispheres experience contrasting seasons, a phenomenon resulting from one hemisphere being tilted toward the sun and the other away from the sun. The earth's seasons have had a significant impact on plant and animal evolution and ecology, decreasing biodiversity with increasing distance from the equator, as seasonal variations become more extreme. In terms of evolution, the selective pressure on plants and animals that have to adapt to changing light cycles and temperatures throughout the year (such as those at higher northern or southern latitudes) would be far greater than those adapted to a single light and temperature cycle (such as organisms at the equator). Though this selective pressure has resulted in some very interesting seasonal adaptations in animals that live at these higher latitudes, I will demonstrate in this dissertation that it appears that aspects of the mammalian nervous system have not fully adapted to the seasonal environments they inhabit.

1.1: Seasonality and Circadian Rhythms

Many multicellular organisms exhibit seasonality or changes in their physiology due to changes in light cycles or temperature. Seasonality is often studied in the laboratory by altering an organism's light cycle or melatonin profile. Melatonin is only synthesized in darkness and is considered a photoperiodic hormone because, as the length of the dark period an organism experiences changes, so does the amount of melatonin being produced. By showing that lizards are able to entrain (or lock onto environmental cycles such as light/dark) their locomotor rhythms to daily melatonin injections during a summer, but not winter, light cycle, Bertolucci and colleagues demonstrated that seasonality can be observed in lower vertebrates. This suggests that the at least part of the reptilian circadian system is dependent on the duration of light the animals receives and therefore can be considered seasonal[6].

Many birds also exhibit seasonality due to photoperiodic changes. Breeding, molting and even song qualities are all influenced by changes in their photoperiodic environment[7]. An increases in the duration of light signals the onset of spring and the coming of summer, with this environmental cue the avian brain increases the production of gonadotropin-releasing hormone (GnRH), increasing the likelihood of breeding. Bird songs also respond to lengthening light periods with an increase in song frequency and volume during light cycles typical of spring and summer months. Both of these physiological and behavioral changes reverse themselves as the light cycle starts to shorten signaling the start of fall and the coming of winter[7].

Interestingly, some mammals have the reproductive reaction to increasing or decreasing light that is opposite to that of birds. For example, Goldman and colleagues demonstrated that hamsters developed on a winter-like light cycle have rapid testicular maturation and growth in anticipation of breeding in the spring. Hamsters developed on a summer-like light cycle will delay their testicular maturation and growth in anticipation of the oncoming winter and the accompanying need to conserve energy[8]. However, in adult pinealectomized hamsters an artificial melatonin profile corresponding with a winter-like light cycle will result in testicular regression much like birds[9]. While most mammals breed during the spring or summer months, there are mammals that breed during the winter months as well. One example of winter breeding mammals is the common sheep. Animals such as sheep have the opposite reaction of hamsters to a winter-like melatonin profile. Ewes that were kept on a summer-like light cycle (16 hours of light, 8 hours of darkness) but were given daily oral doses of melatonin corresponding to what they would produce in a winter-like light cycle initiate their estrous cycle 2-8 weeks before untreated ewes[10]. These data suggest that while the breeding schedule may be different in different mammals many of them time their breeding using photoperiodic signals such as melatonin profile.

Mammals detect these photoperiodic signals through the retina in the eye which is the only area of the mammalian body that contains photoreceptive cells[11-13]. This information is

then transmitted to the circadian system in the brain. The circadian system is the body's internal clock that controls the timing of multiple behavioral outputs such as the sleep/wake cycle, feeding and breeding. To do this the circadian system communicates with many other parts of the brain that are involved in the generation of behavioral outputs. One of the main environmental signals that the circadian system uses to keep time is light detected through the retina.

There has been a significant amount of evidence on how light can affect the circadian system in many organisms, including mammals[14,15]. Mammals have a master circadian oscillator, the suprachiasmatic nuclei (SCN), situated in the hypothalamus directly above the optic chiasm[16]. The SCN receive light input through the retinohypothalamic tract (RHT) via afferent projections from photoreceptive cells in the retina. The SCN receives light information from typical photoreceptors such as rods and cones, but also from specialized intrinsically photosensitive retinal ganglion cells (ipRGCs)[17]. These cells contain the photopigment melanopsin, which unlike normal photopigments reacts to light with a slower, sustained cellular response[18]. In mice photoreceptors are partially functional during development and there is even some evidence that they can detect and respond to light signals through the uterine wall[19]. Therefore it is possible that in mice as well as other mammals, the photoperiod experienced around the time of birth could play a role in development through direct perception of light in the uterus.

Our lab has shown that perinatal photoperiod does indeed have an effect on the circadian gene expression rhythms in the SCN as well circadian behavior[20]. Our lab previously showed that exposure to a long photoperiod perinatally causes a decrease in the duration of the neuronal peak of *Per1*::GFP (a gene involved in the molecular clock, a core clock gene) expression in SCN neurons. There was also a significant decrease in the neuronal period and a trend toward a decrease in the SCN period of mice developed on a long photoperiod compared to a short photoperiod. This translated to circadian wheel running behavior as a decrease in the

behavioral period of animals developed on a long photoperiod compared to short. These effects were not reversed by switching the animals to the opposite photoperiod at weaning and maintaining them on that photoperiod for several weeks[20]. This suggests that there is a critical window during development where environmental light signals can substantially alter SCN circadian gene expression patterns as well as circadian behavior.

1.2: Melatonin, Serotonin and Their Interactions

While our lab did not further pursue the question of how perinatal photoperiod may be causing these changes in both the SCN and behavior, there was much discussion in the field after those results were published. One of the leading theories was that melatonin (MT) may be playing a significant role.

Melatonin is synthesized exclusively during darkness and levels can be rapidly decreased with even a short light pulse[21,22]. How melatonin- which is not produced by the pineal gland until two weeks after birth- could be playing a role in the perinatal photoperiodic programming of neural systems, however, is an important question. The answer is provided by studies done in hamsters showing that the maternal melatonin profile is transferred to the fetus across the placenta and is able to program the fetal circadian clock and reproductive development during a sensitive period late in gestation[23]. Because pups do not produce melatonin during the first two weeks after birth, this maternal melatonin profile must be the main factor in the photoperiodic programming during development[24]. To demonstrate this point, hamster mothers were pinealectomized and given timed daily injections of melatonin representative of different photoperiods (i.e., an extended injection, representing a short photoperiod and a shortened injection representing a long photoperiod). The reproductive maturation of these pups reflected the length of the timed infusions of melatonin. Pups that experienced a “short photoperiod” during development exhibited accelerated reproductive

maturation as if they were developing during winter and should be ready to breed early in life during the spring. Conversely, pups that experienced a "long photoperiod" during development had significantly delayed reproductive maturity as if they were developing during the summer and would wait until next spring to be ready for breeding[23,24]. These experiments, along with the fact that melatonin receptors are present in the SCN as early as E18[25], suggest that melatonin is the key factor in the perinatal photoperiodic programming of the circadian clock and reproductive development. This dissertation will examine if it is also the critical neurochemical signal underlying the photoperiodic programming of the serotonergic system and affective behaviors later in life.

This idea originated from work done in our lab by Drs. Chris Ciarleglio and Holly Resuehr. They demonstrated that perinatal photoperiod can alter depression- and anxiety-related behaviors in C3H/C57 mixed-background mice (which are melatonin proficient[26]) by subjecting mice to forced swim (FST) and tail suspension tests (TST) to measure behavioral despair or depression-related behaviors as well as the open field test (OFT) and the elevated zero maze (EZM) to test anxiety-related behaviors. They showed that mice developed on a long photoperiod spent less time immobile in the FST and TST and decreased time spent in the closed arm of the EZM and decreased thigmotaxis in the OFT compared to mice developed on a short photoperiod. All of these results suggest that mice developed on a long photoperiod exhibit anti-depressant and anxiolytic phenotypes compared to mice developed on short photoperiods[26]. This led us to consider the possibility that perinatal photoperiod may be affecting the serotonin system.

Serotonin, or 5-hydroxytryptophan (5-HT), is one of the major neurotransmitters involved in the regulation of affective behaviors[27]. The rate limiting enzyme in 5-HT production, tryptophan hydroxylase (TPH), catalyzes the conversion of the basic amino acid tryptophan into 5-hydroxytry-L-tryptophan. TPH1, the first of two TPH isoforms, is mainly localized to peripheral tissues that produce 5-HT, such as the immune system and gut endothelial cells. TPH2 is

located almost exclusively in the central nervous system and therefore produces the majority of 5-HT found in the brain[28]. *Tph2* expression, and therefore 5-HT levels, in the brain exhibit a robust circadian rhythm. In rats, *tph2* expression exhibits its highest expression around two hours after light onset (Light onset: Zeitgeber time [ZT] 0*, tph2* peak expression: ZT 2) and ending around six hours later (ZT8)[29-32]. The 5-HT rhythm shows a characteristic lag of about 2 hours behind *Tph2* expression with a crest ranging from ZT 4.5 to ZT10.5 hours and a trough from ZT 13.52030 to ZT14.5[33].

Many drugs used to treat depression and anxiety act on the 5-HT system, and one of the most widely used classes of drugs among these are selective serotonin reuptake inhibitors (SSRIs)[34]. These drugs inhibit the serotonin transporter (SERT), allowing 5-HT to stay in the synapse longer and therefore act on post-synaptic 5-HT receptors for an extended period of time. This action has been shown to decrease depression and anxiety related behaviors in mice, as well as alleviate the symptoms of affective disorders in humans[34]. The effect of these drugs suggests that moderate increases in the level of free extracellular 5-HT can significantly affect mood and mood-related disorders. Considering this information and the fact that melatonin has been shown to directly act on 5-HT neurons, we decided to determine if perinatal photoperiod affects the 5-HT system in a melatonin- dependent manner.

The serotonergic system in the central nervous system is located in the raphe nuclei in the midbrain[35], consisting of the dorsal (DRN) and medial raphe nuclei (MRN). These two nuclei have distinct anatomical, functional, and physiological characteristics. The DRN is located directly below the cerebral aqueduct and serotonergic neurons there project to throughout the brain, including brain areas involved in the control of mood and emotions such as the amygdala, hippocampus, and forebrain [36-39]. The MRN has strong reciprocal connections with the SCN and other locations involved in behavioral arousal, among a collection of other targets[40-43]. Though in human adults, light information is transduced through the retina, down the RHT to the SCN, then delivered to the raphe[44,45] there is some evidence that rodents may

have a direct retina-raphe connection to aid in the serotonergic systems perception of this information[46,47]. The circadian system exerts control over the serotonergic system, but the reverse is also true: 5-HT has been implicated heavily in the non-photic shifting of the circadian system as well as behavioral arousal[48-50]. In the following chapters, the data I present will center around neurons in the DRN in keeping with our goal of finding connections between perinatal photoperiod and affective behaviors made through the serotonergic system.

Because serotonin is low in the dark phase when melatonin is high, it was logical for researchers to search for a link between the two systems. A recent study showed that diurnal administration of melatonin significantly decreased the basal firing rate of serotonergic neurons in the rat DRN (Figure 2). This effect was reversed by the melatonin one and two (MT1/MT2) receptor antagonist luzindole but not by the selective MT2 receptor antagonist 4P-PDOT, suggesting this effect is mediated through the inhibitory action of the MT1 receptor[51]. There is also evidence that melatonin has an effect on the metabolism of serotonin, as in pinealectomized rats daily melatonin treatment increased the 5-HT metabolite 5-HIAA as compared to rats without melatonin treatment in cerebral spinal fluid and other nervous tissues[52]. This neural and metabolic modulation suggests the potential for maternal melatonin crossing the placenta during development to have a significant effect on programming of the serotonergic system later in life.

1.3: Photoperiod and Mood Disorders in Humans

There is accumulating epidemiological and molecular evidence that perinatal photoperiod alters neurological systems in both laboratory animals and in humans. There have been a couple of epidemiological studies in England focused on the correlation between month of birth and prevalence of psychiatric disorders[53,54], demonstrating that people born in the winter months (November-February) have an increased incidence of schizophrenia, bipolar disorder,

and recurrent or major depression compared to those born in the summer months (May-August)[54]. England, being at a relatively high latitude (London- 51.5º N), has an average of about an 8 hour difference in natural light exposure between the summer and winter solstices. This would mean that people born during the winter months in London are exposed to about a 50% shorter duration of light during development than those who are born during the summer months. However, it is not known if the duration of light is the direct cause of this phenomenon. There may other factors that could result in the correlation between season of birth and the prevalence of affective disorders later in life such as changes in the total number of photons detected by the retina during different seasons or change in the seasonal entrainment of the circadian clock.

A number of studies have investigated whether there may be a molecular or genetic basis for this correlation between season of birth and affective disorders. In fact, people who are born in the late winter or spring months show an increase in the level of the 5-HT metabolite 5-HIAA in their cerebral spinal fluid (CSF), and those born in the late summer or fall months show in increase in the dopamine (DA) metabolite HVA in their CSF compared to those born during the remainder of the year[55]. Additionally, there may be a genetic component to this correlation. There are significant ties between season of birth and the prevalence of polymorphisms in genes associated with affected disorder, such as *tph2, sert* and DA receptor D4 (*drd4*). The *tph* and *drd4* gene variants are increased in people born in the winter months while the *sert* variant is elevated in people born in the summer months[56].

In addition to affective disorders and monoamine-associated molecular and genetic factors, season of birth is is correlated with suicide and suicide-related factors. A Hungarian study examined the correlation between season of birth and suicide rates and, interestingly, found the opposite relationship as those studying affective disorders. They showed that in both male and female cohorts, suicide rates were significantly increased in people born in the summer months compared to the winter months[57]. Another group showed that season of birth

can actually affect the method of suicide as well: people born in the spring months preferred violent methods of suicide such as shooting or hanging while people born in the fall months preferred passive methods such as poisoning[58].

Finally, there are significant correlations between season of birth and disorders less related to personality or mood. Season of birth appears to have an effect on the human circadian system, with people born in the summer months having a later chronotype (meaning they naturally go to bed and wake up later) than people born during other times of the year[59]. There has also been some research that suggests season of birth can effect learning with a higher incidence of dyslexia and attention deficit hyperactive disorder (ADHD) in people born during the summer months[60,61], as well as higher incidence of autism spectrum disorders present in people born during March and August in Israel[62,63].

This research, taken together, certainly shows that season of birth in humans can affect a number of physiological and genetic factors involved in a wide range of changes in behavior. The variables associated with season of birth in humans, however, have not been isolated and studied independently. Changes researchers have observed between seasons of birth could be due to changes in the duration of light people receive, changes in temperature, changes in vitamin D levels, exposure to the flu virus and countless other variables associated with the changing of seasons. For this reason, it is important to study each of these variables in controlled laboratory settings to determine which may be most associated with this photoperiodic programing.

1.4: Animal Models

While there are many different types of common laboratory mice, many strains do not produce melatonin in biologically relevant quantities because of a point mutation in the rate limiting enzyme aralkylamine N-acetyltransferase (AANAT) that converts serotonin into N-acetyl-

serotonin, which is then further converted into melatonin by the enzyme ASMT[64]. I required a strain of mouse that produces melatonin (melatonin competent) because of our hypothesis that melatonin may be playing a significant role in the photoperiodic programming of the serotonergic system. Therefore, in these experiments I used a modified version of the common C3H strain of mouse. C3H mice have a retinal degeneration mutation that causes the rods and cones in the retina to degenerate by the time the mice are two months old[65]. I used a version of the C3H mouse referred to as $C3Hf^{+/+}$ that lack this retinal degeneration mutation(Figure 1) and therefore maintain their sight for the entirety of their lives. We obtained melatonin receptor 1 (MT1 KO) and melatonin receptor 1 and 2 (MT1/2 KO) knock-out mice from Dr. Gianluca Tosini, who developed these mice on the $C3Hf^{+/+}$ background that serves as our control mice[66].

$C3Hf^{+/+}$, MT1 KO and MT1/2 KO mice were developed and raised to maturity on an Equinox (Eq, 12 hours of light 12 hours of darkness), Short (S, 8 hours of light and 16 hours of darkness), or Long (L, 16 hours of light and 8 hours of darkness) photoperiod. These light cycles correspond with naturally occurring summer, spring/fall or winter-like light cycles at latitude of about 50 degrees. With some of the world's major cities located around this latitude, including London, Vancouver, Berlin, and Warsaw, millions of people experience these light cycles.

We also were interested in the possible photoperiodic programming of the serotonergic system was perinatal or proximal (a result of the photoperiod the mice were on at the time of testing). Therefore, at P30 some mice were switched to the opposing photoperiod - S to L, L to S, etc.(Figure 2). Experimental assays were performed between P50 and P90 (considered young adulthood in the mouse), with the exception of one L to Eq cohort in which the animals were tested 5 months after switching to examine the longevity of any perinatal effects observed. Tissues were extracted and behavior assays were performed at the mid-day point on all light cycles to account for the circadian aspect of 5-HT production in the brain. Experiments were

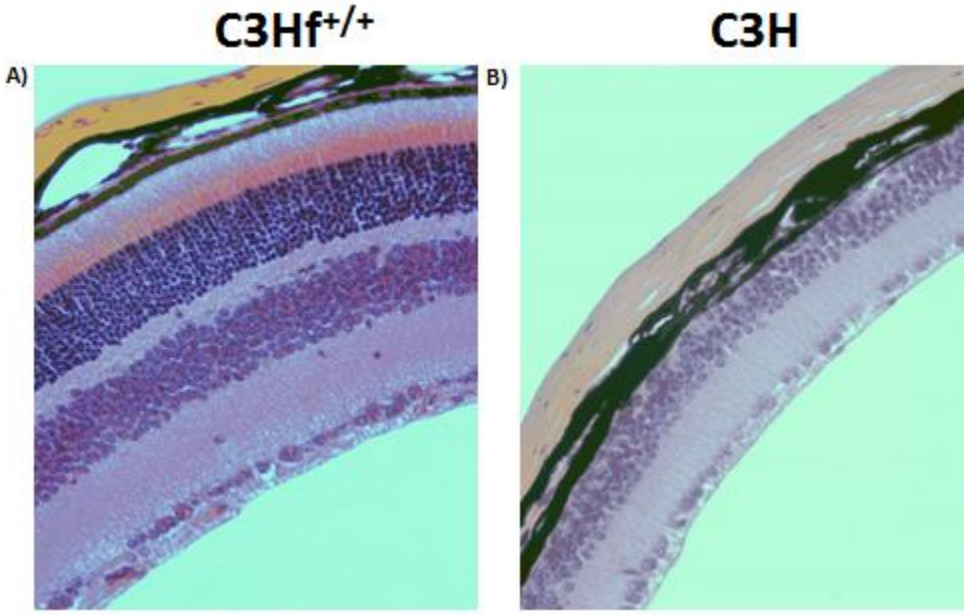

Figure 1: Cross section of retinas from a four month old C3Hf$^{+/+}$ and C3H mouse. A) Cross section of a C3Hf$^{+/+}$ mouse retina with an intact photoreceptor layer. B) Cross section of a C3H mouse retina with a completely degenerated photoreceptor layer (pictures taken by Chad Jackson)

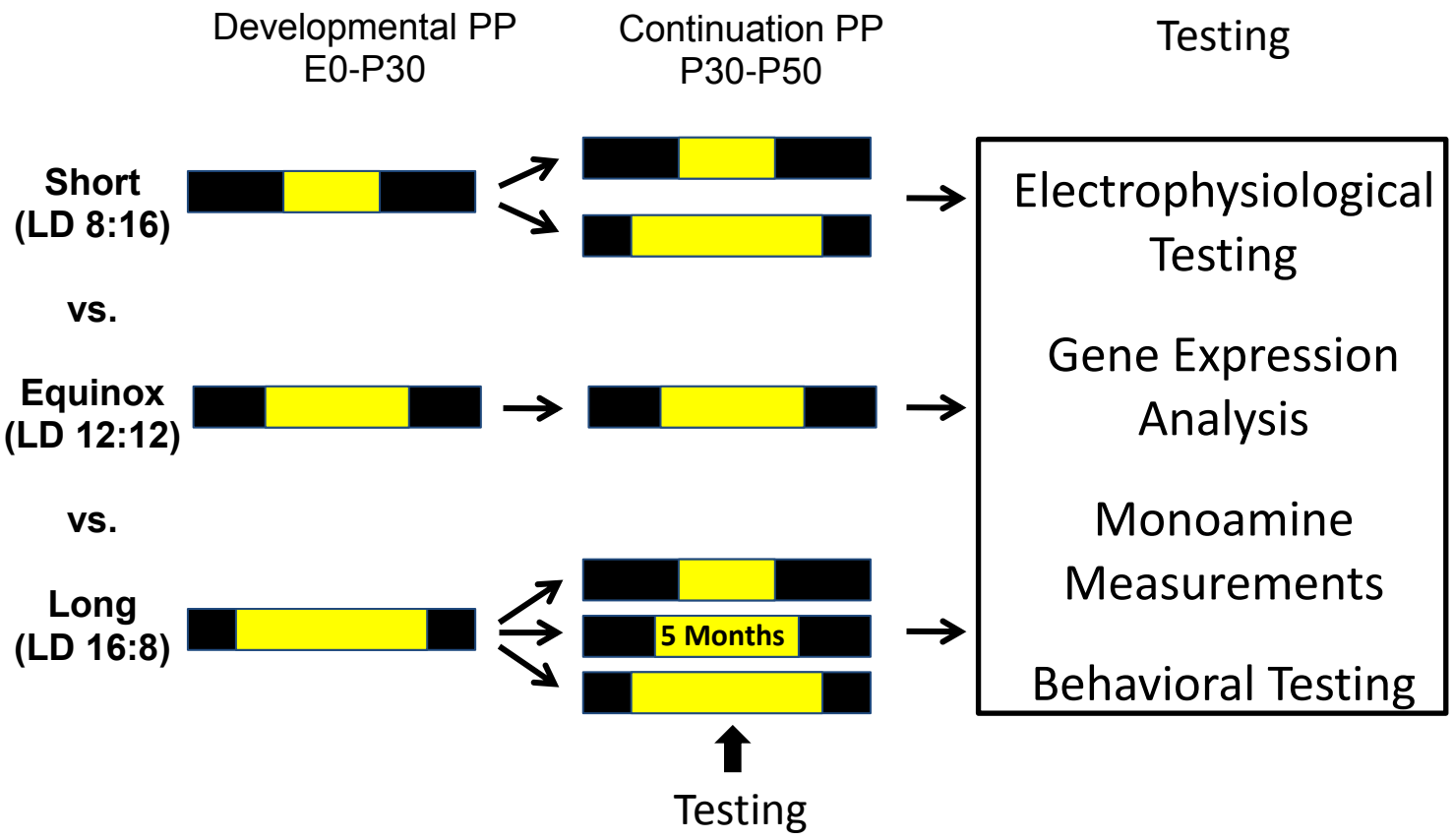

Figure 2. Diagram of our Photoperiod Paradigm.

performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

1.5: Objectives and Relevance to Human Health

Changes in human physiology and behavior have been linked to season of birth, and the question of what could be causing the observed changes is challenging. As stated above, there are countless factors that could result in these correlations. However, because the duration of light experienced during development is able to exert enduring changes on the central nervous system[67,68], perinatal photoperiod is one of the most logical places to begin the search for factors involved in behavioral programming by season of birth. In this dissertation I address whether or not perinatal photoperiod may play an integral role in the photoperiodic programming of the serotonergic system and therefore depression and anxiety behaviors.

The studies in this dissertation examine several significant questions regarding how perinatal photoperiod may be affecting the serotonergic system and therefore depression and anxiety related behaviors. Chapter 2 will examine if perinatal photoperiod exerts any effects on serotonergic neuronal physiology in a melatonin receptor dependent manner using the following methods: Multielectrode array slice recordings to observe the firing rate of 5-HT neurons in the DRN, dose response curves to the main excitatory and inhibitory neurotransmitter agonists of 5-HT receptors to observe receptor-mediated effects of perinatal photoperiod, whole cell patch clamp recordings to determine if there are any changes in the intrinsic electrical properties of these cells, and binding assays to determine if perinatal photoperiod may change the function of excitatory receptors on the surface of 5-HT cells in the DRN.

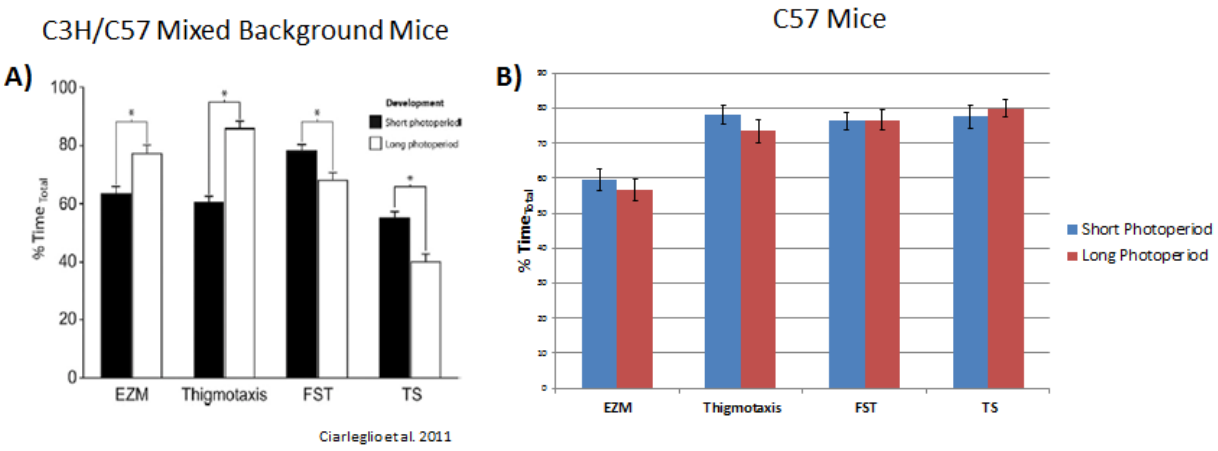

Figure 3: Depression and anxiety related behavioral testing in melatonin competent vs. incompetent mice. A) Melatonin producing C3H/C57 mixed background mice show anti-depressive and anxiolytic effects of the long photoperiod compared to the short. B) Melatonin deficient C57 mice do not show these differences between photoperiod. From left to right, elevated zero maze (EZM), open field test, forced swim test (FST) and tail suspension test (TS). Work done by Drs. Chris Ciarleglio and Holly Resuhr.

Chapter two will also address if perinatal photoperiod affects monoamine concentrations in the midbrain and hippocampus in a melatonin-dependent manner using HPLC to determine mono amine levels including 5-HT, norepinephrine (NE), and dopamine (DA), along with their metabolites, in the midbrain and hippocampus of mice developed on different photoperiods. This will allow us to observe if perinatal photoperiod is changing the neurochemical inputs and outputs of the 5-HT system and therefore could possibly be affecting behavior through a monoamine-dependent mechanism. These monoamine measurements were done in conjunction with Drs. Raymond Johnson and Benlian Gao in the Vanderbilt neurochemistry core.

Next, I wanted to determine if perinatal photoperiod may be affecting the expression of key serotonergic and serotonergic related genes in a melatonin related manner in the midbrain. In chapter three, we used quantitative real-time PCR (done in conjunction with Dr. Chad Jackson) to observe possible changes in serotonergic genes such as *pet-1, tph2*, and *slc6a4* (the gene encoding the SERT protein), as well as genes that are not involved in the generation or transport of 5-HT but act on their cellular physiology or metabolism, such as the $adra_{1b}$ receptor, the glucocorticoid receptors, and mao_a. In accordance with this goal, I wanted to determine if the protein prevalence of genes directly involved in 5-HT synthesis or transport is changed by perinatal photoperiod. To that end, I conducted immunohistochemical staining for TPH2 in the DRN of mice developed on different photoperiods.

I also examined if perinatal photoperiod affects depression and anxiety-related behaviors in a melatonin-dependent manner. I conducted the EZM, OFT, TST and FST on mice developed on different photoperiods. These behavioral tests are not only able to provide a tried and true behavioral output linked to the 5-HT system but could also corroborate the previous results produced in our lab that perinatal photoperiod influences depression and anxiety-related behaviors in mice that are melatonin proficient[26] but not mice that do not produce neurologically relevant amounts of melatonin such as C57Bl/6 mice (unpublished data) (Figure 3). Finally, I

conducted electrophysiological experiments to narrow the critical window for the photoperiodic programming of serotonergic neuronal physiology.

The information generated by the experiments outlined above not only provide us with a further understanding of how developmental light cycles are interacting with our developing brain but will also outline an effective animal model and photoperiodic paradigm for studying such questions. These experiments also provide invaluable information on how the duration of light during development can alter the mammalian serotonergic system and even depression and anxiety related behaviors (Figure 4). It is my hope that the results presented in this dissertation inspire researchers to continue with the characterization of how environmental signals during neurological development can cause enduring changes in the adult brain and behavior.

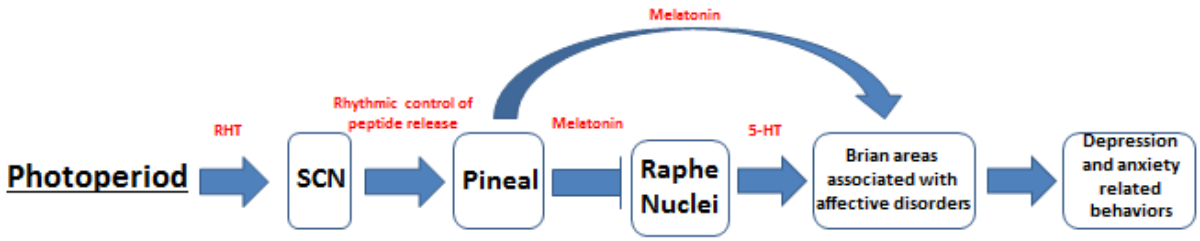

Figure 4: Proposed model of how photoperiod may be affecting the serotonergic system.

Chapter 2

Circadian Photoperiod Programs Dorsal Raphe Serotonergic Neurons and Affective Behaviors

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2.1: Summary Abstract

The serotonergic raphe nuclei of the midbrain are principal centers from which serotonin neurons project to innervate cortical and sub-cortical structures. The dorsal raphe nuclei receive light input from the circadian visual system [43] and indirect input from the biological clock nuclei [69,70]. Dysregulation of serotonin neurotransmission is implicated in neurobehavioral and neurodevelopmental disorders, such as depression, anxiety and autism [71,72], and alterations in the serotonergic phenotype of raphe neurons has dramatic effects on affective behaviors in rodents [73]. Here, we demonstrate that day length (photoperiod) during development induces enduring changes in mouse dorsal raphe serotonin neurons - programming their spontaneous neural activity, their responsiveness to noradrenergic stimulation, their intrinsic electrical properties, serotonin and norepinephrine content in the midbrain, as well as depression/anxiety related behavior in a melatonin receptor 1 (MT1) dependent manner. Our results establish mechanisms by which seasonal photoperiods may dramatically and persistently alter the function of serotonin neurons.

2.2: Introduction

Light as an environmental and neural stimulus transduced through the retina can have profound effects in shaping the development and maturation of light-driven neural circuits[68]. In addition to the neural circuits for image-forming vision, non-imaging forming pathways encode luminance and day length for synchronization of the hypothalamic circadian clock to the external environment [74]. The day length, or circadian photoperiod, experienced perinatally programs the gene rhythms and cellular organization of the hypothalamic circadian pacemaker, the suprachiasmatic nuclei [75], and can influence the neurotransmitter phenotype of developing neurons [76]. In addition, perinatal circadian photoperiod has enduring effects on depression and anxiety behaviors in rodent models [26,77].

The serotonergic Raphe nuclei of the midbrain are principal centers from which serotonin neurons project to widely innervate both cortical and sub-cortical structures. The dorsal Raphe nuclei receive light input from the circadian visual system [43] and polysynaptic input from the biological clock nuclei [69,70]. Dis-regulation of serotonin neurotransmission is implicated in a range of neurobehavioral and neurodevelopmental disorders, such as depression, anxiety and autism [71,72], and alterations in the serotonergic phenotype of Raphe neurons has dramatic effects on affective behaviors in rodents [73]. Here, we have tested whether circadian photoperiods experienced during development and maturation can have long-term, lasting effects on the physiology, signaling and function of serotonergic neurons as well as on depression and anxiety related behaviors.

2.3: Materials and Methods

Animals and Housing

Group housed male and female C3Hf$^{+/+}$ mice were used in these studies. Sex differences were analyzed for all tests and no significant differences were observed. C3Hf$^{+/+}$ mice are melatonin-producing and lack the retinal degeneration alleles of the parent C3H strain. They were developed and raised to maturity on photoperiods designated Equinox (12 hours of light 12 hours of darkness, EE), Short (8 hours of light and 16 hours of darkness, SS), or Long (16 hours of light and 8 hours of darkness, LL). At P30 some mice were switched to the opposing photoperiod - Short to Long (SL), Long to Short (LS) or Long to Equinox (LE) photoperiod. Experimental assays were performed at P50 to P90, with the exception of the Long to Equinox cohort in which the animals were tested 5 months after switching. Tissues were isolated or behavior tested at the mid-day point on all light cycles. MT1KO mice, on a C3Hf$^{+/+}$ background [78], were also developed on the light cycles stated above. Light cycles were aligned so that the testing times on all cycles were at the same time local time corresponding to the middle of the light period for each photoperiod, between 1100 and 1300 (except the forced swim test which ranged from 1100-1500 due to equipment scheduling constraints). Experiments were performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Ex vivo culture

Mice were euthanized by cervical dislocation, brains were extracted and mounted in cold, oxygenated (95%O_2-5%CO_2) dissecting media (in mM: 114.5 NaCl, 3.5 KCL, 1 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 D(+)-glucose, and 35.7 NaCHO3), and 235 μm thick coronal slices were taken using a Vibroslicer (Campden Instruments). Dissecting media was frozen and chunks of dissecting media ice were added to the bath to maintain the low temperature of the bath while slicing. The dorsal raphe nuclei were isolated by removing the extraneous cortical tissue and

placed sample in a slice chamber full of room temperature, oxygenated, extracellular recording media (in mM: 124 NaCl, 3.5 KCl, 1 NaH2PO4, 1.3MgSO4, 2.5 CaCl2,10 D(+)-glucose, and 20 NaHCO3).

Multielectrode Array Electrophysiological Recording

Dorsal raphe nucleus slices were placed on a perforated electrode arrays and immobilized with a harp for recording. 40 µM tryptophan and 3 µM phenylephrine were added to the recording solution which was perfused (1.3 mL/min) over the slice once in the recording chamber. Serotonin neurons were identified by eliciting 5HT1a-mediated suppression of spontaneous firing rate. Serotonin at a concentration of 40 µM (for all general firing rate measurements) or 8-OH-DPAT at a concentration of 1 µM (for dose response experiments) was perfused (1.3 mL/min) over the slice for 5 minutes after 4 to 6 minutes of recording. After 5 minutes of serotonin or 8-OH-DPAT, normal ACSF was started again and recording continued until recovery was observed.

As there are differences in the electrophysiological properties of neurons in the medial and lateral wing subfields of the DR, placement on the array and the dimensions of the electrode grid were used to ensure that only the ventromedial DR neurons (vmDR) are recorded from. Mid-DRN slices of 280 micron thickness are taken between -4.5 mm and -4.75 mm back from bregma. We use a 6X10 perforated array with electrodes that have a diameter of 30 microns and 100 microns spacing between electrodes. The slice is placed so the electrodes cover an area spreading 1200 microns down from the cerebral aqueduct in the ventral direction and 340 microns laterally on either side of the midline (for a 680 micron total recording width). Putative 5HT neurons were identified by feedback inhibition evoked by application of 5-HT (40µM) (for general firing rate experiments) or 8-OH-DPAT (for dose response experiments) for 5 min, with those cells demonstrating a minimum of 50% spike rate suppression being included in the analysis, as we have done in previously published studies [79,80]

Although there is overlap in the electrophysiological characteristics of dorsal raphe serotonergic and non-serotonergic neurons [81,82], these selection characteristics, as well as the placement of the array to record from the ventromedial DR region, assured that a high proportion of cells in our recordings were serotonergic. The vmDR is highly enriched in serotonergic neurons. In the rat this has been carefully quantified with 75-95% of the neurons containing immunoreactivity to 5-HT, and 70-95% of neurons expressing the 5-HT_{1A} receptor also expressing 5-HT [82]. In addition, spike duration is significantly broader in 5-HT vs non-5-HT neurons in the dorsal raphe and the 5-HT_{1A} mediated response is significantly greater in 5-HT neurons [81]. The mouse DRN is thought to be similarly highly enriched for serotonergic neurons in the vmDR region, and 84% of genetically identified 5-HT neurons respond with 5-HT_{1A} inhibition [83]. In our dose-response experiments 156 out of 198 total cells decreased their firing rate by at least 50% upon application of 8-OH-DPAT, indicating that ~80% of the neurons recorded from express functional 5-HT_{1A} receptors and therefore are likely serotonergic. Thus, while we cannot be sure that each and every neuron we putatively identified as serotonergic indeed expressed serotonin, the combination of our anatomical recording site and physiological criteria assures that the overwhelming majority of neurons in our study were serotonergic.

Data files were saved as .mcd files and analyzed in offline sorter. For analysis a Besel filter with a 150 Hz frequency cut off was applied to the raw data traces. The threshold for detection was set manually to a level that will include all legitimate spikes with the least amount of unipolar noise spikes included (between 13 μV and 35 μV). Once spikes have been detected they were sorted by a combination of a K means scan method and manual verification. The manual verification was conducted after the K means scan was run and divided spikes into groups based on criterion such as amplitude, power under the curve and spike duration (for full list of criterion see offline sorter V3 manual under the K means scan, Plexon Inc.). Once waveforms were sorted into groups and judged to be biologically relevant each spike was validated by eye and spikes that did not fit the average waveform shape were invalidated. Also all unsorted

spikes were visualized manually and any spikes that matched the average waveform shape in the relevant group were added to that group. Once the total number of spikes was determined for the period of the recording before the application of 5-HT or 8-OH-DPAT that number was divided by the total time in which those spikes occurred to produce a measure of spikes per second.

Dose-Response Experiments

Dose Response Curve to PE- DRN slices were prepared and placed on the multielectrode array as stated above. No PE and 40 µM tryptophan was added to the ACSF perfused over the slice while settling. The first dose of PE (333 nM) was perfused over the slice for at least 3 minutes. The slice was then switched back to ACSF without PE until the firing rate silenced once more before perfusing subsequent doses of PE. This procedure was repeated for each subsequent dose.

Dose Response Curve to 8-OH-DPAT- DRN slices were prepared and placed on the multielectrode array as previously stated. ACSF containing 40 µM tryptophan and 3 µM PE was perfused over the slice while settling. The lowest dose of 8-OH-DPAT (50 nM) was perfused over the slice after at least 4 minutes of recording the SFR. Each subsequent does was added in a stepwise fashion after at least 2 minutes exposure to the previous dose. Spikes were categorized and sorted as stated above.

Whole Cell Electrophysiological Recordings

Whole cell current clamp were performed with the same ex vivo culture procedure as stated above. The external solution contained: 124 NaCl, 2.8 KCl, $2CaCl_2$, 2 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 Glucose (in mM), 40µM tryptophan, pH7.4, 300 mmol/kg Osm. Putative serotonergic neurons were selected based on 8-OH-DPAT (1µM) response. Spontaneous excitatory and inhibitory inputs were inhibited by 10µM CNQX and 10µM GABAZINE respectively during the recording. The recording pipette (5MΩ) was filled with 135 K Gluconate,

5 NaCl, 10 HEPES, 1 EGTA, 2 $MgCl_2$, 2.5 Na_2ATP, 0.2 Na_2GTP, 10 sucrose (in mM), pH7.4, 290 mmol/kg Osm. Perfusion rate was 1 mL/min at 32C°. The access resistance was limited to less than 50MΩ. The calculated liquid Junction potential (15mV) was adjusted. Series resistance was 90% compensated with 60μs lag time. The data were treated with unpaired two-tailed t-test.

Gene Expression Detection and Analysis

Mouse mid-brains were removed between 11am-1pm, and then frozen in a 1.5 mL tube in liquid nitrogen. Samples were stored at -80°C until RNA extraction. Total RNA was extracted using a Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA, USA, Cat. No. 74104), measured by a Nanodrop system (ThermoScientific), and reverse-transcribed (~200ng) into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA, Cat. No. 205311). qRT-PCR reactions were performed in 20 µL total volume with 2 µL cDNA, 10µL of SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA), 6µL sterile water and 1µL of 300 nM intron-spanning gene specific forward and reverse primers in a Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Quantification of transcript levels were performed by comparing the threshold cycle for amplification of the unknown to those of six concentrations of standard cDNAs for each respective transcript, then normalizing the standard-calculated amount to hypoxanthine guanine phosphoribosyl transferase (*Hprt*) in each sample. Each sample was assayed in duplicate. Statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant.

Adra1b receptor binding

Mice were killed by brief isoflurane anesthesia exposure and decapitation. Brains were quickly dissected and placed into a cold, clean brain matrice, and mibrain was cut free coronally using clean razor blades. The midbrain section was then dissected by making two cuts 2mm each side of the dorsal midline to the aqueduct of sylvius to further isolate dorsal and median raphe regions. The tissue was homogenized in ice-cold 50-mM Tris assay buffer (50 mM Tris–HCl, 10

mM MgCl2, 0.1 mM EDTA (pH 7.3)). The homogenate was centrifuged at 20,000 g for 20 min at 4°C. The supernatant was discarded, and the pellet was resuspended in assay buffer, briefly homogenized, and protein concentration was measured by Bradford assay (Bio-Rad). A single point saturating concentration was chosen based on pilot experiments of complete saturation binding curves for [^{3}H] prazosin. Homogenized midbrain membranes (60 µg/tube) were incubated with [^{3}H] prazosin [6 nM] in the presence or absence of prazosin [10 µM] to measure non-specific and total binding, respectively. Reaction buffer included the alpha 1A receptor antagonist WB 4101 [10 µM]. Specific binding was calculated by subtracting non-specific binding from total binding and expressed as bound ligand (fmol) per mg protein. All experiments were performed in duplicate. Following 30 min incubation at 37°C, free radioligand was separated from bound by vacuum filtration through Whatman GF/B glass filters (Brandel, Gaithersburg, MD, USA). Filters were placed in vials with scintillation cocktail and counted 12 h later in a liquid scintillation counter

Monoamine analysis

Mouse mid-brains were removed between 1100-1300. brains were quickly dissected and placed into a cold, clean brain matrice, and mibrain was cut free coronally using clean razor blades. The midbrain section was then dissected by making two cuts 2mm each side of the dorsal midline to the aqueduct of sylvius to further isolate dorsal and median raphe regions. These sections were then frozen in a 1.5mLtube in liquid nitrogen. Samples were stored at -80°C until the tissue was homogenized, using a tissue dismembrator, in 100-750 µl of 0.1M TCA, which contained 10^{-2} M sodium acetate, 10^{-4} M EDTA, 5ng/mL isoproterenol (as internal standard) and 10.5 % methanol (pH 3.8). Samples were spun in a microcentrifuge at 10000 x g for 20 minutes. The supernatant was removed and stored at –80°C . The pellet was saved for protein analysis. Supernatant was then thawed and spun for 20 minutes. Samples of the supernatant were then analyzed for biogenic monoamines. Biogenic amines were determined by a specific HPLC assay utilizing an Antec Decade II (oxidation: 0.4) (3mm GC WE, HYREF) electrochemical

detector operated at 33°C . Twenty µl samples of the supernatant were injected using a Water 2707 autosampler onto a Phenomenex Kintex (2.6u, 100A) C18 HPLC column (100 x 4.60 mm). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8). Solvent was delivered at 0.6 mL/min using a Waters 515 HPLC pump. Using this HPLC solvent the following biogenic amines eluted in the following order: noradrenaline, Adrenaline, DOPAC, Dopamine, 5-HIAA, HVA, 5-HT, and 3-MT (2). HPLC control and data acquisition were managed by Empower software. For C3Hf$^{+/+}$ mice 5-HT and NE measurements, statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant. For MT1 KO mice 5-HT and NE measurements, statistical significance was determined by a one-way Kruskal-Wallis ANOVA on ranks because the data was determined not to be normal with a p value less than 0.05 considered significant.

Behavioral Testing

Mice- Male and female mice were group housed and transferred from our housing facility to light controlled boxes in the Vanderbilt Neurobehavioral Core. After a week of acclimation the elevated zero maze was conducted on the first day of testing, then the open field test, the tail suspension test and the forced swim test each conducted on successive days with ca. 24 hours between each test. They were tested in the middle of their light phase (1100-1500). No mouse underwent more than one test daily and no mouse underwent the same test more than once. The experimenter was the same for all tests conducted. There was no pseudo-randomization in the order of the mice tested, they were tested on a cage by cage basis. That is to say all the mice in one cage (and therefore one genotype and photoperiod) were tested in succession and then the next cage was tested. Mice were allowed to acclimate to the testing room for 30 minutes prior to all tests. Sex differences in behavioral results within photoperiods were not observed (t-test with Holm-Sidak correction for multiple comparisons).

Forced swim test- Mice were exposed to the FST for 6 min during the light phase 1100-1500 hours. The FST container was a Plexiglas cylinder 45 cm high and 20 cm in diameter filled with

30-35 cm of water (21 ± 2˚C). Testing was conducted in normal room light. During the entire duration of the task, an experimenter was present and watching the mice. The videos were then scored by the same experimenter as performed the tests. Before scoring the scorer was blinded to the genotypes and photoperiods of the mice in each video. Time immobile was defined as any period of time the animal was not making any active escape movements or floating without struggling. Statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant. For all post hoc analysis Holm-Sidak pair-wise comparisons were performed.

Tail suspension test- The tail of a mouse was taped to a force meter attached to the top of an open chamber so that it cannot escape. The mouse was suspended for 6 min. Latency to stop struggling, and amount of time spent struggling were measured through the force meter with a threshold of 7 (arbitrary units) as the limit for struggling. Testing was conducted in normal room light. Mice used in this experiment were similar ages (50 to 90 days old) and there were no large weight discrepancies between mice tested. Data was gathered and processed by Med Associates Inc. tail suspension software. Statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant. For all post hoc analysis Holm-Sidak pair-wise comparisons were performed.

Open field test- Exploratory locomotor activity was measured in an open field measuring 27 x 27 cm, with a light intensity between 90-110 lux across all chambers, over a 60 minute period. Infrared beams and detectors automatically record movement in the open field. Thigmotaxis was defined as time spent on the outer rim of the open field, which was defined as the area 4.25 cm from the wall of the open field. This area constitutes 50% of the total area of the open field. Thigmotaxis was measured throughout the 60 minute period in 5-minute blocks. The average thigmotaxis within a 5-minute block was calculated from all 12 blocks within 60 minutes of testing which is what is represented in the data. Total distance traveled was also measured, there was no difference between photoperiods in C3Hf$^{+/+}$ mice. MT1 KO mice developed on an

EE photoperiod showed an increase in total distance traveled over both SS and LL mice. Statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant. For all post hoc analysis Holm-Sidak pair-wise comparisons were performed.

Elevated zero maze- The zero maze consisted of one circular platform approximately 50 cm. Two discontinuous portions of the maze has walls, approximately 20 cm high with a light intensity within these arms of 71-91 lux and 344-355 lux outside these arms. The remaining portions have no walls. Mice were placed in one of the on-walled arms at the beginning of the session. Number of entries into the closed arms, and amount of time spent in closed arms was used as a measure of anxiety. The zero maze task was conducted once per mouse for 6 min. Statistical significance was determined by a one-way Kruskal-Wallis ANOVA on ranks because the data was determined not to be normal with a p value less than 0.05 considered significant. For all post hoc analysis Dunn's method pair-wise comparisons were performed.

Data Analysis

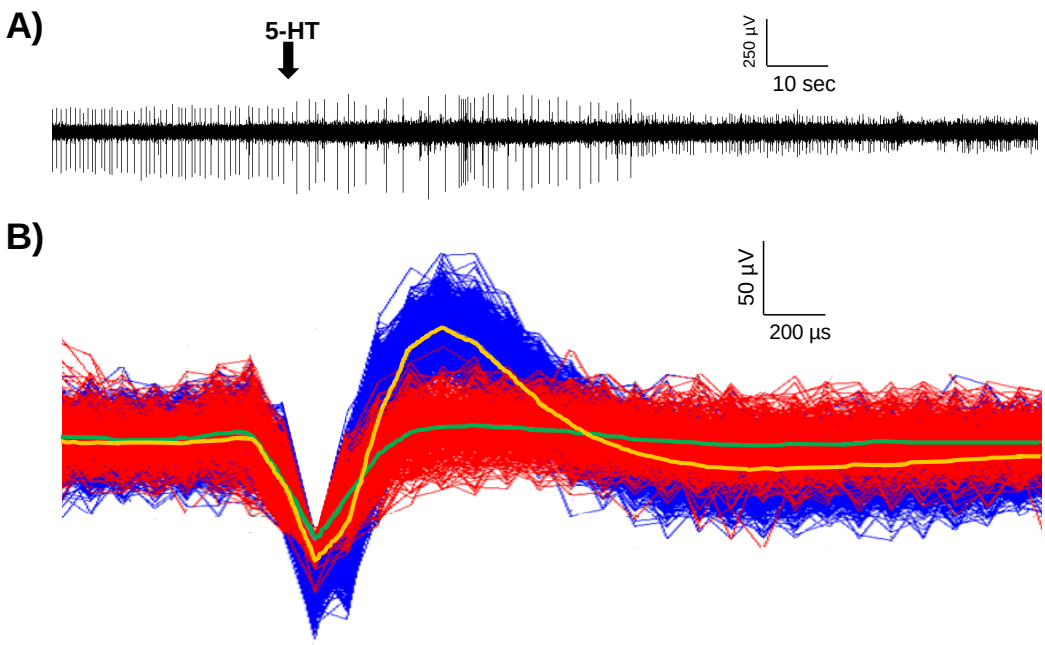
Spike traces from multielectrode arrays were analyzed using offline sorting (Plexon) and spikes were sorted using a combination of manual identification and automatic K means based sorting software. For spontaneous firing rate data of wt C3Hf+/+ mice, where cell/mouse counts were unequal between mice, two-level nested one-way ANOVA was used to rule out significant within-photoperiod variability (mice within photoperiods, $p = 0.3609$, $F(13,394) = 1.0813$, among photoperiods, $p < 0.0001$, $F(13,4) = 23.5042$). All cells within each mouse were then averaged, and those values were used for ordinary One-Way ANOVA. Holm-Sidak's Multiple Comparisons test was used post-hoc for One-Way ANOVA, and Tukey's Multiple Comparisons test for Two-Way ANOVA. All statistical analyses were performed with $\alpha = 0.05$. Monoamine measurements values exceeding $\pm$ 1.5 SD from the mean were excluded. DRC data were analyzed with Two-Way ANOVA (factors: concentration, photoperiod, interaction) and with non-linear regression modeling to identify unshared parameters and plot curves. Parameter values for individual mice

were determined using least-squares non-linear regression constraining “Bottom” values greater than zero. Parameter values for each photoperiod were then analyzed using One-Way ANOVA (Tables 1 and 2). For intrinsic excitability curves regression analysis and slope significance testing was calculated as per [84].

2.4: Results

Multi-electrode array recordings were performed on acute dorsal Raphe nucleus (DRN) slices to measure the firing rate of serotonin neurons in the dorsomedial portion of the DRN (Fig. 5). These recordings demonstrated clear differences in the firing rate of dorsal raphe serotonin neurons of mice exposed to different photoperiods during development (Fig. 6A). Neurons from Long photoperiod mice (LL) exhibited significantly increased firing rates, compared to Equinox (EE) and Short (SS) photoperiod groups (LL = 1.24 Hz $\pm$ 0.084, n=6 mice, 153 cells; EE = 0.83Hz $\pm$ 0.058, n=6 mice, 92 cells; SS = 0.69Hz $\pm$ 0.024, n=6 mice, 70 cells; EE vs. LL: $p = 0.0005$, LL vs. SS: $p < 0.0001$, $F(2,15) = 22.75$, One-Way ANOVA; Fig. 6B). Noradrenergic excitatory input mediated by $Adra_{1b}$ receptors, and serotonergic auto-inhibition from 5HT1a autoreceptors are critical regulators of raphe neuron spontaneous spike frequency [85]. Dose-response curves performed with the adrenergic agonist phenylephrine (PE) revealed that serotonergic neurons in DRN from LL photoperiod mice (n=4 mice, 57 cells), exhibited significantly higher firing rates in response to a range of PE concentrations compared to neurons from EE (n=3 mice, 19 cells) and SS (n=3 mice, 23 cells) mice (Fig. 6C, Table 1). Thus, increased response to the $Adra_{1b}$ agonist PE, present in the recording medium at 3 μM to simulate the *in vivo* noradrenergic input that activates serotonin neurons, likely contributes to increased firing rate in serotonin neurons observed *in vitro* from mice developed in LL photoperiods. In contrast, dose response curves for 8-OH-DPAT, a 5HT1a agonist which activates the inhibitory 5HT1a autoreceptor, suppressed ongoing spike activity with similar

concentration dependence in all groups (Fig. 6D). The baseline firing rate before 8-OH-DPAT inhibition was significantly elevated in LL as in Fig. 6, however, IC50 values for each photoperiod were not significantly different (Table 2). These data indicate that LL photoperiods increase responsiveness of raphe serotonin neurons to adrenergic stimulation but do not significantly affect the responsiveness to 5HT1a negative feedback. The increase in responsiveness of LL photoperiod raphe neurons to adrenergic stimulation could result from increased adrenergic receptor expression or activation, or from changes in the intrinsic excitability of serotonin neurons that may amplify the effects of adrenergic input. Neither $Adra_{1b}$ receptor mRNA expression nor $Adra_{1b}$ receptor binding, nor the EC50 values for PE was found to be different in the mid-brain across photoperiods (Fig. 7A, B; Table 1), although given the widespread expression of this receptor in that region, this does not rigorously exclude the possibility of changes in expression limited to serotonin neurons. To test for changes in intrinsic excitability, we measured electrophysiological variables from LL and SS DRN serotonin neurons by whole cell recording. No EE group was included here as EE and SS groups were not different in their baseline spike rates (Fig. 6). The resting membrane potential of neurons from LL photoperiod mice was significantly depolarized compared to those from SS photoperiods (LL -61.73 + 2.42 n=12 cells, SS -69.99 + 1.99 n=13 cells, mV, p=0.01, Fig. 6E) and neurons from the LL group exhibited a trend toward lower amplitude after-hyperpolarization following action potentials compared to SS (LL -21.03 + 1.09 n=12 cells, SS -24.22 + 1.15 n=13 cells, mV, p=0.06, Fig. 6F). Serotonin neurons from LL photoperiod also exhibited increased spike rate for a given amount of membrane current injection compared to SS, with the slope of the current/spike relationship being significantly increased in LL (LL slope 0.052, n=12 cells, SS slope 0.039, n=13 cells, *p*=0.001, Fig. 6G). Thus, photoperiod acts to alter the intrinsic properties of serotonin neurons, with LL photoperiods producing changes that increase excitability. The depolarized resting potential and increased excitability of serotonin neurons



<u>Figure 5.</u> Representative traces of multielectrode array recordings in DRN slices. (A) Representative spike train from a multielectrode array recording in the DRN. Large amplitude spikes represent 5-HT neuronal firing. 5-HT was added after at least 4 minutes of recording to verify serotonergic neuronal firing by 5-HT_{1A} mediated autoinhibition, indicated by arrow. (B) Spikes were sorted in offline sorter using a combination of a K-means scan automated sorting software and manual verification. Blue traces indicate individual sorted serotonergic spikes, the bold yellow line indicates the average waveform of all serotonergic spikes. Red traces indicate individual sorted spikes not classified as serotonergic, the bold green line indicates the average waveform of all non-serotonergic spikes.

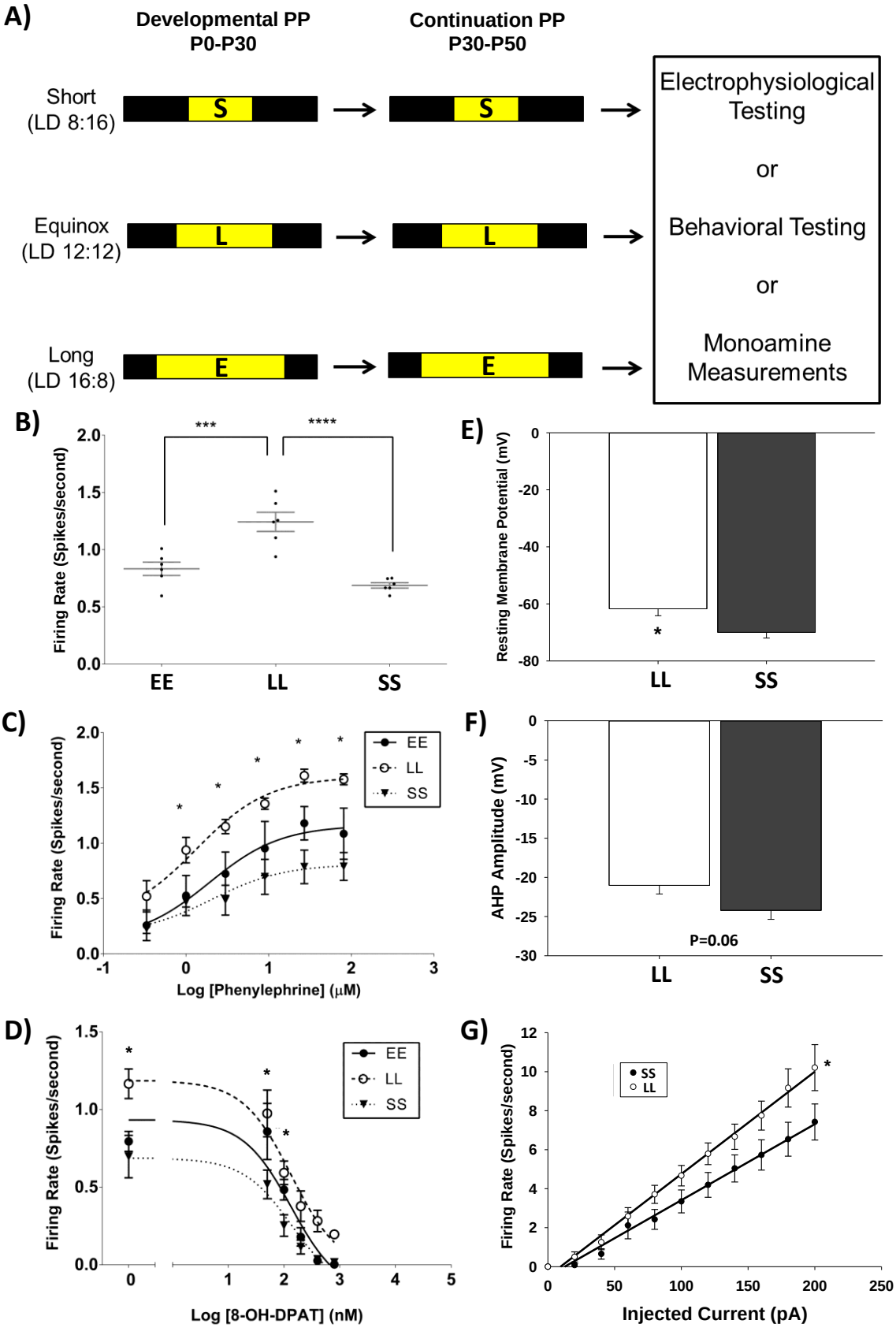
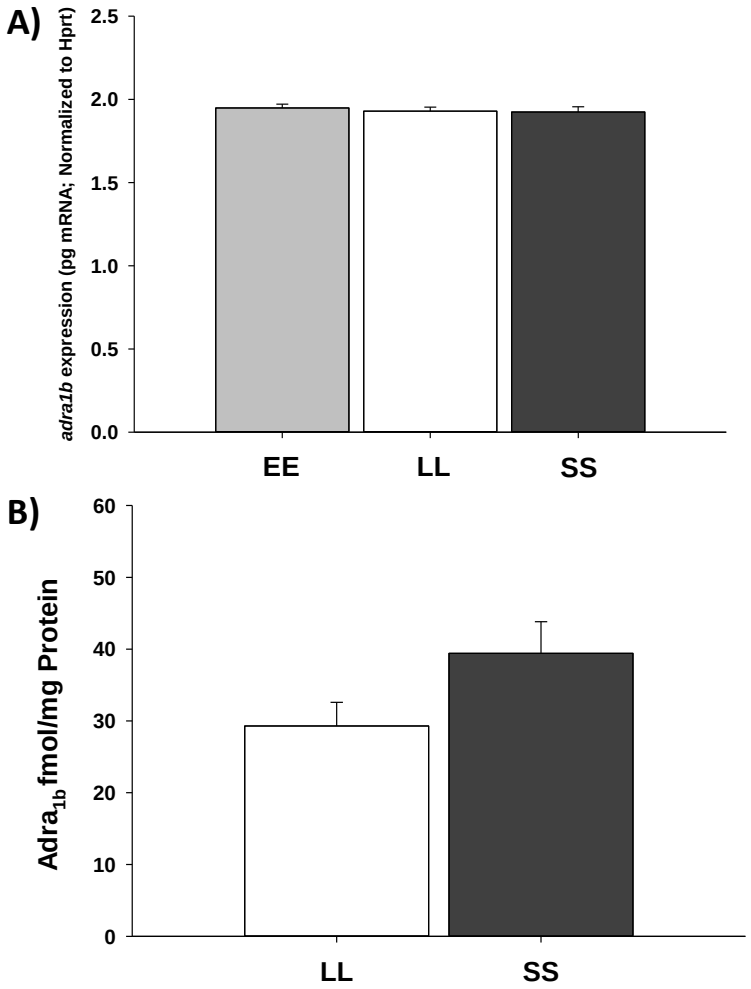

A)
Developmental PP
P0-P30
Continuation PP
P30-P50
Short
(LD 8:16)
S
S
Equinox
(LD 12:12)
L
L
Long
(LD 16:8)
E
E
Electrophysiological Testing
or
Behavioral Testing
or
Monoamine Measurements
B)
Firing Rate (Spikes/second)
***
****
EE
LL
SS
E)
Resting Membrane Potential (mV)
LL
SS
C)
Firing Rate (Spikes/second)
EE
LL
SS
Log [Phenylephrine] (μM)
F)
AHP Amplitude (mV)
P=0.06
LL
SS
D)
Firing Rate (Spikes/second)
EE
LL
SS
Log [8-OH-DPAT] (nM)
G)
Firing Rate (Spikes/second)
SS
LL
Injected Current (pA)

Figure 6. Photoperiod shapes the physiological properties of 5-HT neurons. (**A**) Photoperiod paradigm. (**B**) Firing rate of serotonergic neurons in DRN slices from mice exposed to different photoperiods (Equinox, EE; Long, LL; Short, SS; $p < 0.001$, One-Way ANOVA, EE vs. LL: adj. $p = 0.0005$, LL vs. SS: adj. $p < 0.0001$, Holm-Sidak Multiple Comparison Test). (**C**) Dose response curve to phenylephrine (PE). Neurons from LL mice (open circles) display an increased firing rate compared to SS mice (closed triangle) across most doses of PE (1 µM: p=0.0379, 3 µM: $p = 0.0022$, 9 µM: $p = 0.0023$, 27 µM: $p = 0.0002$, 81 µM: $p = 0.0003$, Mixed Design Two-Way ANOVA with Tukey's MC test) and compared to EE mice at 81 µM ($p = 0.0280$). (**D**) Dose response curve to 8-OH-DPAT. Neurons from LL mice display an increased firing rate compared to SS mice at baseline and at 2 doses of 8-OH-DPAT (0 nM: p=0.0006, 50 nM: p=0.0008, 100 nM: p=0.00151, Mixed Design Two-Way ANOVA with Tukey's Multiple Comparison test). (**E**) Resting membrane potential is significantly different across photoperiods (p=0.01 LL vs. SS, t-test). (**F**) After-hyperpolarization amplitude across photoperiod shows a trend toward reduction in Long photoperiod (p=0.06 LL vs. SS, t-test). (**G**) Neurons from LL mice have increased intrinsic excitability compared to SS mice (p=0.001)



<u>Figure 7.</u> No change across photoperiods in the mRNA expression of the *adra1b* receptor or ADRA1b receptor binding in the midbrain. (A) No change across photoperiods in *adra1b* receptor expression. n= 6 mice for each photoperiod (p= 0.583). (B) No change between Short and Long in ADRA1b receptor binding. n=7 mice for LL and 5 mice for SS (p=0.089).

	EE	LL	SS	F	p
Max (Spikes/Sec)	1.18 ± 0.19	1.60 ± 0.04	0.86 ± 0.10	F (2, 7) = 10.91	0.0071*
Min (Spikes/ Sec)	0.18 ± 0.17	0.29 ± 0.12	0.16 ± 0.05	F (2, 7) = 0.3005	0.7496
EC50 (µM)	2.93 ± 0.97	1.34 ± 0.53	7.13 ± 5.69	F (2, 7) = 0.9872	0.4191

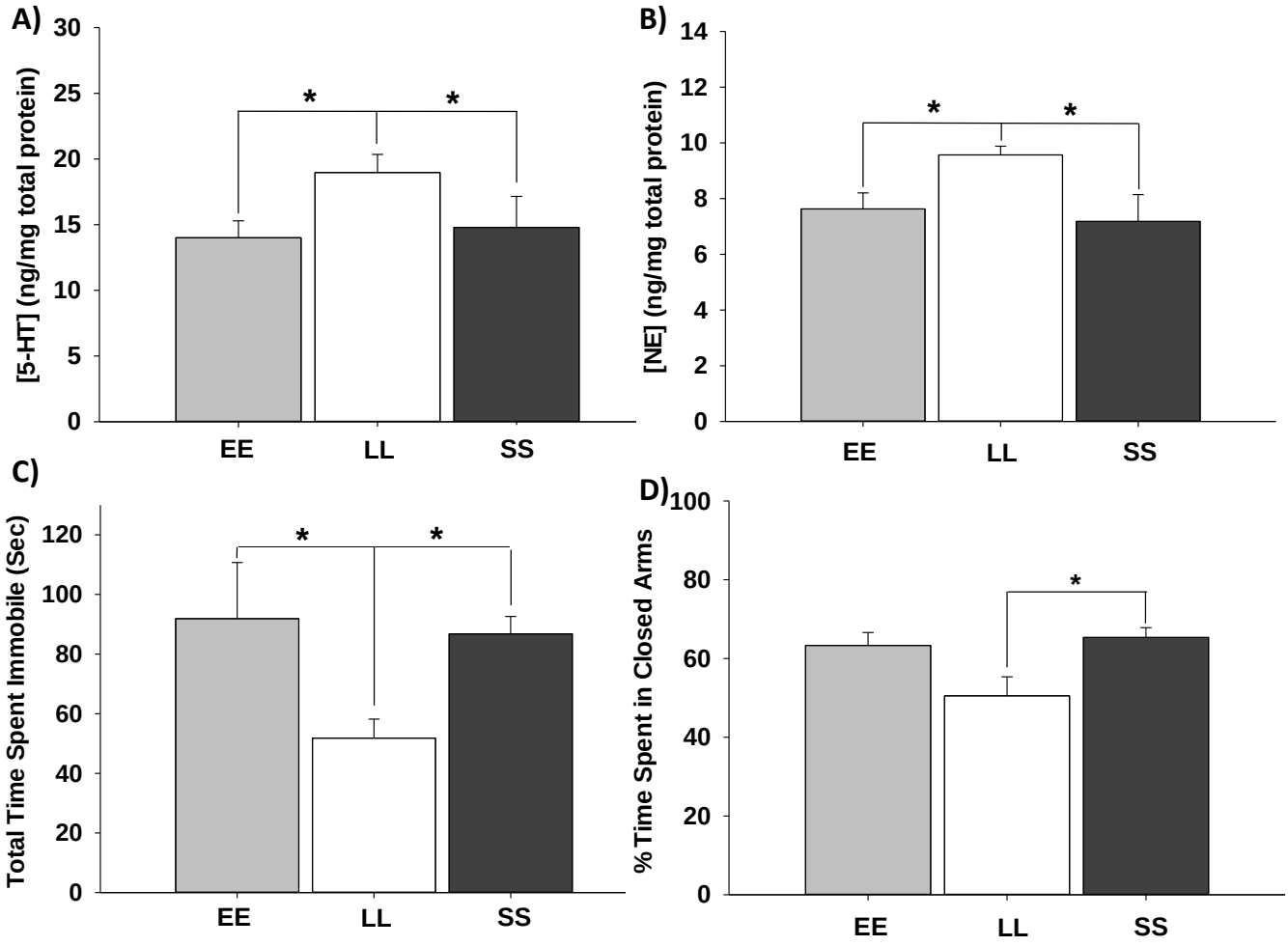
Table 1. Fitted curve values for the dose response curve to Phenylephrine. The maximum is higher in the LL photoperiod compared to EE and SS photoperiods (p=0.0071). However, there is not a significant difference between the minimum (p= 0.7496) and EC50 values (p=0.4191), One-Way ANOVA.

	EE	LL	SS	F	p
Max (Spikes/Sec)	0.897 ± 0.09	1.205 ± 0.11	0.718 ± 0.14	F (2, 8) = 4.486	0.0494*
Min (Spikes/Sec)	0	0.04 ± 0.03	0	F (2, 8) = 0.8485	0.4633
IC50 (nM)	208.3 ± 56.5	140.4 ± 31.3	95.4 ± 10.9	F (2, 8) = 2.354	0.1571

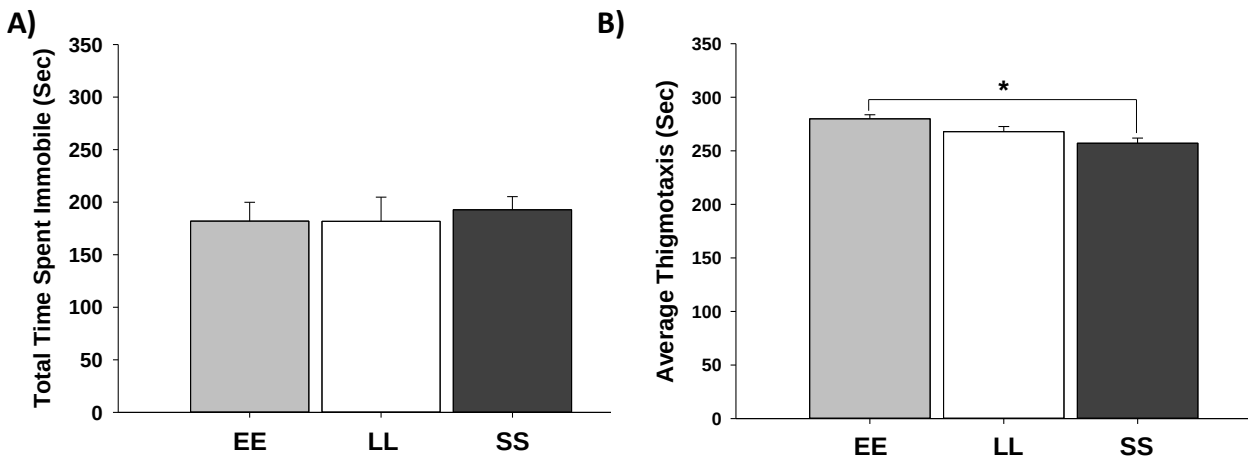
Table 2. Fitted curve values for the dose response curve to 8-OH-DPAT. The maximum spike rate before adding 8-OH-DPAT is higher in the LL photoperiod compared to EE and SS photoperiods (p= 0.494), reflecting the LL photoperiod-induced increase in spike rate (Fig. 6).

from LL photoperiod mice are similar to those of the juvenile state of dorsal raphe neurons [86], suggesting that photoperiod is an environmental stimulus that may influence the functional maturation of serotonin neurons, with LL photoperiods preserving an immature-like state that is more highly excitable.

Photoperiod also altered serotonin signaling and affective behavior. Midbrain 5-HT content was increased in LL mice compared to EE and SS groups (LL 18.97+ 1.38 n=12; EE 14.00 + 1.29 n=12; SS 14.80 + 2.36 n=6, ng/mg total protein, p=0.047, df=2, F=3.42 Fig 8A). In addition, midbrain norepinephrine content was also increased in LL mice compared to EE and SS (LL 9.57 $\pm$ 0.32 n=12; EE 7.63 $\pm$ 0.58 n=12; SS 7.18 + 0.96 n=6, ng/mg total protein, p=0.013, df=2, F=5.13 Fig 8B). Thus both the effectiveness of NE on DRNs and the strength of its input are increased in LL photoperiods. Mice from LL photoperiods showed significant reductions in time spent immobile in the forced swim test (EE 91.46 $\pm$ 12.57 n=8, LL 51.89 $\pm$ 6.39 n=15, SS 86.77 $\pm$ 5.86 n=20, p=0.002, df=2, F=7.22 Fig. 8C), and in time spent in the closed arm of the elevated z-maze (EE 63.34 $\pm$ 3.27 n=12, LL 50.54 $\pm$ 5.81 n=15, SS 65.35 $\pm$ 2.50 N=20, p=0.047, df=2, H=6.10 Fig. 8D), while no effects of photoperiod were observed in the tail suspension test (Fig. 9). Both LL and SS groups showed reductions in thigmotaxis in open field tests compared to EE (Fig. 9). A number of previous studies in multiple rodent models have shown that photoperiod manipulation may produce variable results in rodent thigmotaxis in the open field test while displaying consistent results in the forced swim test [77,87-89]. This suggests the possibility that thigmotaxis may be an insensitive measure of rodent anxiety-related behaviors in photoperiod manipulation or that photoperiod may more consistently impact depression-related behaviors. Taken together our results here indicate that prior photoperiod influences serotonin content and norepinephrine input to the DRN, and reinforce previous findings that photoperiod can drive alterations in depression/anxiety-like behaviors [26,77].



<u>Figure 8</u>. Long photoperiod increases midbrain monoamines and decreases depression/anxiety behaviors. (**A**) LL mice exhibit ~20 % greater 5-HT concentration in the midbrain over both EE and SS (p= 0.047). (**B**) LL mice exhibit a ~25% increase in NE concentration in the midbrain over both EE and SS (p=0.013). (**C**) LL mice display significantly less time spent immobile than EE and SS in the forced swim test (p=0.002). (**D**) LL mice display significantly less time spent in the closed arms of the elevated zero maze than EE and SS (p=0.047).



<u>Figure 9.</u> Open Field Test and Tail Suspension Test. (A) No significant differences across photoperiod in the TST. EE; n= 12, LL; n=15, SS; n=20 (p=0.244). (B) The EE group displays an increase in thigmotaxis over the SS group but not the LL group (p=0.011). There is no difference between the SS and LL groups. OFT: EE; n=10, LL; n=15, SS; n=20.

The first 3-4 weeks of postnatal life have been proposed to be a critical window for environmental input and plasticity governing the physiological maturation of DRN serotonin neurons in rodents [86]. To test if photoperiodic effects on dorsal raphe neuron neurophysiology are due to enduring effects of photoperiod exposure during development and maturation, cohorts of mice were switched from Long to Short (LS) or Short to Long (SL) photoperiods at P30 until testing at P50-P90 (Fig. 10A). DRN neuron spike rates from LS and SL mice were compared with those from mice that were continued on the same photoperiod from P0 to P50-P90 (LL and SS, Figure 6). Effects of developmental photoperiod on neuronal firing rate were sustained despite reversal of the photoperiod for 3-7 weeks. The firing rate of raphe neurons from LS mice was similar to LL mice (LS: 1.12±0.09, n=6 mice, 54 cells, LL: 1.24±0.08, Hz±SEM), and the firing rate of SL neurons was similar to SS (SL: 0.72±0.06, n=6 mice, 63 cells, SS: 0.69±0.02, Hz±SEM). Analysis of the effects of developmental and continuation photoperiod revealed a significant effect of developmental photoperiod on firing rate, with mice raised on a Long developmental photoperiod (LL, LS) having an increased firing rate compared to mice raised on a Short developmental photoperiod (SS, SL), regardless of continuation photoperiod (Development: $p < 0.0001$, Continuation: $p = 0.17$, Interaction: $p = 0.42$; Two-Way ANOVA, Fig. 10B). To test the duration of developmental programming of dorsal raphe neurons, a cohort of Long developmental photoperiod mice was switched to Equinox photoperiod at P30 and maintained in that photoperiod for 5 months (LE). The spontaneous firing rate of DRN neurons from these mice was essentially identical to LL mice (LL: 1.24±0.08, n=6 mice, 153 cells, LE: 1.21±0.07, n = 6 mice, 33 cells, EE = 0.84±0.06, n= 6 mice, 93 cells, Fig. 10C), well elevated from EE ($p = 0.0053$, One-Way ANOVA). Thus, developmental photoperiod can have enduring effects on the activity of dorsal raphe neurons that are not reversed by weeks, or even months, of subsequent photoperiod change.

The hormone melatonin is a key photoperiodic signal that can acutely inhibit the activity of dorsal raphe neurons through MT1 receptor signaling [51]. In addition, maternal-fetal melatonin

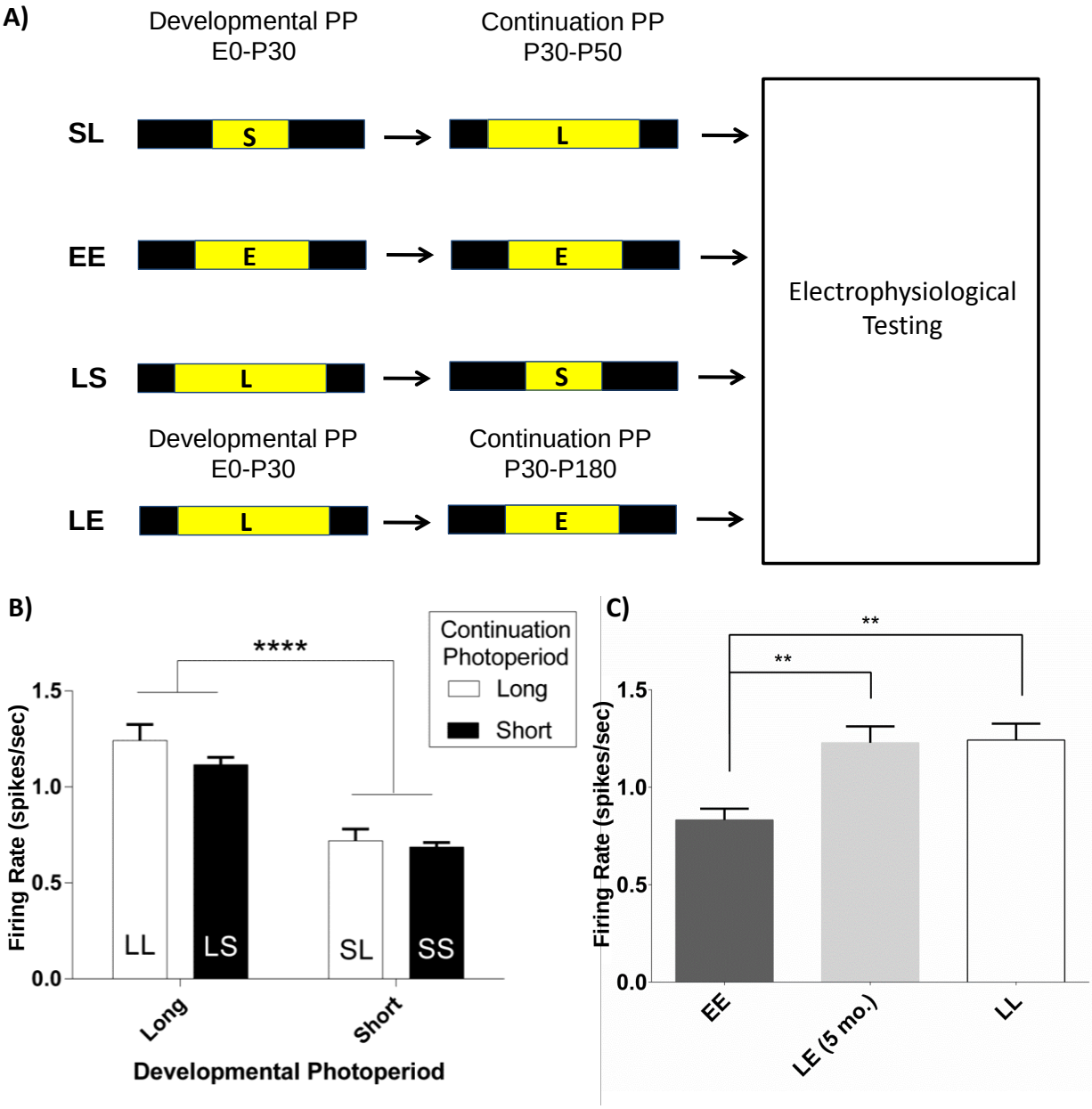


Figure 10. Switched photoperiod paradigm reveals persistence of developmental photoperiod effects on serotonergic neurons. (**A**) Switched photoperiod paradigm. Mice raised on Short or Long photoperiods were switched at P30 (Short to Long, SL; Long to Short, LS). (**B**) Effects of developmental photoperiod. Left bars, developmental Long photoperiod groups; Right bars, developmental Short photoperiod groups; Black bars, Long photoperiod continuation photoperiod; Grey bars, Short continuation photoperiod (Developmental effect: $p < 0.0001$, continuation effect: $p = 0.1744$, interaction: $p = 0.4167$, Two-Way ANOVA). (**C**) Persistence of developmental photoperiod effects on firing rate. Firing rate in LE neurons (right) is similar to LL but not EE (LE vs EE, middle, $p = 0.0028$, One-Way ANOVA with Holm-Sidak Multiple Comparison Test) and LL vs EE mice (right, $p = 0.0037$).
0.72±0.06, n=6 mice, 63 cells, SS: 0.69±0.02, Hz±SEM).

signaling of photoperiod in late gestation can program gonadal development later in life in rodents suggesting that melatonin signaling during development can have enduring effects [23]. We tested whether melatonin signaling was involved in photoperiodic programming of raphe neurons using MT1 receptor knockout mice. In the absence of MT1 receptor signaling there were no photoperiod-specific differences in spike rates, and the spontaneous firing rate of serotonin neurons was elevated above 1 Hz across all photoperiods (Fig. 11A), similar to LL photoperiod wild-type mice. In addition, there were no differences across photoperiods in 5-HT or NE concentrations in the midbrain in MT1 KO mice (Fig. 11B, 8C). Finally, MT1 KO mice displayed no changes in depression and anxiety related behaviors across photoperiods in the OFT, TST or FST (Fig. 11D-F). In the EZM MT1 KO EE mice spent significantly less time in the closed arm of the maze compared to MT1 KO LL and MT1 KO SS mice ($p<0.001$, df=2, F=14.91, One-Way ANOVA, Fig. 11G), but also showed significantly elevated distance traveled in the OFT. Therefore, the MT1 receptor plays a critical role in photoperiodic programing of the serotonergic neurons as well as depression and anxiety related behaviors in mice. The effects of the MT1 receptor on serotonin neuron programming could be direct, or could be mediated indirectly through melatonin effects on the development of the adrenal[90] or gonadal[23] systems which have hormonal input to the raphe

2.5 Discussion

Our results demonstrate that circadian light cycles experienced during development and maturation have enduring effects on dorsal Raphe serotonin neuron excitability, responsiveness to adrenergic stimulation, monoamine concentrations in the midbrain and depression and anxiety related behaviors. In particular, developmental exposure to long-day photoperiods, induces significant increases in spontaneous firing rate, the excitatory effects of adrenergic input and serotonergic content of Raphe neurons. Moreover, long-day photoperiods substantially

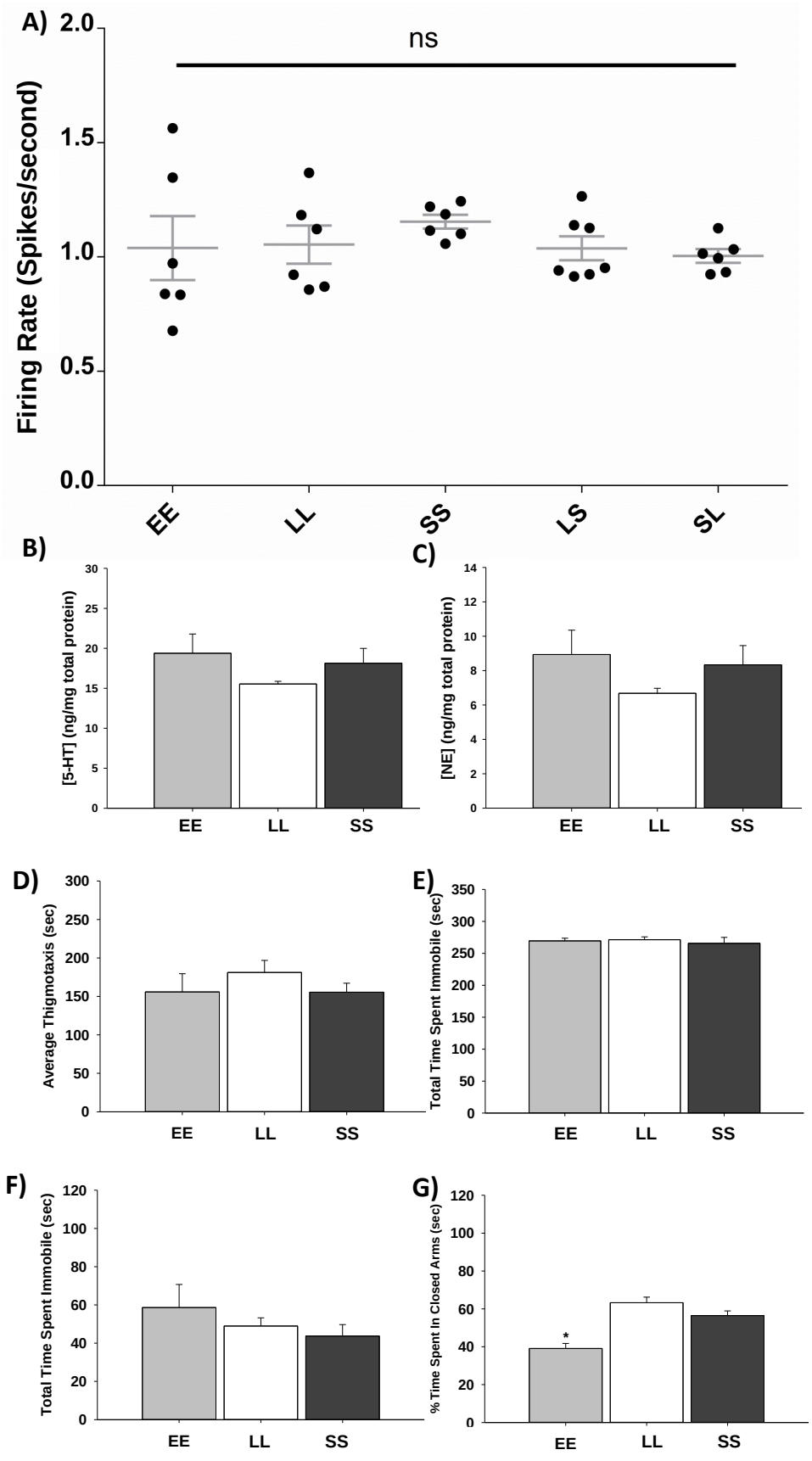

A)
ns
Firing Rate (Spikes/second)
2.0
1.5
1.0
0.5
0.0
EE
LL
SS
LS
SL
B)
[5-HT] (ng/mg total protein)
C)
[NE] (ng/mg total protein)
D)
Average Thigmotaxis (sec)
E)
Total Time Spent Immobile (sec)
F)
Total Time Spent Immobile (sec)
G)
% Time Spent In Closed Arms (sec)
*

Figure 11. MT1KO negates circadian photoperiod's effects. (**A**) No difference in spontaneous firing rate across the five photoperiods tested. EE: 64 cells, LL: 66 cells, SS: 81 cells, SL: 56 cells, LS: 98 cells, n=6 mice for each photoperiod (p=0.7183). (**B**) No difference in 5-HT concentration in the midbrain. EE: 5 mice, LL: 6 mice, SS: 6 mice (p=0.355). (**C**) No difference in NE concentration in the midbrain. n=6 for each photoperiod (p=0.281). (**D**) No difference in thigmotaxis in the OFT (p=0.466). (**E**) There is no difference in time spent immobile in the TST (p=0.761). (**F**) No difference in time spent immobile in the FST (p=0.399). (**G**) EE mice spend significantly less time in the closed arm of the EZM than LL or SS mice but also exhibit increased overall locomotor activity (p<0.001, F=14.91, all tests were compared via a One-Way ANOVA).

increase the concentration of serotonin and norepinephrine in the midbrain. The long-day photoperiod also exhibits an anti-depressive and anxiolytic effect in behavioral testing. The effects on dorsal raphe neuronal activity endure for weeks to months in the face of subsequent photoperiod change. A culminating finding here is that melatonin signaling through the MT1 receptor is necessary for photoperiod programming of serotonin neuron physiology as well as 5-HT and NE concentrations in the midbrain.

The programming of dorsal Raphe serotonin neuron activity is observed in *ex vivo* de-afferented brain slices, indicating that these neurophysiological effects are intrinsic to the Raphe nucleus. This assessment is corroborated by a less negative resting membrane potential, increased intrinsic excitability and a strong trend toward smaller afterhyperolarization (Fig. 6) in Raphe neurons from long photoperiod mice, all of which increase the ease of action potential initiation and recovery. These characteristics are similar to the juvenile state of dorsal raphe neurons [86], suggesting that photoperiod is an environmental stimulus that shapes the maturation of raphe neurons and that developmental exposure to long summer-like photoperiods may partially maintain raphe neurons in an immature state. Interestingly, although the photoperiodic programming of increased adrenergic responsiveness of dorsal Raphe serotonin neurons is intrinsic to the nucleus, there may be additional circuit effects on adrenergic input *in vivo* as the norepinephrine content of the midbrain is also increased in Long photoperiod mice (Fig. 7).

Photoperiodic programming of Raphe activity, monoamine levels and affective related behaviors is dependent on melatonin signaling through the MT1 receptor. Maternal-fetal melatonin signaling of photoperiod in late gestation can program gonadal development later in life in rodents [23], suggesting a mechanism by which perinatal photoperiod may have lasting influence on serotonin neurons. Melatonin MT1 receptors are expressed in the Raphe nuclei and melatonin can act acutely to suppress serotonergic neuron firing [51]. In addition, melatonin MT1 receptors are also present on the intrinsically photoreceptive retinal ganglion cells that mediate circadian light signaling to the brain[91]. Thus, there are multiple cellular locations in

which the genomic knockout of MT1 receptors may have affected circadian photoperiodic regulation of raphe neurons. The overall pattern of changes in serotonin neuronal activity, monoamine content and affective behavioral changes suggest that developmental photoperiod induces a combination of regulatory changes in the serotonin neuraxis. Increased serotonin neuron spike rate and increased mid-brain 5-HT and NE content indicate that developmental photoperiod programs multiple aspects of dorsal raphe function, changing serotonergic tone through actions at the cellular and molecular levels.

Seasonal circadian photoperiods can acutely impact depression and anxiety behaviors in rodents (Fig. 3, 7) and in humans, as seen in Seasonal Affective Disorder [92]. In addition, our results indicate that in mice circadian light cycles have enduring effects on serotonin neuron activity that persist in adulthood even after substantial intervals of photoperiod reversal. Serotonin metabolism, as measured in the cerebral spinal fluid, varies seasonally in humans, and also varies in adulthood according to season of birth, both in healthy volunteers and neurospychiatriatric patients [93,94]. There is also accumulating epidemiological evidence of a 10-15% increased risk of mood and affective disorders in human winter birth cohorts [53,54], with a variety of potential seasonal signals proposed, including photoperiod, exposure to flu virus and vitamin D deficiency. Our results establish mechanisms by which seasonal circadian photoperiods may dramatically and persistently alter the function of the serotonin neuraxis, and institute an experimental model for further exploring the role of developmental programing by light experience in the seasonality of neurobehavioral disorders.

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Conflicts of Interests: None of the Authors have conflicts of interest pertaining to this work.

2.7: Author Contributions

NHG and DMG conceived the project and experiments with input and advice from CRJ. NHG, CRJ and HI performed the experiments and compiled the results. NHG and MCT performed data and statistical analysis. NHG and DGM wrote the paper with CRJ and HI providing critical commentary and input.

Chapter 3

Photoperiod affects the expression of key serotonergic genes and TPH2 positive neuronal ratio in the DRN without affective monoamine concentrations in the hippocampus

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3.1: Summary Abstract

Day length, or circadian photoperiod, influences mood and mood disorders in humans and depression- and anxiety-like behaviors in animal models. The neurobiological basis for the influence of photoperiod on affective behaviors, however, is not known. The serotonergic raphe nuclei of the midbrain receive photic input through the circadian visual system, and alteration of Raphe serotonin signaling is implicated in depression and anxiety disorders. Our lab has previously demonstrated that circadian light cycles experienced during early life can critically shape the function of midbrain serotonin neurons, monoamine concentrations, and depression- and anxiety-related behaviors in a melatonin receptor-dependent manner. Here, we demonstrate that differing circadian photoperiods can induce corresponding changes in the expression of key serotonergic genes in the midbrain, such as genes related to synthesis and transport, while not affecting many genes related to its signaling and metabolism. In addition, knocking out the MT1 receptor negates these changes, exhibiting a distinct gene expression pattern across photoperiods. Changes in photoperiod also result in changes in the ratio of TPH2 positive cells to total cells in the DRN. We also demonstrate that the changes we saw in monoamine concentrations in the midbrain due to photoperiod are not present in the hippocampus of control or MT1 KO mice developed on distinct photoperiods. Next we show

that the lack of both the MT1 and MT2 receptors negates photoperiodic changes in depression and anxiety related behaviors. Finally, we demonstrate that the critical period for photoperiodic programming of 5-HT neuronal physiology appears to occur before birth. Thus, circadian light cycles shape serotonergic gene expression in a melatonin receptor-dependent manner and change the ratio of TPH2 positive cells to total cells in the DRN while not affecting monoamine concentrations in the midbrain.

3.2: Introduction

The serotonergic system has been implicated in the control of many neuropsychiatric disorders such as depression, bipolar disorder and schizophrenia[27,71,95-98]. In general, increased 5-HT signaling alleviates the symptoms of these disorders. Free 5-HT content in the brain can be altered through multiple mechanisms including increases in the activity or concentration of the rate limiting 5-HT synthesis enzyme, TPH2 and/or increases in the activity of 5-HT releasing neurons in the DRN[99-101]. Serotonergic neurons in the raphe nuclei can be influenced by multiple neurochemical mechanisms, including the photoperiodic peptide melatonin. Melatonin is produced by the pineal gland and its synthesis is under circadian control via the SCN. In mammals the SCN is significantly influenced by light input from the retina and therefore melatonin is indirectly controlled by light input from the retina as well. Melatonin is synthesized exclusively in the dark phase of nocturnal and diurnal animals and its synthesis can be rapidly interrupted by a light pulse during the dark phase[23,102]. The melatonin profile (the duration of melatonin produced in a 24 hour period) is therefore significantly influenced by photoperiod.

During development, melatonin receptors are expressed in the SCN as early as day E18 in mice[103]. However, the fetal pineal gland does not produce melatonin, suggesting that all melatonin signaling in the fetus is provided by the mother. To emphasize this point, hamster mothers were pinealectomized and given timed daily injections of melatonin representative of

different photoperiods (i.e., an extended injection, representing a short photoperiod and a shortened injection representing a long photoperiod). The reproductive maturation of these pups reflected the length of the timed infusions of melatonin[23,24].

Our lab has previously demonstrated that circadian light cycles experienced during development and maturation have enduring effects on dorsal Raphe serotonin neuron excitability, responsiveness to adrenergic stimulation, monoamine concentrations in the midbrain, and depression- and anxiety-related behaviors of C3Hf$^{+/+}$ mice in a MT1 receptor-dependent manner. In particular, developmental exposure to long-day photoperiods induces significant increases in spontaneous firing rate, the excitatory effects of adrenergic input, and serotonergic content of Raphe neurons. Moreover, long-day photoperiods substantially increase the concentration of serotonin and norepinephrine in the midbrain. The long-day photoperiod also exhibits an anti-depressant and anxiolytic effect in behavioral testing. The effects on dorsal raphe neuronal activity endure for weeks to months in the face of subsequent photoperiod change. However, we have not yet established if this photoperiodic programming occurs *in utero* or during the weaning period after birth. In addition, we demonstrated that melatonin signaling through the MT1 receptor is necessary for photoperiod programming of serotonin neuron physiology, 5-HT and NE concentrations in the midbrain, as well as affective-related behaviors.

These results gave rise to a myriad of additional inquiries about how perinatal photoperiod may be affecting the serotonergic system. Previous research has demonstrated that environmental factors such as stress can have a significant and enduring effect on the epigenetic programming, and therefore expression of genes, in the serotonergic system[104]. Here we investigate if perinatal photoperiod is influencing the gene expression of key serotonergic genes or genes involved in the regulation of the 5-HT system, as well as if perinatal photoperiod affects the relative number of TPH2 positive cells in the DRN. We were also curious to see if monoamine content changes in areas associated with the modulation of depression and anxiety

disorders (outside of the midbrain), such as the hippocampus. We also aimed discover if the critical period for the photoperiodic programming of 5-HT neuronal physiology is before or after birth having already found that it is a developmental effect. Finally, we set out to determine if the changes in depression- and anxiety-related behaviors we observed in our previous paper are influenced by the lack of both the melatonin one and two receptors (MT1/2 KO). The answers to these questions will provide significant insight into how perinatal light cycles are altering the function and physiology of the serotonergic system.

3.3: Materials and Methods

Animals and Housing

C3Hf$^{+/+}$ mice, which produce melatonin and lack the retinal degeneration alleles of the C3H strain, were developed and raised to maturity on photoperiods designated Equinox (12 hours of light 12 hours of darkness), Short (8 hours of light and 16 hours of darkness), or Long (16 hours of light and 8 hours of darkness). For critical period experiments, breeders were taken from the equinox light cycle and placed into a long light cycle and the first generation of offspring was used in order to avoid any genetic or epigenetic programming of the parents of the experimental mice. On the day they were born, the pups and their parents were transferred to a short light cycle- additionally, one experimental cohort was transferred back to long at 30 days old in order to isolate the *in utero*, post-natal weaning, and post-weaning developmental periods. Another cohort, also switched to a short photoperiod at birth, was left in this photoperiod until experimentation to isolate pre-natal from all post-natal development. Tissues were isolated or behavior was tested at the mid-day point on all light cycles. MT1 KO mice, on a C3Hf$^{+/+}$ background[78], were also developed on the light cycles described above. All light cycles were aligned such that their midpoints (and therefore testing times) occurred at the same time

local time. Experiments were performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Gene Expression Detection and Analysis

Mouse mid-brains were removed between 11am and 1pm, then frozen in a 1.5 mL tube in liquid nitrogen. Samples were stored at -80°C until RNA extraction. Total RNA was extracted using a Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA, USA, Cat. No. 74104), measured by a Nanodrop system (ThermoScientific), and reverse-transcribed (~200ng) into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA, Cat. No. 205311). qRT-PCR reactions were performed in 20 µL total volume with 2 µL cDNA, 10µL of SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA), 6µL sterile water and 1µL of 300 nM intron-spanning gene specific forward and reverse primers in a Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Quantification of transcript levels were performed by comparing the threshold cycle for amplification of the unknown to those of six concentrations of standard cDNAs for each respective transcript, then normalizing the standard-calculated amount to hypoxanthine guanine phosphoribosyl transferase (*Hprt*) in each sample. Each sample was assayed in duplicate.

Monoamine analysis

Mouse mid-brains were collected and frozen as described above. Samples were stored at -80°C until the tissue was homogenized, using a tissue dismembrator, in 100-750 ul of 0.1M TCA, which contained 10^{-2} M sodium acetate, 10^{-4} M EDTA, 5ng/mL isoproterenol (as internal standard) and 10.5 % methanol (pH 3.8). Samples were spun in a microcentrifuge at 10,000 g for 20 minutes. The supernatant was removed and stored at –80°C. The pellet was saved for protein analysis. Supernatant was then thawed and spun for 20 minutes. Samples of the supernatant were then analyzed for biogenic monoamines. Biogenic amines were determined by a specific HPLC assay using an Antec Decade II (oxidation: 0.4; 3mm GC WE, HYREF) electrochemical detector operated at 33°C. Twenty µl samples of the supernatant were injected

using a Water 2707 autosampler onto a Phenomenex Kintex (2.6u, 100A) C18 HPLC column (100 x 4.60 mm). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8). Solvent was delivered at 0.6 mL/min using a Waters 515 HPLC pump. Using this HPLC solvent the following biogenic amines eluted in the following order: noradrenaline, Adrenaline, DOPAC, Dopamine, 5-HIAA, HVA, 5-HT, and 3-MT (2). HPLC control and data acquisition were managed by Empower software.

Multielectrode Array Electrophysiological Recording

Dorsal raphe nucleus slices were placed on a perforated electrode arrays and immobilized with a harp for recording. 40 µM tryptophan and 3 µM phenylephrine were added to the recording solution which was perfused (1.3 mL/min) over the slice once in the recording chamber. Serotonin neurons were identified by eliciting 5HT1a-mediated suppression of spontaneous firing rate. Serotonin at a concentration of 40 µM (for all general firing rate measurements) or 8-OH-DPAT at a concentration of 1 µM (for dose response experiments) was perfused (1.3 mL/min) over the slice for 5 minutes after 4 to 6 minutes of recording. After 5 minutes of serotonin or 8-OH-DPAT, normal ACSF was started again and recording continued until recovery was observed.

As there are differences in the electrophysiological properties of neurons in the medial and lateral wing subfields of the DR, placement on the array and the dimensions of the electrode grid were used to ensure that only the ventromedial DR neurons (vmDR) are recorded from. Mid-DRN slices of 280 micron thickness are taken between -4.5 mm and -4.75 mm back from bregma. We use a 6X10 perforated array with electrodes that have a diameter of 30 microns and 100 microns spacing between electrodes. The slice is placed so the electrodes cover an area spreading 1200 microns down from the cerebral aqueduct in the ventral direction and 340 microns laterally on either side of the midline (for a 680 micron total recording width). Putative 5HT neurons were identified by feedback inhibition evoked by application of 5-HT (40µM) (for

general firing rate experiments) or 8-OH-DPAT (for dose response experiments) for 5 min, with those cells demonstrating a minimum of 50% spike rate suppression being included in the analysis, as we have done in previously published studies[79,80]

Although there is overlap in the electrophysiological characteristics of dorsal raphe serotonergic and non-serotonergic neurons[81,82], these selection characteristics, as well as the placement of the array to record from the ventromedial DR region, assured that a high proportion of cells in our recordings were serotonergic, as the vmDR is highly enriched in serotonergic neurons. In the rat this has been carefully quantified with 75-95% of the neurons containing immunoreactivity to 5-HT, and 70-95% of neurons expressing the 5-HT_{1A} receptor also expressing 5-HT[82]. In addition, spike duration is significantly broader in 5-HT vs non-5-HT neurons in the dorsal raphe and the 5-HT_{1A} mediated response is significantly greater in 5-HT neurons[81]. The mouse DRN is thought to be similarly highly enriched for serotonergic neurons in the vmDR region, and 84% of genetically identified 5-HT neurons respond with 5-HT_{1A} inhibition[83]. In our dose-response experiments, 156 out of 198 total cells decreased their firing rate by at least 50% upon application of 8-OH-DPAT, indicating that ~80% of the neurons recorded from express functional 5-HT_{1A} receptors and therefore are likely serotonergic. Thus, while we cannot be sure that each and every neuron we putatively identified as serotonergic indeed expressed serotonin, the combination of our anatomical recording site and physiological criteria assures that the overwhelming majority of neurons in our study were serotonergic.

Data files were analyzed in Offline Sorter (Plexon Inc.). For analysis, a Besel filter with a 150 Hz frequency cut off was applied to the raw data traces. The threshold for detection was set manually to a level that includes all legitimate spikes with the least amount of unipolar noise spikes (between 13 µV and 35 µV). Once spikes were detected they were sorted by a combination of a K means scan method and manual verification. The manual verification was conducted after the K means scan was run and divided spikes into groups based on criterion such as amplitude, power under the curve, and spike duration (for full list of criterion see Offline

Sorter V3 manual under the K means scan, Plexon Inc.). Once waveforms were sorted into groups and judged to be biologically relevant each spike was validated by eye and spikes that did not fit the average waveform shape were discarded. All unsorted spikes were visualized manually and any spikes that matched the average waveform shape of a relevant group were added to that group. Once the total number of spikes was determined for the period of the recording before the application of 5-HT or 8-OH-DPAT, that number was divided by the total time in which those spikes occurred to produce a measure of spikes per second.

Behavioral Testing

Mice. Male and female mice were group housed and transferred from our housing facility to light controlled boxes in the Vanderbilt Neurobehavioral Core. After a week of acclimation, the elevated zero maze was conducted on the first day of testing, followed by the open field test, the tail suspension test, and the forced swim test- each conducted on successive days with about 24 hours between each test. They were tested in the middle of their light phase (1100-1500). No mouse underwent more than one test daily and no mouse underwent the same test more than once. The experimenter was the same for all tests conducted. There was no pseudo-randomization in the order of the mice tested, they were tested serially by cage. Mice were allowed to acclimate to the testing room for 30 minutes prior to all tests. Sex differences in behavioral results within photoperiods were not observed (t-test with Holm-Sidak correction for multiple comparisons).

Forced swim test. Mice were exposed to the FST for 6 min during the light phase 1100-1500 hours. The FST container was a Plexiglas cylinder 45 cm high and 20 cm in diameter filled with 30-35 cm of water (21 ± 2˚C). Testing was conducted in normal room light. Throughout the task, an experimenter was present and watching the mice. The videos were then scored by the same experimenter that performed the tests. Before scoring, the scorer was blinded to the genotypes and photoperiods of the mice in each video. Time immobile was defined as any period of time the animal was not making any active escape movements or floating without struggling.

Statistical significance was determined by a one-way ANOVA with α = 0.05. For all post hoc analysis, Holm-Sidak pair-wise comparisons were performed.

Tail suspension test. The tail of a mouse was taped to a force meter attached to the top of an open chamber so that it cannot escape. The mouse was suspended for 6 min. Latency to stop struggling, and amount of time spent struggling were measured through the force meter with a threshold of 7 (arbitrary units) as the limit for struggling. Testing was conducted in normal room light. Mice used in this experiment were similar ages (50 to 90 days old) and there were no large weight discrepancies between mice tested. Data was gathered and processed by Med Associates Inc. tail suspension software. Statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant. For all post hoc analysis Holm-Sidak pair-wise comparisons were performed.

Open field test. Exploratory locomotor activity was measured in an open field measuring 27 x 27 cm, with a light intensity between 90-110 lux across all chambers, over a 60 minute period. Infrared beams and detectors automatically recorded movement in the open field. Thigmotaxis was defined as time spent on the outer rim of the open field, which was defined as the area 4.25 cm from the wall of the open field, constituting 50% of the total area of the open field. Thigmotaxis was measured throughout the 60 minute period in 5-minute blocks. The average thigmotaxis within a 5-minute block was calculated from all 12 blocks within 60 minutes of testing. There was no difference between photoperiods in C3Hf$^{+/+}$ mice in total distance traveled. MT1 KO mice developed on an EE photoperiod showed an increase in total distance traveled over both SS and LL mice. Statistical significance was determined by a one-way ANOVA with α = 0.05. For all post hoc analysis, Holm-Sidak pair-wise comparisons were performed.

Elevated zero maze. The zero maze consisted of one circular platform approximately 50 cm across. Two discontinuous portions of the maze have walls, approximately 20 cm high with a light intensity within these arms of 71-91 lux compared to 344-355 lux outside these arms. The

remaining portions have no walls. Mice were placed in one of the open arms at the beginning of the session. Number of entries into the closed arms and amount of time spent in the closed arms was used as a measure of anxiety. The zero maze task was conducted once per mouse for 6 min. Statistical significance was determined by a one-way Kruskal-Wallis ANOVA on ranks, as the data was not normally distributed, with $\alpha = 0.05$. For all post hoc analysis, Dunn's method pair-wise comparisons were performed.

Data Analysis

Significance was established using a one or two-way ANOVA with $\alpha = 0.05$. For monoamine measurements values exceeding $\pm$ 1.5 SD from the mean were excluded.

3.4: Results

To observe the effects of developmental photoperiod on the expression of key genes involved in 5-HT production and signaling, qRT-PCR was used to measure the mRNA levels of *Tph2, Slc6a4* (SERT)*, Pet-1,* and *5-HT$_{1A}$* in the midbrain of mice developed on an Eq, L or S photoperiod. *Tph2* and *sert* exhibited significant decreases of about 50% in mRNA abundance in L compared with both the Eq and S groups, with no difference between the Eq and S groups (Fig. 12 A, B). *Pet-1* showed a similar trend to that of *Tph2* and *Slc6a4*, however the difference was not statistically significant (p= 0.27, Fig. 12C). In contrast, the *5-HT$_{1A}$* receptor transcript showed no change between photoperiods (Fig. 12D). Levels of *5-HT$_{1b}$*, *Adra$_{1a}$*, *Adra$_{1b,}$* *GABA$_{1a}$R*, *GABA$_{1b}$R*, *Nr3c1* (the glucocorticoid receptor), *Lmx1b*, and *MAO* expression did not exhibit significant differences across photoperiods (n=6 mice in all groups, Table 3 left column).

To determine if photoperiod may influence the number or percentage of cells containing the TPH2 protein, as well as to get an initial idea of if there is a change in TPH2 protein levels, we conducted immunohistochemical staining for TPH2 in the DRN of mice developed on a short or long photoperiod (Fig. 13 A, B). We observed that there is a trend towards an increase in the

total number of TPH2 positive cells in long mice compared to short mice (L= 108.17 ± 5.84 n=13, S= 90.29 ± 13.27 n= 7, *p*= 0.18, Fig. 14A) but there is a significant increase in the ratio of TPH2 positive cells to total cells in long photoperiod mice compared to short photoperiod mice in the portion of the DRN measured (L= 0.155 ± 0.01 n=13, S= 0.102 ± 0.01 n=7, *p*= 0.04, Fig. 14B). We also measured the fluorescence intensity of each of the TPH2 cells identified and then compared the average fluorescence intensity of long and short mice to get a preliminary idea of whether there is an increase in TPH2 protein present in these cells. We did not see a difference in the fluorescence intensity between long and short mice (*p*=0.507, Fig. 14C). However, it should be noted that this result is not a direct measure of TPH2 levels since fluorescence intensity can vary due to variation in experimental quality from animal to animal. We mention it here are a possible indicator of relative TPH2 levels.

mRNA levels of the same key serotonergic genes measured in C3Hf$^{+/+}$ mice were also measured in MT1 KO mice. mRNA concentrations in *Pet-1, Tph2* and *Sert* were all elevated by about 50% in S compared to L and Eq photoperiods (n=6 mice in all groups, Fig. 15 A-C). There were no changes in mRNA levels in any of the other genes across photoperiods except an increase of about 10% in the L over S photoperiods in *lmx1b* and an increase of about 30% in Eq over S in *5-HT$_{1b}$* receptor (Table 3, right column).

To determine if perinatal photoperiod influences monoamine concentrations in raphe terminal areas associated with the modulation of affective behaviors we used HLPC to measure the monoamine concentrations in the brain. Interestingly, we did not see any change in the levels of 5-HT(n: Eq= 11 mice, L= 12 mice, S= 6 mice), NE (n: Eq, L= 12 mice, S=6 mice), or their metabolites (5-HIAA- n: Eq, L= 12 mice, S=6 mice; HVA- n: Eq, L=12 mice, S= 6 mice) in the hippocampus of mice developed on different photoperiods (Fig. 16 A-D). However, we did see significant changes in dopamine levels in the hippocampus with the levels in S mice being elevated above L mice with Eq mice exhibiting an intermediate concentration of DA (Eq= 0.17 ± 0.03, L= 0.12 ± 0.02, S= 0.28 ± 0.05; n: Eq, L= 11 mice, S= 6 mice, *p*= 0.032) (Fig. 16 E).

To test if developmental photoperiodic programming may extend to affective behaviors in MT1/2 KO mice, we conducted the same four behavioral tests on these mice as those conducted on C3Hf$^{+/+}$ and MT1 KO mice. There were no behavioral differences across photoperiods in MT1/2 KO (n: L=15 and S=10, Fig. 17 A-C) mice in the OFT, TST, or FST. However, in the EZM S MT1/2 KO mice spent significantly less time in the closed arms than L mice (p= 0.031, Fig. 17 D).

Finally, having demonstrated that the effect of photoperiod on 5-HT neuronal physiology is developmental (Fig. 10), we set out to narrow down our estimate of the critical window in which this photoperiodic programming is occurring. The first generation of offspring of breeders moved from the equinox photoperiod to a long photoperiod was used in order to avoid any genetic or epigenetic programming of the parents of the experimental mice. We were therefore able to isolate the photoperiod on which the mice were gestated and developed as the only dependent variable in establishing the critical period. On the day they were born, the pups and parents were transferred to a short light cycle and one experimental cohort was transferred back to long at 30 days old, to isolate the prenatal, postnatal/pre-weaning, and postnatal/post-weaning developmental periods. We observed that the firing rate of 5-HT neurons in mice developed under this photoperiodic paradigm was much closer to those of mice that had been developed on a long photoperiod than on an equinox or short light cycle (LL = 1.24 Hz $\pm$ 0.084, n=6 mice, 153 cells; L to S (P0-P30) back to L= 1.15 Hz $\pm$ 0.104, n=5 mice, 34 cells, L to S (P0-P50) = 1.18 Hz + 0.076, n=5 mice, 30 cells, EE = 0.83Hz $\pm$ 0.058, n=6 mice, 92 cells; SS = 0.69Hz $\pm$ 0.024, n=6 mice, 70 cells; EE vs. L to S (P0 to P30) to L: p = 0.019, SS vs. L to S (P0 to P30) to L : p= 0.004, LL vs. L to S (P0 to P30) to L: p= 0.521, One-Way ANOVA). Another cohort was left to remain in a short light cycle until experimentation after being switched at birth to isolate the *in utero* developmental period from all postnatal development, ensuing proximal photoperiod was still counteracted by developmental photoperiod before birth. We observed that

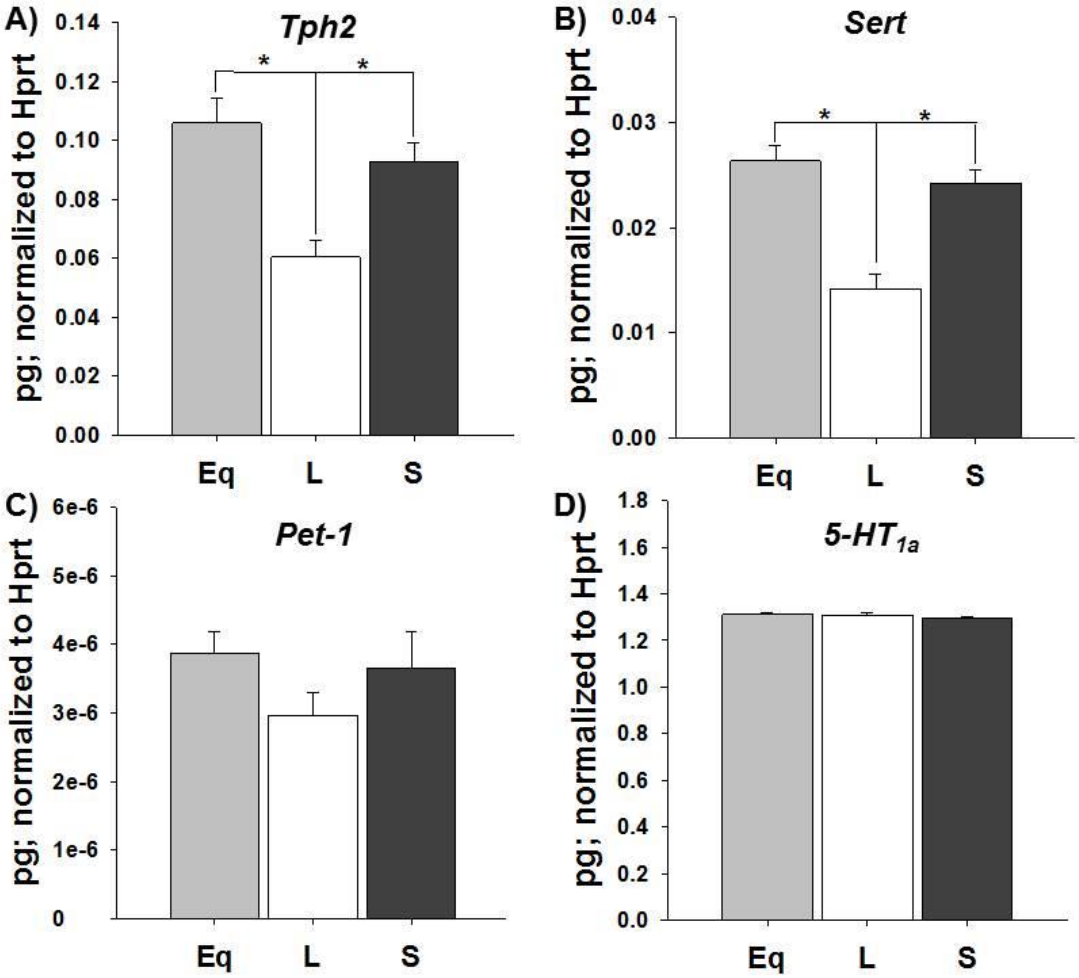


Figure 12. Gene Expression of key 5-HT synthesis and signaling genes in the midbrain across photoperiods. (**A**) *Tph2* gene expression is significantly decreased in L compared to Eq and S. (**B**) *Sert* gene expression is significantly decreased in L compared to Eq and S. (**C**) There is a trend toward a significant decrease in *Pet*-1 gene expression in L compared to Eq and S. (**D**) There is no change in *5-HT$_{1A}$* gene expression across photoperiods. * = $p < 0.05$.

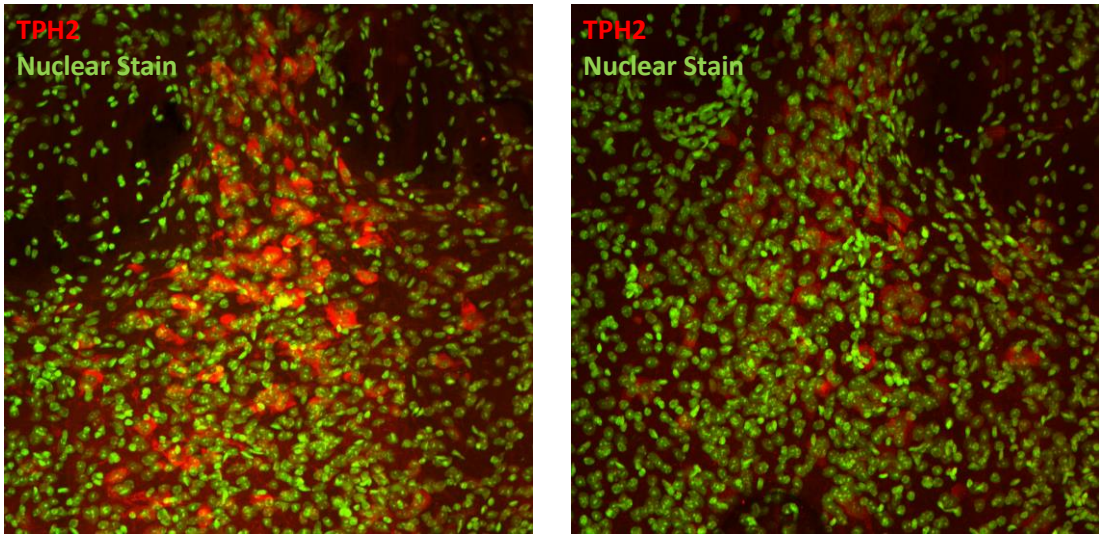
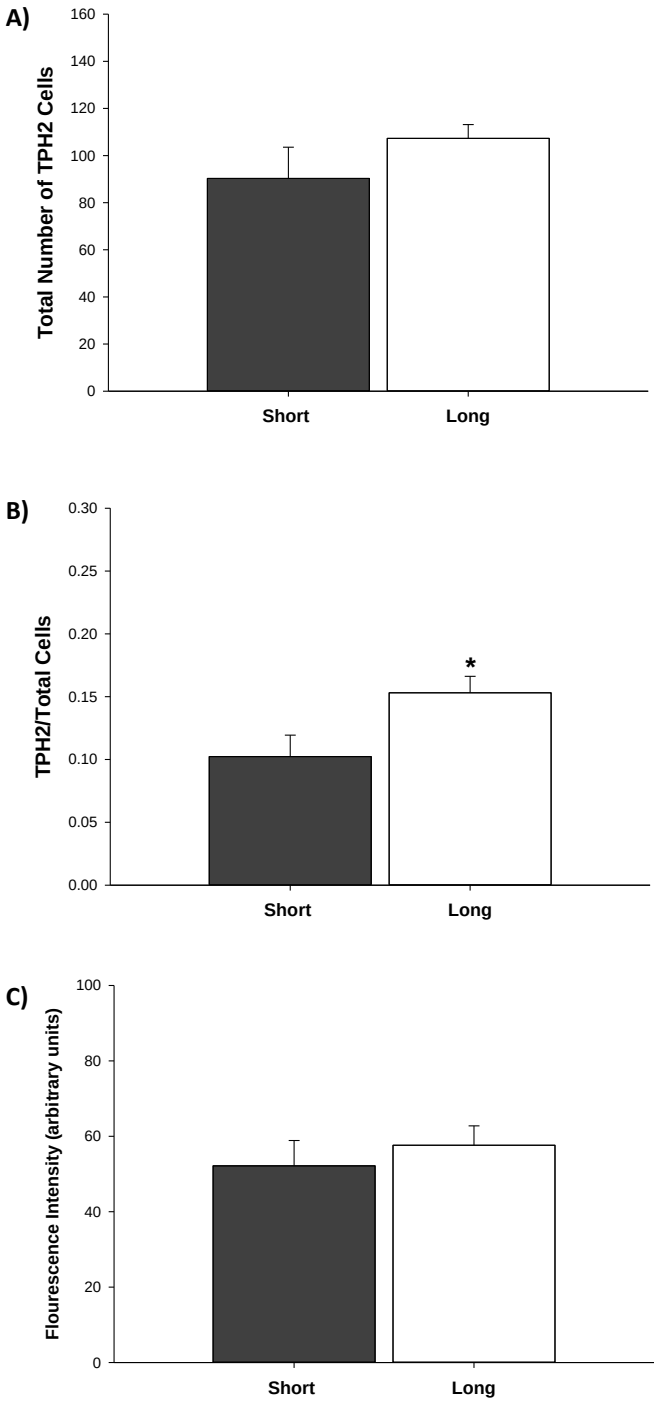


Figure 13. Representative images of the dorsal portion of the DRN stained for nuclei and TPH2. Red stain indicates TPH2, green stain indicates nuclei.



<u>Figure 14</u>. Quantified TPH2 positive cell numbers, proportions and fluorescence intensity. (**A**) Total number of TPH2 positive cells. (**B**) Proportion of TPH2 positive cells to total cells. (**C**) Average fluorescence intensity of TPH2 positive cells. * = $p < 0.05$.

the firing rate of 5-HT neurons in mice developed under this photoperiodic paradigm was also much closer to that of mice developed on a long photoperiod than on an equinox or short light cycle (LL = 1.24 Hz $\pm$ 0.084, n=6 mice, 153 cells; L to S at P0= 1.18 Hz $\pm$ 0.076, n=5 mice, 30 cells, EE = 0.83Hz $\pm$ 0.058, n=6 mice, 92 cells; SS = 0.69Hz $\pm$ 0.024, n=6 mice, 70 cells; EE vs. L to S at P0: p = 0.005, SS vs. L to S at P0: p= 0.004, LL vs. L to S at P0: p= 0.605, One-Way ANOVA, Fig. 18).

3.5: Discussion

Our results demonstrate that circadian light cycles affect the expression of key genes involved in the production and transport of 5-HT, but not genes involved in the modulation of the 5-HT system. Specifically, we found that long-day photoperiods decrease the expression of *pet-1, tph2* and *sert* in the midbrain compared to equinox and short photoperiods. PET-1, an ETS transcription factor that acts as the master regulator of the 5-HT system[105,106], is required for the normal maturation of 5-HT neurons and the expression of key 5-HT related genes during development [73,107]. It is also involved in the maintenance and upkeep of the 5-HT system throughout adult life in the mouse[108]. Therefore, it would make sense that if *pet-1* expression was decreased in mice developed on a long photoperiod that genes that are under its control such as *tph2* and *sert* would follow suit and decrease their expression patterns as well. However, this is an interesting result owing to the fact that we see an increased proportion of TPH2 positive cells in the DRN as well as our previous results demonstrating an increase in 5-HT concentrations in the midbrain which may result in the expectation of *pet-1* and *tph2* to be elevated to produce this increase. However, it appears that this decrease in key 5-HT gene expression may be compensation for the amount of TPH2 protein and 5-HT already present and being released in mice developed on long photoperiod. Since our previous results demonstrate

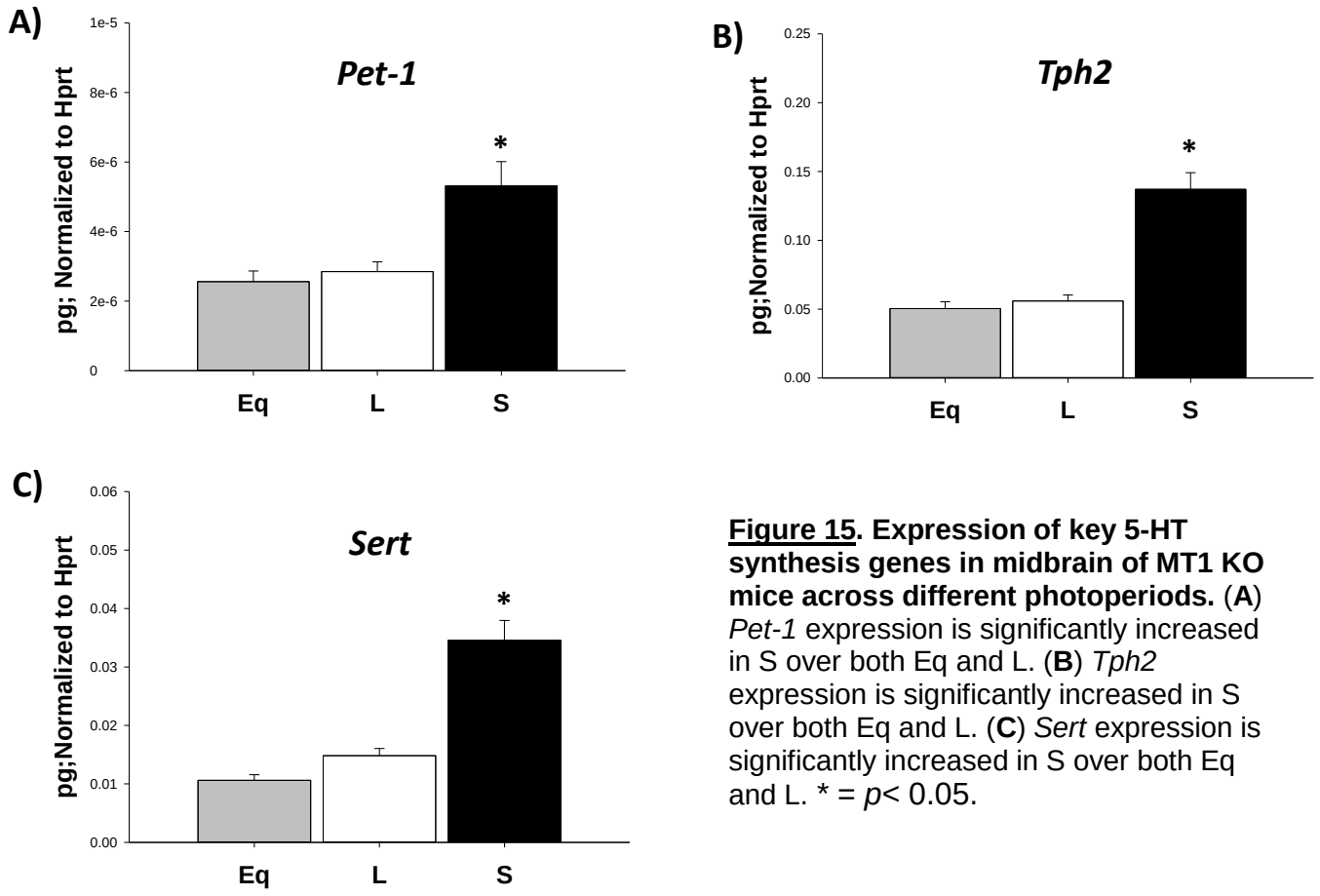


Figure 15. Expression of key 5-HT synthesis genes in midbrain of MT1 KO mice across different photoperiods. (**A**) *Pet-1* expression is significantly increased in S over both Eq and L. (**B**) *Tph2* expression is significantly increased in S over both Eq and L. (**C**) *Sert* expression is significantly increased in S over both Eq and L. * = $p < 0.05$.

C3Hf$^{+/+}$	**MT1 KO**
5-HT$_{1a}$ – No Change	*5-HT$_{1a}$* – No Change
5-HT$_{1b}$ – No Change	*5-HT$_{1b}$* – ca. 30% inc. in Eq over S
Adra$_{1a}$ – No Change	*Adra$_{1a}$* – No Change
Adra$_{1b}$ – No Change	*Adra$_{1b}$* – No Change
GABA$_{1a}$R – No Change	*GABA$_{1a}$R* – No Change
GABA$_{1b}$R – No Change	*GABA$_{1b}$R* – No Change
Nr3c1 – No Change	*Nr3c1* – No Change
Lmx1b – No Change	*Lmx1b* – ca. 10% inc. in L over S
MAO – No Change	*MAO* – No Change

Table 3. Expression of genes involved in regulating the signaling and release of 5-HT in the midbrain. Left column from the midbrains C3Hf$^{+/+}$ mice. Right column from the midbrains MT1 KO mice Six animals for each gene in each genotype. MT1 KO: *5-HT$_{1b}$* - p=0.01, *Lmx1b* - *p*= 0.012.

that 5-HT neurons in mice developed on a long photoperiod have increased intrinsic excitability and a higher firing rate[109], there is a significant chance that more 5-HT is being released from the these neurons which could then feedback on the system and turn down the expression of these genes. Another possibility is that perinatal long photoperiods cause epigenetic programming of the promotors of these genes, causing their decreased expression throughout development and adult life. This decrease results in the prevention of full maturation of 5-HT neurons in the DRN, which instead maintain their juvenile state[107].

We do not see these same changes in gene expression in MT1 KO mice. Instead we see a vast increase in the expression of *pet-1, tph2*, and *sert* in the short photoperiod compared to the equinox and long photoperiods. Due to the widespread nature of the MT1 receptor it is difficult to determine the exact cause of this increase in gene expression in the short photoperiod. However, it is possible that decreasing the duration of light causes altered circadian expression of these genes which could be mediated by the MT1 receptor. Therefore without MT1 receptor signaling there is dysregulation of expression of these genes within the DRN.

We also demonstrate that there are no changes in the concentration of 5-HT, NE, or their metabolites in the hippocampus of mice developed on different photoperiods, suggesting that while we saw an increase in 5-HT and NE content in the midbrain of mice developed on a long photoperiod[109] there is not increased concentrations of these monoamines in the terminal region of neurons projecting from the raphe to the hippocampus. Taken together, these results could be interpreted in multiple ways: Mice developed on long photoperiods may only have increased 5-HT in their midbrains and not the rest of the brain, there may be increased release of 5-HT in the hippocampus therefore depleting terminal stores and causing increased 5-HT_{1A} feedback, or increased 5-HT in the midbrains of long photoperiod mice may be packaged, transported and released in other areas associated with depression and anxiety disorders, such

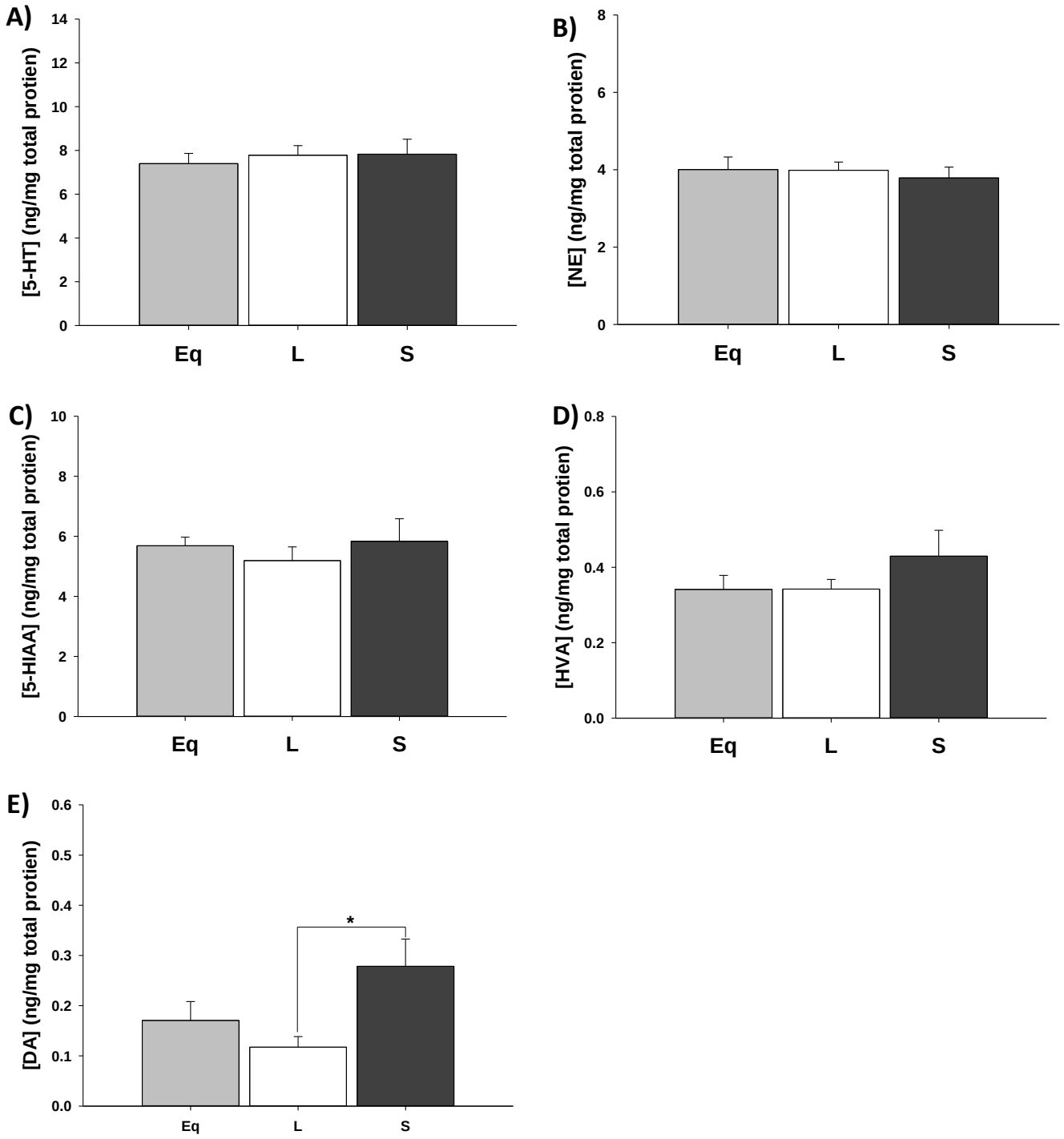
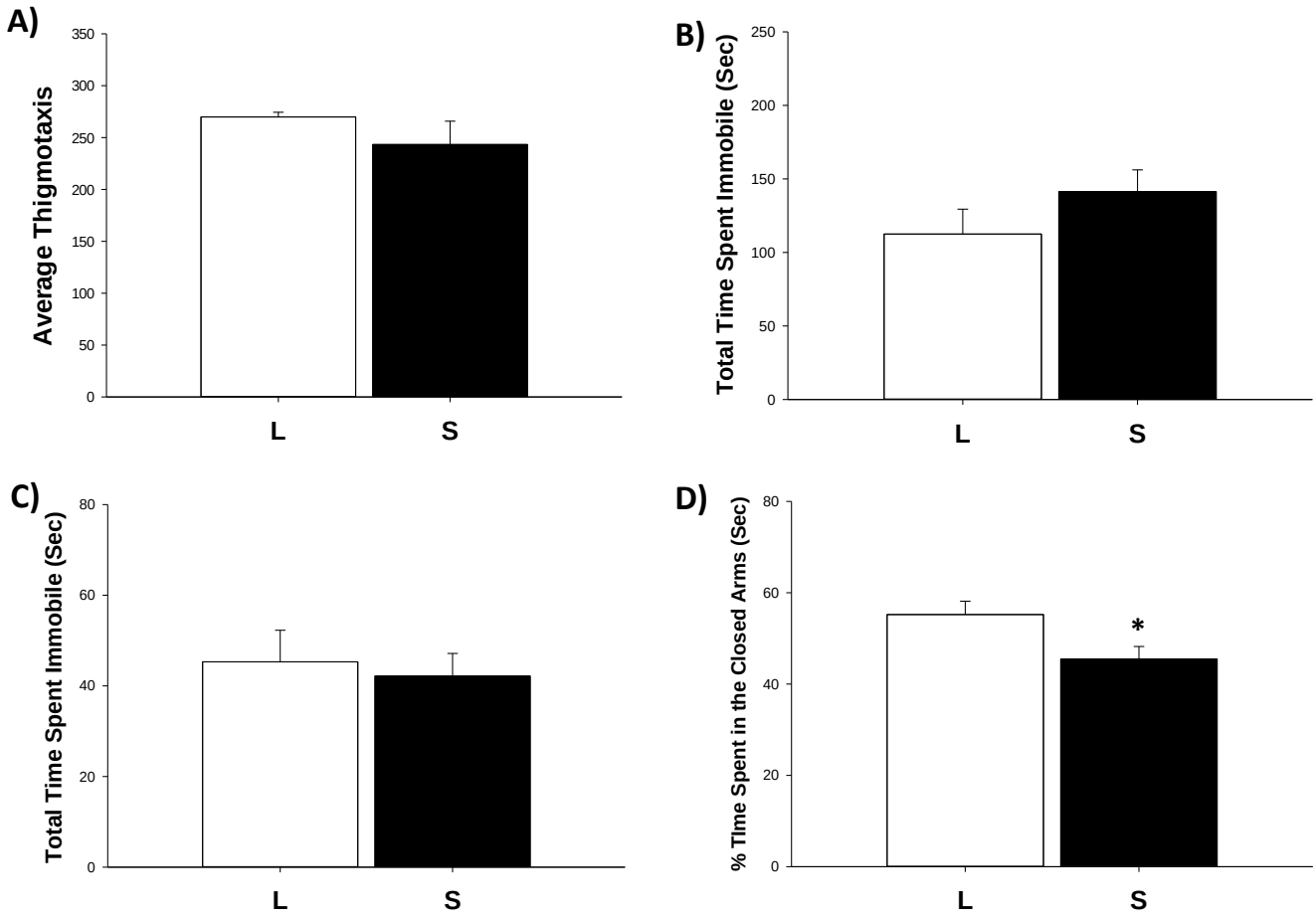


Figure 16. Monoamine and metabolite levels in the hippocampus of C3Hf$^{+/+}$ mice. (**A-D**) There is no change in 5-HT, NE or their metabolites in the hippocampus across photoperiods. (**E**) There is a significant increase in DA levels in the hippocampus of S mice compared to L mice (p=0.032). * = p< 0.05.

as the forebrain and amygdala, but not in the hippocampus. Regardless of how the increase in 5-HT in the midbrain acts on its terminal areas, it appears that this photoperiodic effect on the serotonergic system translates into melatonin receptor-dependent changes in depression- and anxiety-related behaviors. Our previous results demonstrate that developing mice on a long photoperiod imparts anti-depressant and anxiolytic behaviors. In this study, we demonstrate that these effects are melatonin receptor-dependent. We ran MT1 KO and MT1/2 KO mice developed on distinct photoperiods through the same battery of tests performed in our previous work and demonstrated that knocking out the melatonin receptors negates the photoperiodic changes we see in control animals, with the exception of the elevated zero maze. Previous work has demonstrated that manipulation of 5-HT neurons with optogenetics can alter depression- and anxiety-related behavior, demonstrating that increasing the firing rate of these neurons decreases affective related behaviors[99,110]. This suggests that it is most likely the changes in the physiological effects of 5-HT neurons that are producing the changes we see in affective behaviors, rather than the changes in gene expression described in this study. Seasonal circadian photoperiods can acutely impact depression and anxiety behaviors in rodents (Fig. 3,11) and in humans, as seen in Seasonal Affective Disorder [92]. In addition, our results indicate that this phenomenon appears to be melatonin receptor-dependent. This photoperiodic influence does not seem to require an increase in 5-HT or NE concentration in the hippocampus. Serotonin metabolism, as measured in the cerebral spinal fluid, varies seasonally in humans and also varies in adulthood according to season of birth, both in healthy volunteers and neurospychiatriatric patients [93,94]. There is also accumulating epidemiological evidence of a 10-15% increased risk of mood and affective disorders in human winter birth cohorts [53,54], with a variety of potential seasonal signals proposed, including photoperiod, exposure to flu virus, and vitamin D deficiency. Our results establish mechanisms by which seasonal circadian photoperiods may dramatically and persistently alter the function of the serotonin neuraxis, and



<u>Figure 17</u>. Depression and anxiety related behaviors in MT1/2 KO mice developed on a long or short photoperiod. (**A**) There is no change in time spent around the perimeter of the open field across photoperiods. (**B**) There is no change in total time spent immobile in the tail suspension test. (**C**) There is no change in total time spent immobile in the forced swim test. (**D**) Mice developed on a short photoperiod spend less time in the closed arms of the EZM than mice developed on a long photoperiod. * = $p < 0.05$.

institute an experimental model for further exploring the role of developmental programing by light experience in the seasonality of neurobehavioral disorders.

Finally, we wanted narrow down our estimate of the critical window for when photoperiod is acting during development to cause changes in 5-HT neuronal physiology. Our results indicate that this critical period most likely occurs before birth, because both groups of mice developed on a long photoperiod before birth and then switched to short for varying amounts of time exhibited 5-HT neuronal firing rates similar to mice developed on a long photoperiod and not short or equinox. Further experiments are needed to verify these results, such as developing mice on a short light cycle until birth and then switching to a long light cycle to determine if those mice display firing rates similar to that of a short or long photoperiod. Because melatonin can cross the placenta, and we demonstrated that 5-HT neuronal physiological programming happens most likely happens before birth, it appears that the mother's melatonin profile may be acting on the developing 5-HT neurons in the fetal brain and programming their activity patterns in utero. How this translates to a critical period for photoperiodic programming of the serotonergic system for humans is a complicated question because of the vastly different rates of neurological and physiological development of mice and humans. However, these results set the stage for further exploration of when photoperiod may be acting to program the serotonergic system.

3.6: Acknowledgements

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Conflicts of Interests: None of the Authors have conflicts of interest pertaining to this work.

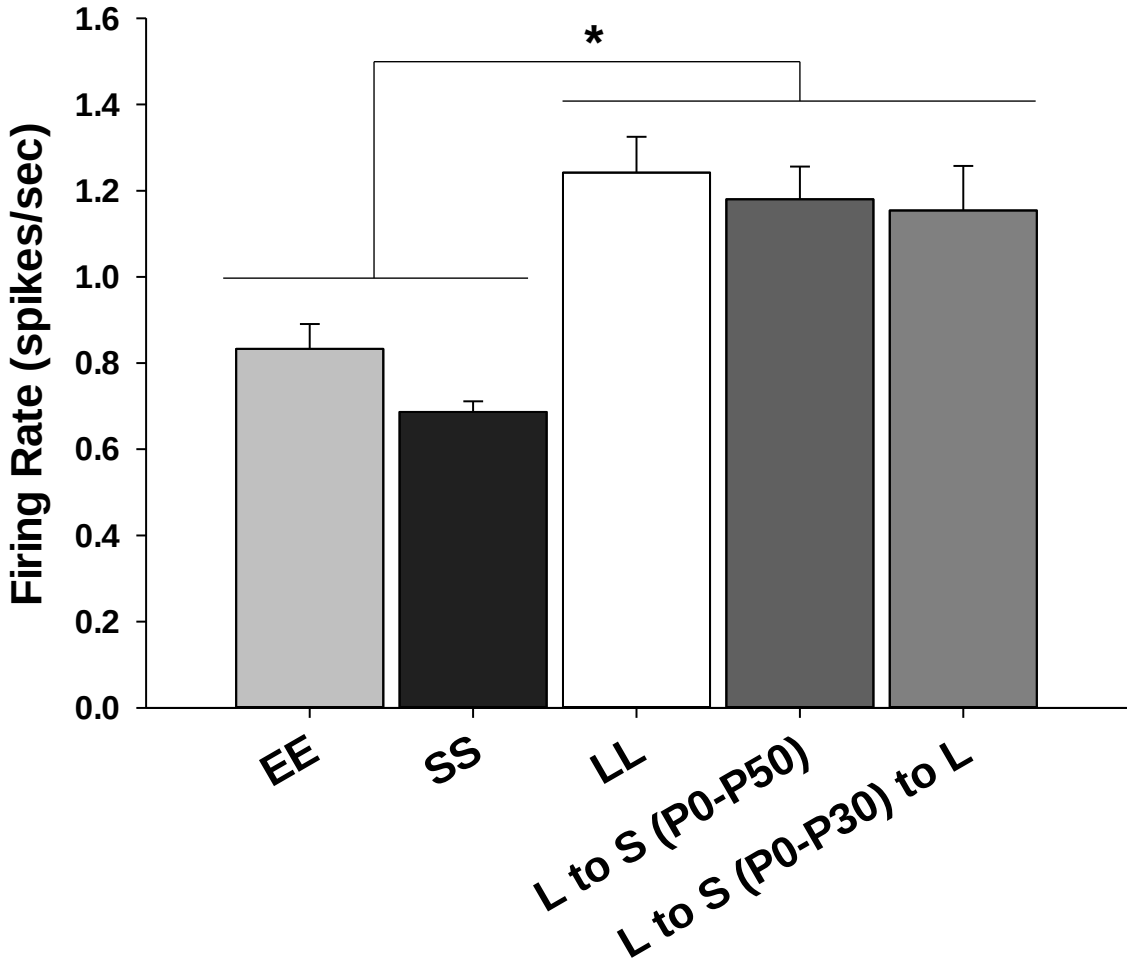


Figure 18. Critical period for 5-HT neuronal physiology in the DRN. Mice developed on a long photoperiod before birth and then switched to a short photoperiod exhibit firing rates closer to that of mice developed and maintained on a long photoperiod than that of short or equinox photoperiods. * = $p < 0.05$.

3.7: Author Contributions

NHG and DMG conceived the project and experiments with input and advice from CRJ. NHG, CRJ performed the experiments and compiled the results. NHG and CRJ performed data and statistical analysis. NHG and DGM wrote the paper with CRJ providing critical commentary and input.

Chapter 4

Summary and Discussion

4.1: Summary

In this dissertation, I have outlined how developmental photoperiod can influence multiple aspects of the serotonergic system in a MT1 dependent manner. I demonstrated that perinatal photoperiod programs several aspects of 5-HT neuronal physiology including firing rate, resting membrane potential and afterhyperpolarization amplitude. I demonstrated that increased light through a long photoperiod increases firing rate, significantly depolarizes the resting membrane potential, causes a strong trend toward a smaller afterhyperpolarization amplitude and significantly increases the intrinsic excitability of these cells. The latter three of these 5-HT cell properties will increase the ease and speed at which a neuron can fire an action potential, resulting (at least in part) in the increased firing rate observed in these neurons. Perinatal photoperiod also programs serotonergic and noradrenergic tone in the midbrain. Once again, increased light exposure through a long photoperiod increases the concentrations of 5-HT and norepinephrine in the midbrain, both congruent with the increased excitability and firing rate of 5-HT neurons in the DRN. I was then able to link these increases in 5-HT neuronal activity and content in the midbrain with decreases in depression- and anxiety-related behaviors with an increased duration of light. Finally, I demonstrated that each of these aspects of perinatal photoperiod programming is MT1 receptor-dependent by showing that in MT1 KO mice each of these photoperiodic changes are negated.

Next, I wanted to observe several other aspects of the 5-HT system to determine if perinatal photoperiod programmed expression of key serotonergic genes as well as 5-HT associated genes, TPH2 levels in the DRN, and monoamine concentrations in other areas of the brain, as well as if the lack of both the MT1 and MT2 receptors influenced the photoperiodic

effects on depression- and anxiety-related behaviors. Finally, I set out to determine what the critical period is for the perinatal photoperiodic programming of 5-HT neuronal physiology.

Out of all the genes we measured, we saw changes in three due to photoperiod: *Pet-1, Tph2* and *Sert*. These three genes exhibited a decrease in their expression with increased light due to a long photoperiod- contrary our expectations, as all other measurements of the 5-HT system were upregulated with an increased duration of light. However, this may be compensation due to the increase in excitability of 5-HT neurons and 5-HT content in the midbrain. Another caveat to these findings is mRNA for these genes was extracted from crude midbrain dissections. Therefore, genes in which I did not see a difference but that maintain expression throughout the midbrain, such as the $adra_{1b}$ receptor, may exhibit changes in the DRN that are masked by the noise of their expression in the rest of the midbrain. Therefore, it may be more useful measure the expression of such genes from raphe punches rather than crude midbrain dissections.

These data lead us to investigate if the number of cells containing the TPH2 protein may be altered due to photoperiodic programming. Therefore we stained the dorso-medial section of the DRN for the TPH2 protein as well as a nuclear stain in order to determine if there was a change in the overall number of TPH2 positive cells or a change in the ratio of TPH2 positive cells to total cells in this portion of the DRN due to changes in photoperiod. We did not observe a significant change in the total number of TPH2 positive cells but we did see an increase in the ratio of TPH2 positive cells to total cells in the long photoperiod compared to the short photoperiod. These data reinforce the hypothesis that the decrease in *tph2* mRNA expression in the long photoperiod compared to short and equinox photoperiods may be compensation for the amount of TPH2 protein already present in the DRN.

In order to determine if monoamine concentrations in other areas of the brain involved in the control of affective behaviors were increased due to increase in the duration of light the animals were exposed to, we measured the monoamine concentrations in the hippocampus of

mice developed on different photoperiods. I did not see a change in the monoamine concentrations in the hippocampus across photoperiods. We were also interested to see if the MT2 receptor may be playing a role in the photoperiodic programming of affective related behaviors. I therefore conducted depression- and anxiety-related behavioral testing on MT1/2 KO mice. I did not see a change in their behavioral phenotype compared to MT1 KO mice suggesting the MT2 receptor is most likely not involved in the photoperiodic programming of affective behaviors.

Finally I set out to determine what the critical period for the photoperiodic programming of 5-HT neuronal physiology could be. I conducted multielectrode array recordings on mice that had been maintained on a long photoperiod, switched to a short photoperiod at birth, and then switched back to a long photoperiod at 30 days. I observed that the firing rates of 5-HT neurons in these mice were similar to those of mice developed on a long photoperiod, but not those of mice on a short photoperiod, suggesting that the critical period for photoperiodic programming of 5-HT neuronal physiology occurs before birth. While I have also demonstrated that the proximal photoperiod does not affect 5-HT neuronal photoperiod for up to six months in a separate set of experiments, I also wanted to ensure that proximal photoperiod was not playing a role in these critical period experiments. Therefore, I developed mice on a long photoperiod until birth, at which point I switched them to a short photoperiod, where they were maintained until testing at 50 to 90 days.

These results taken together demonstrate that increasing the duration of light during development, results in increased 5-HT neuronal intrinsic excitability and firing rate, monoamine content in the midbrain, as well as decreases in depression- and anxiety-related behavior, the expression of key 5-HT related genes in the midbrain in a MT1 receptor-dependent manner. In addition, initial experiments suggest that the photoperiodic programming of serotonergic neuronal physiology may be occurring before birth. However, perinatal photoperiod does not seem to affect monoamine concentrations in the hippocampus or the expression of many genes

that are tertiary to, but act on, the 5-HT system. These studies could lead to lines of research that could ultimately reduce the prevalence of neuropsychiatric disorders through the use of light therapy during development.

4.2: Does Light or Lack of Light Affect the Serotonergic System

Seasonal depression or seasonal affective disorder (SAD) is a form of depression in which the expression of depressive symptoms is restricted to the winter, and sometimes fall, months[111]. Research has suggested that this type of depression is most likely due to the decreased duration of full spectrum light during the winter months[92]. Most humans do indeed experience less full spectrum light (light containing the majority of visibly perceptible wavelengths) during the winter months, as light from the sun is full spectrum (as it contains all wavelengths in our visual spectrum as well as those not visible to humans such as UV and infrared)[112]. Interestingly, the majority of incandescent and fluorescent light bulbs used indoors are not full spectrum. These indoor light sources lack light towards the blue end of the spectrum in particular, which has been shown to be the most effective wavelength of light for shifting circadian rhythms and treating depression and anxiety disorders in mammals[113-116]. This is because specialized cells in the retina, intrinsically photosensitive retinal ganglion cells (ipRGCs), are most sensitive to blue light (specifically, light with a wavelength of around 488 nm)[117].

Artificial lighting is categorized by “correlated color temperature” or CCT. Simply stated light sources are assigned a number that is correlated with the temperature (in Kelvin) of a black body radiator that emits light of a comparable hue to the light source. Light sources with higher temperatures contain more blue light and are referred to as “cool” (a psychological association with the color, not referring to the actual temperature) while light sources with lower temperatures contain more red light and are referred to as “warm”. Most indoor lighting

including incandescent blubs and most fluorescent bulbs are considered warm light with temperatures ranging from ~2,700 to 3,350 K and therefore do not contain much blue light.

Therefore, even if we supplement our short wintertime sunlight exposure with most varieties of indoor lighting to match the duration of sunlight exposure from the summer months, we are not experiencing the same amount of 488 nm light as we would be if we were outside in the sunshine. This may seem like too small of a change to cause such a drastic effect; however, research has demonstrated that even a one minute pulse of full spectrum light is enough to shift circadian rhythms and inhibit the production of melatonin[22,118,119]. It has also been demonstrated that full spectrum and blue light therapy is extremely effective in treating SAD[120-124]. In my experiments cool fluorescent light was used with a color temperature of 4,100 K, containing more blue light than most indoor lighting. Therefore my experiments demonstrate that full spectrum light may not be needed to produce photoperiodic effects on the serotonergic system but that "cool" lighting is sufficient.

Though it would seem like a lack of exposure to enough blue light should be the cause of SAD, we have shown here that decreasing the duration of "cool" light from an equinox to short photoperiod does not significantly affect the serotonergic system. However, increasing the duration of light from equinox or short, to a long photoperiod has significant effects on multiple aspects of the serotonergic system. This suggests that it is not the lack of light that is downregulating the serotonergic system but rather that increasing the duration of light during development can upregulate the serotonergic system. While we have shown that this phenomenon is developmental and therefore does not apply directly to SAD, it may influence it indirectly.

I postulate that gene-environment interactions may be involved in the etiology of SAD. The Moffitt group has shown that certain polymorphisms in the *sert* gene can cause increased depressive behavior, but only when combined with stressful life events. Patients with the s/s allele of the promoter of the *Sert* gene do not exhibit an increase in depression over those with

the l/l allele until patients with s/s experience major stressful life events, at which point they show significantly increased depressive behaviors compared to l/l patients that have undergone a similar number of stress events[125,126]. This suggests that an unbalanced serotonergic system makes an organism more susceptible to becoming depressed due to abnormal or stressful life events.

If developmental photoperiod is altering the serotonergic system during adulthood it could unbalance it sufficiently such that the change in duration of light due to the progression of the seasons from summer to fall and fall to winter can alter the 5-HT system enough to cause depression. In fact, in 2004 the Kasper group demonstrated that the incidence of SAD compared to melancholic forms of depression is correlated with season of birth[127]. Therefore season of birth may alter a person's predisposition to developing SAD later in life.

We have also shown that in mice the critical period for the photoperiodic programming of 5-HT neuronal physiology most likely occurs before birth. Due to the vast difference in developmental processes and time course between mice and humans, it is unclear how this critical period may translate to humans. However, if this critical period were to be confirmed in mice and humans it presents the possibility that people born during late winter and early spring, who have undergone most of their prenatal development during the fall and winter months, possess an imbalance in their serotonergic system due to the lack of maternal light exposure which could translate into a higher incidence of SAD later in life. In addition it is possible that people who are born during the late summer and early fall, who have undergone most of their development in the spring and summer months, are protected against the symptoms of SAD due to an upregulation of their serotonergic system cause by an increased duration of light experienced by their mother during development.

With this information in mind I would suggest that an increase in the duration of light during development could upregulate the serotonergic system during adulthood and therefore decrease a person's predisposition for developing any number of psychiatric disorders including

SAD. This hypothesis is based on our results that perinatal photoperiodic programming of serotonergic neuronal physiology persists until 6 months of age in mice. However, since mice and humans have such drastically different life spans and developmental progressions, it is possible that the enduring nature of the photoperiodic programming in humans is different in than in mice.

However, It appears photoperiodic programming by an increase in the duration of light may not be the complete story. While we did not see significant changes in the serotonergic system due to a shortened photoperiod, there are clearly neuropsychiatric effects of developing on a shorten photoperiod. This may be due to changes in other neurotransmitters involved in the control of affective behaviors such as dopamine or GABA. In addition, if this is indeed a melatonin signaling-dependent mechanism, which our results suggest, increased melatonin during development due to a short photoperiod could be acting on a number of other systems that interact with the serotonergic system such as the cortisol or gonadotropic systems without directly influencing the serotonergic system. Therefore, I conclude that both the presence and lack of light play can a role in the photoperiodic programming of some affective behaviors, such as SAD, and that this area of research should be further explored.

4.3: How the Interplay Between the Circadian and Melatonergic Systems Could Cause Photoperiodic Programming of the Serotonergic System

Because light information is transduced in mammals exclusively through the retina, it must travel through specific neural pathways to influence multiple brain areas[128]. Light information from the eye is transmitted down the optic nerve, which splits into two main pathways: the retinohypothalamic tract (RHT) which leads to brain areas located in the hypothalamus, including the SCN, and the optic tract which leads to the visual cortex and is involved in image forming vision[129-131]. Photoperiodic information is sent down the RHT from

ipRGCs containing melanopsin, as well as from retinal ganglion cells receiving signals from rods and cones in the retina. Information from both of these sources can shift the circadian clock in the SCN and therefore produce shifts in the neuronal phase in other brain areas, the timing of peptide release from other brain areas such as melatonin from the pineal, as well as the phase of peripheral clocks outside the central nervous system (CNS). My data suggests that melatonin signaling plays a critical role in the photoperiodic programming of the 5-HT system, and therefore I will be concentrating on how photoperiod is affecting the SCN-Pineal-Raphe circuitry resulting in the programming of the 5-HT system through melatonin signaling.

Melatonin release is tightly controlled by the SCN through a multisynaptic pathway terminating with circadian controlled release of norepinephrine onto pinealocytes in the pineal gland to stimulate the release of melatonin in the absence of light stimulation of the SCN[132-135]. Melatonin is then able to feed back onto SCN cells to inhibit their firing rate and peptide release resulting in the continued release of melatonin from the pineal[102,136-138]. Once light is perceived by the retina, glutamate is release by RGC axons terminating in the SCN, stimulating SCN neuronal firing and peptide release. This stimulation rapidly reduces the amount of melatonin released by the pineal by reducing the noradrenergic tone in the pineal and therefore shutting off the positive feedback loop produced by melatonin's action on the SCN. Through this pathway the duration of light during the day can significantly affect the amount of melatonin release, and therefore signaling, in a 24 hour timeframe, suggesting that during the summer months with an increased light period, there is a significantly shorter period of melatonin release compared to the winter months.

Interestingly, inhibitory melatonin receptors are present on SCN neurons by E17 during development in the rat[139]. Therefore the mother's melatonin profile could be acting on the developing circadian clock to enact enduring changes that may play a role in the photoperiodic programming of the 5-HT system. In fact, our lab has previously shown that developmental photoperiod does alter multiple aspects of the circadian system, including the properties (such

as phase) of SCN neurons[20]. While this phenomenon has not been proven to be dependent on melatonin signaling, it stands to reason that melatonin may play a significant part in photoperiodic programming of the circadian system.

This interaction could therefore be playing a major role in the photoperiodic programming of the 5-HT system, given there are such strong functional reciprocal connections between the SCN and the raphe[26]. While it is not precisely known when the SCN-Raphe circuitry is complete during development, we know 5-HT neurons begin to develop and release 5-HT around E13-E16 and that SCN synaptogenesis begins around E18 and continues well after birth[140-142]. Therefore it is feasible that the developing SCN could be communicating with cells in the raphe as early as E17. It is possible that melatonergic input on the SCN during late development could therefore alter circadian input to the raphe during development, and possibly adulthood, causing changes in baseline of circadian activity of 5-HT neurons in the DRN. Because 5-HT neuronal activity and release is high during the day and low at night[31,143,144], increased activity in raphe neurons is correlated with increased activity in SCN neurons. Therefore, with decreased melatonin signaling in the summer months or long photoperiod, developing SCN neurons would exhibit increased neuronal activity for a larger portion of the day. This activity in the SCN could then increase the activity of developing 5-HT neurons in the raphe, setting their baseline physiology and 5-HT content higher for the rest of their life.

It is important to note that while there are melatonin receptors present on raphe neurons during adulthood[51], the ontogeny of these receptors is not yet known. Therefore, it is also possible that melatonin is acting directly on developing 5-HT neurons, setting their physiological and serotonergic tone higher with decreased melatonin signaling in a long photoperiod and vice versa if developing during the winter months. Until the developmental aspects of the connections between these two systems are fully enumerated we can only speculate about melatonin's action on the SCN (and therefore the raphe), melatonin's action directly on the

raphe, or a combination of the two that causes the observed photoperiodic programming of the serotonergic system.

4.4: Other Ways Melatonin Signaling Could Result in Photoperiodic Programming of the Serotonergic System

In this dissertation I have demonstrated that the photoperiodic programming of the 5-HT system is MT1 receptor dependent and that this programming happens during development. However, I have not addressed the question of whether this is a direct effect of melatonin signaling on 5-HT neurons themselves, or an indirect mechanism working through another neuronal or hormonal pathway. Melatonin receptors are expressed in a significant number of distinct brain regions[145-147] as well as many non-neuronal tissues[145]. Therefore, there is good chance that during development, melatonin is crossing the placenta and photoperiodically programming multiple different tissues that may interact with the serotonergic system.

I have shown in this dissertation that increasing the duration of light during development not only increases the serotonergic tone in the midbrain but also increases that of noradrenaline as well. Norepinephrine (NE) is the principle excitatory input for 5-HT neurons in the DRN[148]. Additionally, the melatonergic and noradrenergic systems interact because of the significant noradrenergic role in the synthesis of melatonin[149,150]. Therefore, there is a possibility that melatonin is altering noradrenergic tone into the DRN during development: with a shorter melatonin profile (via more light) during development, the noradrenergic tone would be increased due to the lack of inhibitory input from melatonin. In turn, an increased noradrenergic tone in the DRN during development could increase the excitability of the developing 5-HT neurons by providing more excitatory input at critical periods of development.

Another system that is intimately linked with melatonin production is the cotricosterone (Cort, cortisol in humans) system. Cort is produced in the adrenal cortex and enters the blood

stream to perform many functions, but can have significant effects as a regulator of stress behaviors[151-153]. There are melatonin receptors present in the zona fasciculata where Cort is produced in the adrenals[154], as well as in the SCN which controls part of the HPA axis governing Cort release[155,156]. Glucocorticoid receptors are present on 5-HT neurons in the DRN and have been shown to critically impact the production of 5-HT and key genes in the 5-HT system[32,157,158]. In fact, in the absence of a Cort rhythm, in part provided by melatonergic signaling, the serotonergic system loses circadian rhythmicity in *Tph2* expression in the DRN[32,101,159]. Therefore, another possibility of an indirect action of photoperiodic melatonin signaling on the 5-HT system would be that melatonin is altering the levels of Cort signaling in the DRN during development and therefore altering the 5-HT system. Cort can act on the 5-HT system through several receptors which, in turn, will cause differential effects on the neurons in the DRN[157,158,160,161]. Cort can also change its role from development to adulthood[162-165]. Therefore, if melatonin is programming the Cort system, which is in turn modulating the 5-HT system, it would be interesting to discover which actions of Cort on the DRN are responsible for this regulation and consequently lead to a better understanding of the photoperiod programming of the 5-HT system.

There are many other mechanisms by which melatonin could be indirectly photoperiodically programming the 5-HT system which I will not detail here. I plan to, and would encourage other researchers to, examine and potentially experiment with this concept. The most effective way to determine if melatonergic signaling is having a direct or indirect role on the photoperiodic programming of the 5-HT system would be to knock out the MT1 gene specifically within the raphe nuclei using a Cre-Lox system with *Pet1* as a Cre driver. If photoperiodic effects are rescued without the presence of MT1 receptors on raphe neurons specifically, it would prove that melatonergic signaling is acting directly on the raphe neruons and not through another indirect mechanism.

4.5: Conclusions and Future Directions

In this dissertation, I have outlined how developmental photoperiod can affect the serotonergic system through melatonin signaling. I have also hinted at a mechanism through changes in the intrinsic electrical properties of 5-HT neurons in the DRN. There is still significant work to do in order to completely understand the mechanism behind how photoperiod is achieving this developmental programming. There are several factors that we have not yet explored that may be part of the mechanism of photoperiod influence on the 5-HT system. These factors include epigenetic programming, the timing of the critical period for the observed photoperiodic programming and addressing some of the circadian variables involved in altering the duration of light experienced. I plan to address at least some of these factors in the near future.

There is a significant unanswered question regarding whether developmental photoperiod is altering the epigenome of key genes within the 5-HT system. In this dissertation I have demonstrated that developmental photoperiod alters the gene expression of several key serotonergic genes. *Pet-1*,*Tph2*, and *Sert* all show decreased gene expression in a long light cycle. I would like to investigate if the changes in expression of these genes may be due to epigenetic changes in their promoters. Using bi-sulfite conversion, I will measure the amount of methylation on the promoters of these genes in mice developed on different photoperiods. I would also like to determine if the entire methylome has changed in the midbrain, and the DRN specifically, which may give us an indication of whether there are widespread epigenetic changes occurring in DRN neurons. These experiments could be preceded by an RNAseq study to observe which genes show a change in expression due to photoperiod and therefore give us a better idea of what genes we may want to investigate epigenetically.

While these are both important questions regarding the photoperiodic programming of the 5-HT system, the largest question that remains partially unanswered is that of critical period.

This is a weighty question because if we are to use the knowledge gleaned from these studies, we must understand the timing involved in this photoperiodic programming. My preliminary results suggest that this critical period occurs before birth, with mice developed prenatally on a long photoperiod and switched at birth to a short photoperiod displaying similar firing rates as mice developed pre- and postnatally on a long photoperiod. I must confirm this result by performing a positive control experiment in which I will maintain mice on a short photoperiod before birth and then switch them to long at birth. If I observe that mice developed on a short photoperiod before birth exhibit firing rates closer to that of mice developed on short until weaning, it would confirm that the critical period for this photoperiodic programming of the 5-HT system does indeed occur before birth.

Changing the light schedules of animals during development alters several factors beyond photoperiod lengthening that may play a role in the photoperiodic programming of the serotonergic system. The first, the absolute light exposure to the retina- that is the total number of photons detected by the retina in a 24 hour period- can have significant effects on photoreceptive cells in the retina. This is difficult issue to ameliorate given our experimental set-up, as food and water in our mouse cages are situated over the mice which move around the cage freely. As a result, each mouse may be detecting differing amounts of photons while moving in and out of the shaded area caused by food and water. To address this, cages would need to be modified so that mice receive absolutely consistent light exposure throughout the light phase. The second confounding variable is the possibility of differential circadian entrainment to dawn and dusk in long and short light cycles- the onset and offset of light change with altered photoperiods. By maintaining the same duration of light (long: 16:8, short: 8:16), but altering the timing of light onset and offset, the effect on the serotonergic system of a change in entrainment could be isolated from that of a change in the duration of light.

These experiments will significantly advance our understanding of how a pervasive environmental signal, the duration of light experienced during development can influence the predisposition to developing depression and anxiety disorders later in life. Determining if epigenetics play a role in the photoperiodic programming of the 5-HT system would illuminate another environmental signal that can alter gene expression in a major neuromodulatory center in the brain. Establishing the critical period and how other circadian variables interact with the photoperiodic programming of the serotonergic system would allow future researchers to move forward with a more complete picture of how they may use the findings in this dissertation to improve human health and wellbeing.

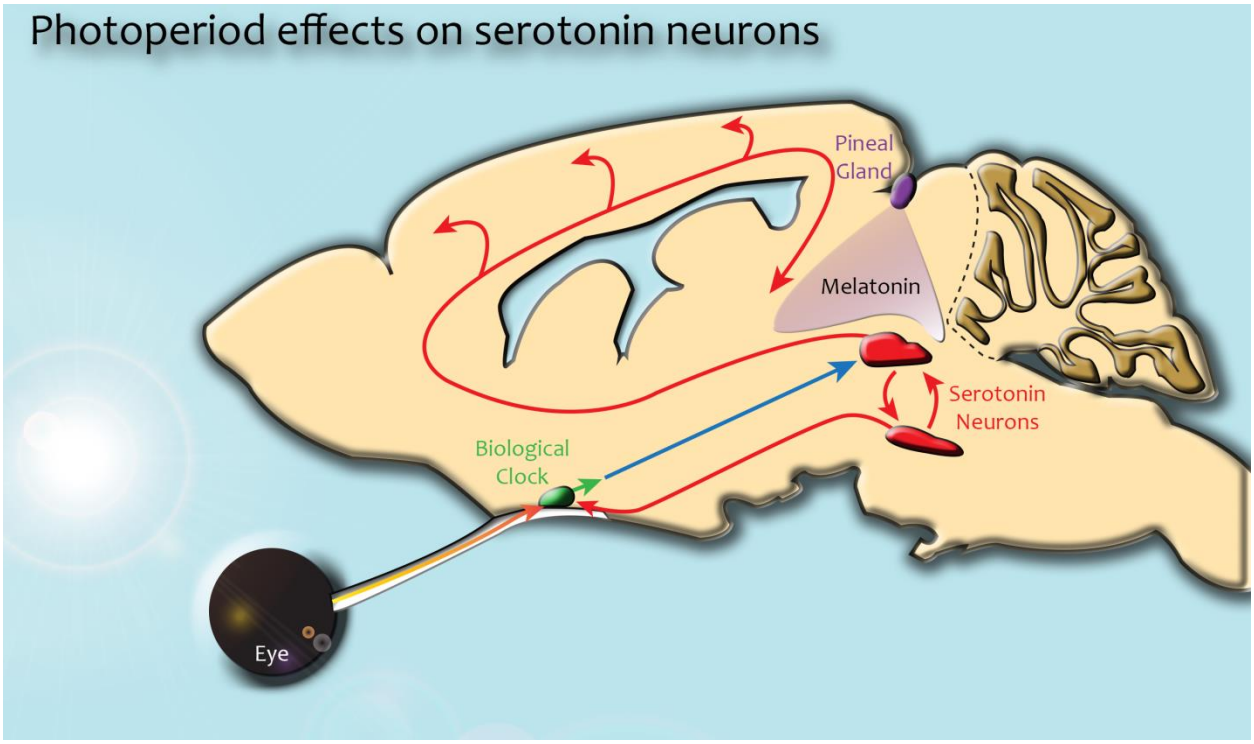


Figure 19. Model of the neural and hormonal pathways that result in the melatonin signaling dependent photoperiodic programming of the serotonergic system.

Appendices

Appendix 1: Impairing retinal function affects 5-HT neuronal physiology

In this dissertation, I have demonstrated that increasing the duration of light during development has lasting effects on multiple aspects of the 5-HT system. It is therefore logical to assume that manipulating the organ that senses this light may cause similar repercussions for the 5-HT system. Accordingly, we decided to examine whether impairing retinal function would have any effect on 5-HT neuronal physiology. Light information is transduced from the retina to the brain by two distinct classes of photoreceptive cells. The first are traditional photoreceptors consisting of rods and cones, which synapse on bipolar cells that either directly or indirectly transmit information to retinal ganglion cells which in turn carry that information to the brain. The second class of photoreceptive cells are melanopsin-containing, intrinsically photoreceptive ganglion cells (ipRGCs) which transmit light information directly to the brain, mainly to the SCN. There is evidence in some rodents of a direct retinal-raphe connection but not in others[46]. Even lacking a direct connection, there is an established polysynaptic pathway from the retina to the raphe nuclei through the SCN[26]. Therefore the signals from the retina may be acting on neurons in the raphe nuclei through a direct or indirect mechanism.

In order to determine if a lack of rods and cones could affect 5-HT neuronal physiology, we used aged C3H mice, which contain a retinal degeneration mutation causing their photoreceptor layer (composed of rods and cones) to completely degenerate by 3 months of age (Fig. 3). We measured the firing rate of 5-HT neurons in the DRN of C3H mice that were at least 6 months old, ensuring that their rods and cones were no longer functional. We compared this to the firing rate of 5-HT neurons in the DRN of age-matched C3Hf$^{+/+}$ which lack the retinal

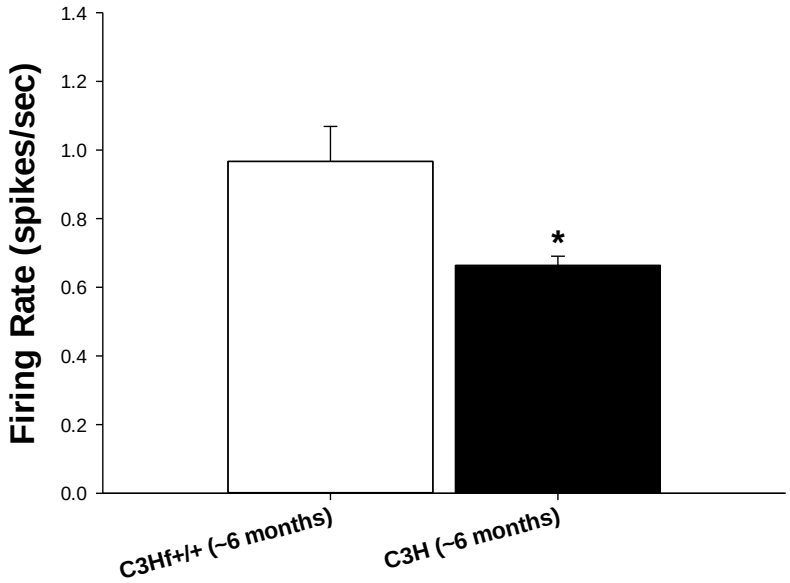


Figure A1. The lack of rods and cones significantly decreases the firing rate of 5-HT neurons in the DRN. C3Hf$^{+/+}$: 3 mice, 33 cells, Firing Rate= 0.97 ± 0.1. C3H: 5 mice, 40 cells, Firing Rate= 0.66 ± 0.03. *p*= 0.011.

degeneration mutation (Fig. 3). We observed that mice who lacked rods and cones demonstrated a significant decrease in 5-HT neuronal firing rate compared to those with functional retinas ($C3Hf^{+/+}$: 0.97 ± 0.1, C3H: 0.66 ± 0.03 spikes/sec, p= 0.01) (Fig. A1).

In order to determine if impaired melanopsin signaling could alter 5-HT neuronal physiology we obtained $Opn4^{-/-}$ mice on a C57-Bl/6 background from Jackson Labs which lack the protein melanopsin while leaving retinal ganglion cells intact. We compared these to age-matched C57 WT mice obtained from Jackson Labs. We measured the 5-HT neuronal firing rate of each of these genotypes and observed that the lack of melanopsin signaling significantly decreases 5-HT neuronal firing rate in the DRN (WT: = 0.77 ± 0.07, $Opn4^{-/-}$: 0.44 ± 0.04, spikes/sec, p= 0.001) (Fig. A2).

These results taken together suggest that impairing either the function of rods and cones or ipRGCs can significantly affect serotonergic neuronal physiology. That impairing retinal function decreases the firing rate of 5-HT neurons in the DRN, and therefore most likely decreases the release of 5-HT from these neurons, is concordant with reports of higher incidences of depression in people with impaired eyesight[166]. Therefore with this data we have provided the first step in linking retinal function and depression and anxiety disorders.

Methods

Animals and Housing

All genotypes of mice were maintained on a 12/12 light/dark cycle and provided with normal chow and water *ad libitum*. C3H and $C3Hf^{+/+}$ mice were tested between the ages of 5 and 7 months old. C57 and $Opn4^{-/-}$ mice were tested between 50 and 90 days old. All genotypes were sacrificed between the hours of 1100 and 1300. Experiments were performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

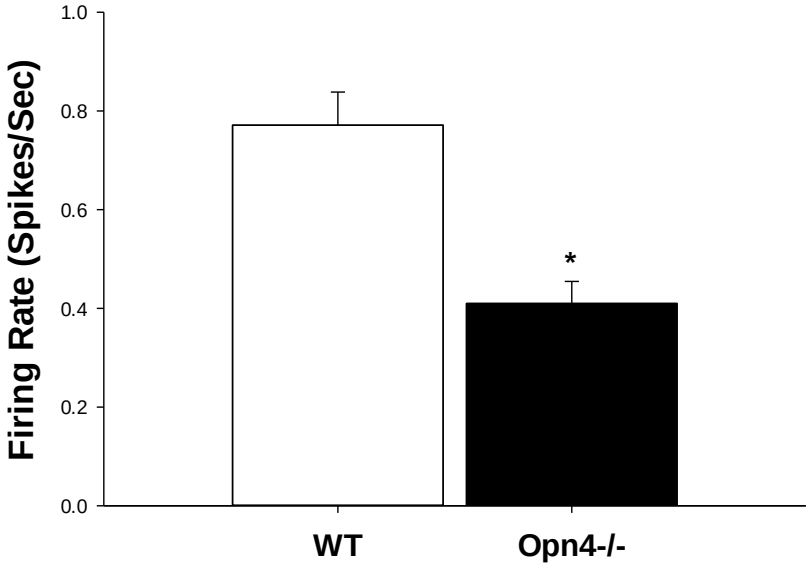


Figure A2. The lack of melanopsin signaling in the retina causes a decrease in the firing rate or 5-HT neurons in the DRN. WT: 7 mice, 28 cells, Firing Rate= 0.77 ± 0.07. *Opn4*$^{-/-}$: 6 mice, 47 cells, Firing Rate= 0.44 ± 0.04. *p*= 0.001.

***Ex vivo* culture**

Mice were euthanized by cervical dislocation. Brains were extracted and mounted in cold, oxygenated (95%O_2-5%CO_2) dissecting media (in mM: 114.5 NaCl, 3.5 KCL, 1 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 D(+)-glucose, and 35.7 NaCHO3), and 235 µm thick coronal slices were taken using a Vibroslicer (Campden Instruments). Dissecting media was frozen and chunks of dissecting media ice were added to the bath to maintain the low temperature of the bath while slicing. The dorsal raphe nuclei were isolated by removing the extraneous cortical tissue and placed in a slice chamber full of room temperature, oxygenated, extracellular recording media (in mM: 124 NaCl, 3.5 KCl, 1 NaH2PO4, 1.3MgSO4, 2.5 CaCl2,10 D(+)-glucose, and 20 NaHCO3).

Multielectrode Array Electrophysiological Recording

Dorsal raphe nucleus slices were placed on perforated electrode arrays and immobilized with a harp for recording. 40 µM tryptophan and 3 µM phenylephrine were added to the recording solution, which was perfused (1.3 mL/min) over the slice in the recording chamber. Serotonin neurons were identified by eliciting 5HT1a-mediated suppression of spontaneous firing rate. Serotonin at a concentration of 40 µM (for all general firing rate measurements) or 8-OH-DPAT at a concentration of 1 µM (for dose response experiments) was perfused (1.3 mL/min) over the slice for 5 minutes after 4 to 6 minutes of recording. After 5 minutes of serotonin or 8-OH-DPAT, normal ACSF was started again and recording continued until recovery was observed.
As there are differences in the electrophysiological properties of neurons in the medial and lateral wing subfields of the DR, placement on the array and the dimensions of the electrode grid were used to ensure that only the ventromedial DR neurons (vmDR) are recorded from. Mid-DRN slices of 280 micron thickness are taken between -4.5 mm and -4.75 mm caudal to bregma. We use a 6X10 perforated array with electrodes that have a diameter of 30 microns and 100 micron spacing between electrodes. The slice is placed so the electrodes cover an area spreading 1200 microns down from the cerebral aqueduct in the ventral direction and 340

microns laterally on either side of the midline (for a 680 micron total recording width). Putative 5HT neurons were identified by feedback inhibition evoked by application of 5-HT (40μM, for general firing rate experiments) or 8-OH-DPAT(1 μM, for dose response experiments) for 5 min, with those cells demonstrating a minimum of 50% spike rate suppression being included in the analysis, as we have done in previously published studies [79,80]

Although there is overlap in the electrophysiological characteristics of dorsal raphe serotonergic and non-serotonergic neurons [81,82], these selection criteria, as well as the placement of the array to record from the ventromedial DR region, assured that a high proportion of cells in our recordings were serotonergic. The vmDR is highly enriched in serotonergic neurons. In the rat this has been carefully quantified with 75-95% of the neurons containing immunoreactivity to 5-HT, and 70-95% of neurons that express the 5-HT_{1A} receptor also expressed 5-HT [82]. In addition, spike duration is significantly broader in 5-HT vs non-5-HT neurons in the dorsal raphe and the 5-HT_{1A} mediated response is significantly greater in 5-HT neurons [81]. The mouse DRN is thought to be similarly highly enriched for serotonergic neurons in the vmDR region, and 84% of genetically identified 5-HT neurons respond with 5-HT_{1A} inhibition[83]. In our dose-response experiments, 156 out of 198 total cells decreased their firing rate by at least 50% upon application of 8-OH-DPAT, indicating that ~80% of the neurons from which we recorded express functional 5-HT_{1A} receptors and therefore are likely serotonergic. Thus, while we cannot be sure that each and every neuron we putatively identified as serotonergic indeed expressed serotonin, the combination of our anatomical recording site and physiological criteria assures that the overwhelming majority of neurons in our study were serotonergic.

Data files were saved as .mcd files and analyzed in offline sorter. For analysis, a Besel filter with a 150 Hz frequency cutoff was applied to the raw data traces. The threshold for detection was set manually to a level that will include all legitimate spikes with the least amount of unipolar noise spikes (between 13 μV and 35 μV). Once spikes have been detected they

were sorted by a combination of a K means scan method and manual verification. The manual verification was conducted after the K means scan was run and divided spikes into groups based on criterion such as amplitude, power under the curve, and spike duration (for full list of criterion see offline sorter V3 manual under the K means scan, Plexon Inc.). Once waveforms were sorted into groups and judged to be biologically relevant each spike was validated by eye and spikes that did not fit the average waveform shape were invalidated. Also all unsorted spikes were visualized manually and any spikes that matched the average waveform shape in the relevant group were added to that group. Once the total number of spikes was determined for the period of the recording before the application of 5-HT or 8-OH-DPAT that number was divided by the total time in which those spikes occurred to produce a measure of spikes per second.

Data Analysis

Spike traces from multielectrode arrays were analyzed using offline sorting (Plexon) and spikes were sorted using a combination of manual identification and automatic K means based sorting software. All cells within each mouse were then averaged, and those values were used for ordinary One-Way ANOVA. Holm-Sidak's Multiple Comparisons test was used post-hoc for One-Way ANOVA. All statistical analyses were performed with $\alpha = 0.05$.

Acknowledgements

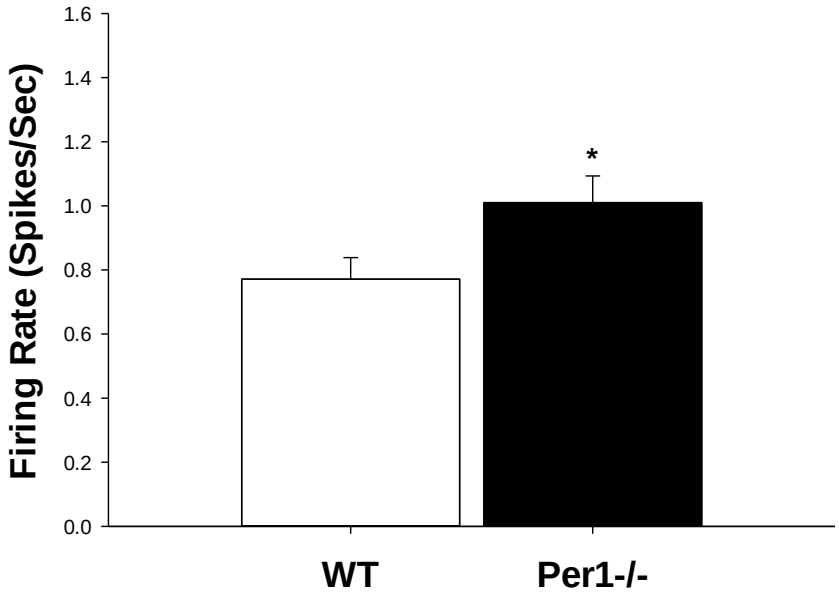
This work was supported by NIH R01 EY015815 (DGM), P50 MH096972 (Randy D. Blakely) and Vanderbilt Conte Pilot Grant (CRJ). The mice and inspiration for this work were provided by Dr. Chad Jackson. I would also like to thank the Vanderbilt Silvio S. Conte Center for all their help and expertise.

Conflicts of Interests: None of the Authors have conflicts of interest pertaining to this work.

Appendix 2: Lack of the Core Circadian Clock Gene: Per1 Affects Serotonergic Neuronal Physiology in the DRN

Most mammalian tissues contain an oscillator known as the molecular circadian clock. This clock is composed of four main proteins that form a transcription/translation negative feedback loop. The positive element of this feedback loop is composed of a heterodimer of CLOCK and BMAL1 proteins. These proteins act on the E-box of numerous genes to promote transcription including those genes involved in the negative arm of this feedback loop. The negative arm of this loop is composed of the period genes, *Period 1 & 2* (*Per1, 2*), and the cryptochrome genes, *Cryptochrome 1 & 2* (*Cry1, 2*). These proteins accumulate in the cytoplasm, dimerize and re-enter the nucleus, where they inhibit the binding of CLOCK and BMAL1 to E-boxes, resulting in decreased expression of genes positively regulated by the CLOCK/BMAL1 heterodimer[167]. PER1 can dimerize with either PER2 or CRY1 to perform its function of inhibiting the CLOCK/BMAL1 complex. Knocking out *Per1* does not cause arrhythmicity because PER2 can compensate for the lack of PER1. The lack of PER1 also does not cause a change in behavioral period[168]. However, our lab has demonstrated that PER1 may be involved in the link between the molecular circadian clock and circadian neuronal firing rate[169]. Therefore we sought to determine if the lack of PER1 can affect the physiology of 5-HT neurons in the DRN.

Using mulitelectrode array recordings we measured the firing rate of 5-HT neurons in the DRN of *Per1* knockout mice on a C57Bl/6 background and WT C57Bl/6 mice both obtained from Jackson labs. We observed that 5-HT neurons from *Per1* knockout mice had a significantly increased firing rate compared to WT mice ($Per1^{-/-}$: 1.01 $\pm$ 0.08, WT: 0.77 $\pm$ 0.07 spikes/sec, p=0.045, Fig A3). This suggests that PER1 does play a role in the neurophysiology of 5-HT



<u>Figure A3</u>. The lack of *Per1* increases the firing rate of 5-HT neurons in the DRN. WT: 7 mice, 28 cells, *Per1*$^{-/-}$: 7 mice, 64 cells, *p*= 0.045.

neurons in the DRN and may play a role in the neurophysiology in other brain areas outside of the SCN and the DRN. This result provides further evidence for the idea that PER1 may be playing an integral role in the interplay between the molecular genetics, specifically the molecular circadian clock, and the physiology of neurons in multiple areas of the brain.

Methods

Animals and Housing

All genotypes of mice were maintained on a 12/12 light/dark cycle and provided with normal chow and water *ad libitum*. C57 and *Pet1*$^{-/-}$ mice were tested between 50 and 90 days old. Both genotypes were sacrificed between the hours of 1100 and 1300. Experiments were performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Ex vivo culture

Mice were euthanized by cervical dislocation. Brains were extracted, mounted in cold, oxygenated (95%O_2-5%CO_2) dissecting media (in mM: 114.5 NaCl, 3.5 KCL, 1 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 D(+)-glucose, and 35.7 NaCHO3), and 235 µm thick coronal slices were taken using a Vibroslicer (Campden Instruments). Dissecting media was frozen and chunks of dissecting media ice were added to the bath to maintain the low temperature of the bath while slicing. The dorsal raphe nuclei were isolated by removing the extraneous cortical tissue and placed in a slice chamber full of room temperature, oxygenated, extracellular recording media (in mM: 124 NaCl, 3.5 KCl, 1 NaH2PO4, 1.3MgSO4, 2.5 CaCl2,10 D(+)-glucose, and 20 NaHCO3).

Multielectrode Array Electrophysiological Recording

Dorsal raphe nucleus slices were placed on perforated electrode arrays and immobilized with a harp for recording. 40 µM tryptophan and 3 µM phenylephrine were added to the recording

solution which was perfused (1.3 mL/min) over the slice in the recording chamber. Serotonin neurons were identified by eliciting 5HT1a-mediated suppression of spontaneous firing rate. Serotonin at a concentration of 40 µM (for all general firing rate measurements) or 8-OH-DPAT at a concentration of 1 µM (for dose response experiments) was perfused (1.3 mL/min) over the slice for 5 minutes after 4 to 6 minutes of recording. After 5 minutes of serotonin or 8-OH-DPAT, normal ACSF was started again and recording continued until recovery was observed.

As there are differences in the electrophysiological properties of neurons in the medial and lateral wing subfields of the DR, placement on the array and the dimensions of the electrode grid were used to ensure that only the ventromedial DR neurons (vmDR) are recorded from. Mid-DRN slices of 280 micron thickness are taken between -4.5 mm and -4.75 mm back from bregma. We use a 6X10 perforated array with electrodes that have a diameter of 30 microns and 100 microns spacing between electrodes. The slice is placed so the electrodes cover an area spreading 1200 microns down from the cerebral aqueduct in the ventral direction and 340 microns laterally on either side of the midline (for a 680 micron total recording width). Putative 5HT neurons were identified by feedback inhibition evoked by application of 5-HT (40µM) (for general firing rate experiments) or 8-OH-DPAT (for dose response experiments) for 5 min, with those cells demonstrating a minimum of 50% spike rate suppression being included in the analysis, as we have done in previously published studies [79,80]

Although there is overlap in the electrophysiological characteristics of dorsal raphe serotonergic and non-serotonergic neurons [81,82], these selection characteristics, as well as the placement of the array to record from the ventromedial DR region, assured that a high proportion of cells in our recordings were serotonergic. The vmDR is highly enriched in serotonergic neurons. In the rat this has been carefully quantified with 75-95% of the neurons containing immunoreactivity to 5-HT, and 70-95% of neurons expressing the 5-HT_{1A} receptor also expressing 5-HT [82]. In addition, spike duration is significantly broader in 5-HT vs non-5-HT neurons in the dorsal raphe and the 5-HT_{1A} mediated response is significantly greater in 5-HT neurons [81]. The mouse DRN

is thought to be similarly highly enriched for serotonergic neurons in the vmDR region, and 84% of genetically identified 5-HT neurons respond with 5-HT_{1A} inhibition [83]. In our dose-response experiments 156 out of 198 total cells decreased their firing rate by at least 50% upon application of 8-OH-DPAT, indicating that ~80% of the neurons recorded from express functional 5-HT_{1A} receptors and therefore are likely serotonergic. Thus, while we cannot be sure that each and every neuron we putatively identified as serotonergic indeed expressed serotonin, the combination of our anatomical recording site and physiological criteria assures that the overwhelming majority of neurons in our study were serotonergic.

Data files were saved as .mcd files and analyzed in offline sorter. For analysis a Besel filter with a 150 Hz frequency cut off was applied to the raw data traces. The threshold for detection was set manually to a level that will include all legitimate spikes with the least amount of unipolar noise spikes included (between 13 µV and 35 µV). Once spikes have been detected they were sorted by a combination of a K means scan method and manual verification. The manual verification was conducted after the K means scan was run and divided spikes into groups based on criterion such as amplitude, power under the curve and spike duration (for full list of criterion see offline sorter V3 manual under the K means scan, Plexon Inc.). Once waveforms were sorted into groups and judged to be biologically relevant each spike was validated by eye and spikes that did not fit the average waveform shape were invalidated. Also all unsorted spikes were visualized manually and any spikes that matched the average waveform shape in the relevant group were added to that group. Once the total number of spikes was determined for the period of the recording before the application of 5-HT or 8-OH-DPAT that number was divided by the total time in which those spikes occurred to produce a measure of spikes per second.

Data Analysis

Spike traces from multielectrode arrays were analyzed using offline sorting (Plexon) and spikes were sorted using a combination of manual identification and automatic K means based sorting

software. All cells within each mouse were then averaged, and those values were used for ordinary One-Way ANOVA. Holm-Sidak's Multiple Comparisons test was used post-hoc for One-Way ANOVA. All statistical analyses were performed with $\alpha = 0.05$ and a p value of < 0.05 was considered significant.

Appendix 3: Lack of Neuroligin 2 Affects the Physiology of Serotonergic Neurons in the DRN

Neuroligin 2 is a type 1 integral membrane protein that acts as a cell adhesion protein in the CNS. It is a postsynaptic protein that helps facilitate the creation and maintenance of synaptic connections[170,171]. Neuroligin 2 is concentrated on inhibitory synapses while neuroligin 1 is concentrated on exctitory synapses in the CNS[172]. Neuroligins have been demonstrated to have a link to affective behaviors and autism through the synaptic maintenance function. There are several genetic polymorphism in the neuroligin 3 and 4 genes that have been correlated with autism[173]. However, neuroligin 2 is more associated with affective disorders and aggression than autism spectrum[174-176]. Therefore, we decided to investigate if the lack of neuroligin 2 has an effect on 5-HT neuronal physiology in the DRN.

Using mulitelectrode array recordings we measured the firing rate of 5-HT neurons in the DRN of neuroligin 2 knockout mice on a C57 Bl/6 mouse background and WT C57 Bl/6 littermates both obtained from Dr. Randy Blakley's laboratory. Knocking out neuroligin 2 significantly decreased the firing rate of 5-HT neurons in the DRN compared to WT littermates (WT: 1.01 $\pm$ 0.11, neuroligin 2 KO: 0.73 $\pm$ 0.09 spikes/sec, p=0.038) (Fig. A4). This suggests that the lack of neuroligin 2 plays a role in the maintenance of 5-HT neuronal physiology in the DRN. This decrease in firing rate could result from a decrease in synaptic formation or maintenance between excitatory noradrenergic axonal terminals and 5-HT neurons in the DRN during development. Since these recordings were performed in deafferented slices and therefore are being driven by external phenylephrine in the bath rather than intrinsic noradrenergic stimulation from other brain areas. As a result the changes we see are due to the intrinsic properties of the neurons rather than synaptic input which has been largely removed (discounting the effects of small interneurons within the slice). Therefore, this decrease in

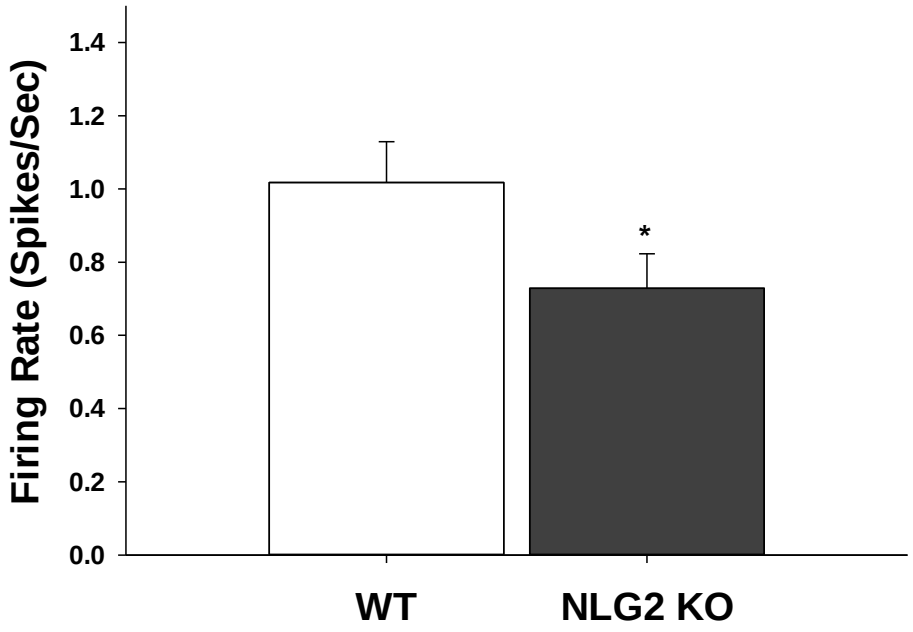


Figure A4. The lack of neuroligin 2 decreases the firing rate of 5-HT neurons in the DRN. WT: 6 mice, 23 cells, neuroligin 2 KO (NLG2 KO): 6 mice, 23 cells, p= 0.038.

neuronal firing rate is most likely due to developmental effects of the lack of neuroligin 2 instead of proximal effects that would largely negated in deafferented slices.

Methods

Animals and Housing

All genotypes of mice were maintained on a 12/12 light cycle and provided with normal chow and water ad libitum. C57 and *NLG2* KO mice were tested between 50 and 90 days old. Both genotypes were killed between the hours of 1100 and 1300. Experiments were performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Ex vivo culture

Mice were euthanized by cervical dislocation, brains were extracted and mounted in cold, oxygenated (95%O_2-5%CO_2) dissecting media (in mM: 114.5 NaCl, 3.5 KCL, 1 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 D(+)-glucose, and 35.7 NaCHO3), and 235 µm thick coronal slices were taken using a Vibroslicer (Campden Instruments). Dissecting media was frozen and chunks of dissecting media ice were added to the bath to maintain the low temperature of the bath while slicing. The dorsal raphe nuclei were isolated by removing the extraneous cortical tissue and placed sample in a slice chamber full of room temperature, oxygenated, extracellular recording media (in mM: 124 NaCl, 3.5 KCl, 1 NaH2PO4, 1.3MgSO4, 2.5 CaCl2,10 D(+)-glucose, and 20 NaHCO3).

Multielectrode Array Electrophysiological Recording

Dorsal raphe nucleus slices were placed on a perforated electrode arrays and immobilized with a harp for recording. 40 µM tryptophan and 3 µM phenylephrine were added to the recording solution which was perfused (1.3 mL/min) over the slice once in the recording chamber. Serotonin neurons were identified by eliciting 5HT1a-mediated suppression of spontaneous firing rate. Serotonin at a concentration of 40 µM (for all general firing rate measurements) or 8-OH-DPAT at a concentration of 1 µM (for dose response experiments) was perfused (1.3

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application of 8-OH-DPAT, indicating that ~80% of the neurons recorded from express functional 5-HT_{1A} receptors and therefore are likely serotonergic. Thus, while we cannot be sure that each and every neuron we putatively identified as serotonergic indeed expressed serotonin, the combination of our anatomical recording site and physiological criteria assures that the overwhelming majority of neurons in our study were serotonergic.

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Data Analysis

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Acknowledgements

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Conflicts of Interests: None of the Authors have conflicts of interest pertaining to this work.

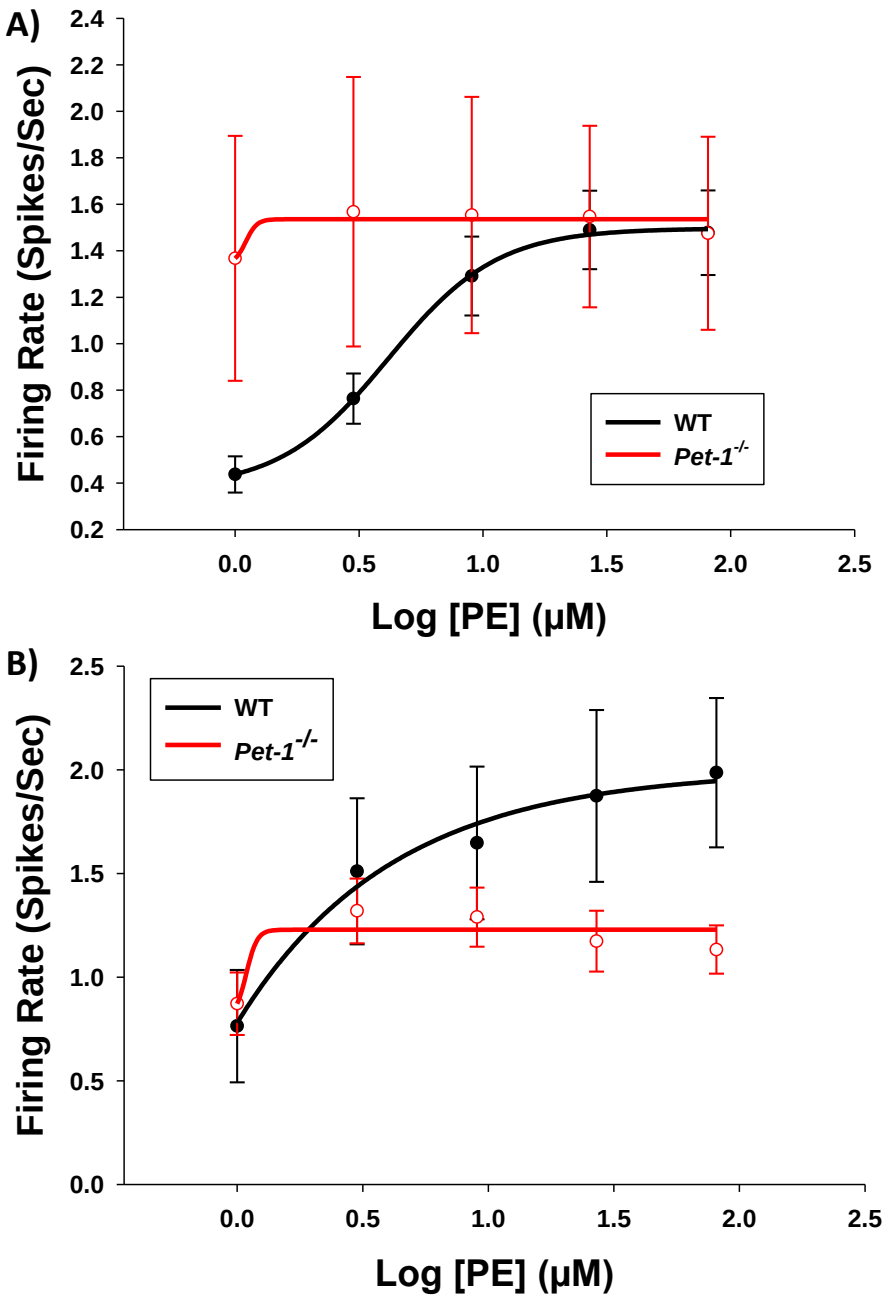
Appendix 4: Effects of the loss of *Pet-1* on 5-HT Neuronal Sensitivity to Pharmacological Stimulation of the $ADRA_{1b}$ and LPAR1 receptors

Pet-1 is an ETS transcription factor that is responsible for the induction during development, and maintenance during adulthood, of many, if not all, key serotonergic genes[107]. ETS domain transcription factors play a significant role in the specification of multiple different types of hematopoietic cells[177]. Deneris and colleagues have demonstrated that *Pet-1* expression is present in 5-HT cells in the developing brain slightly before 5-HT immunoreactivity can be detected, with *Pet-1* expression initiating around E12.75 in rats and E11 in mice and 5-HT around E13 in most rodents. They have also observed that *Tph2*, *Sert*, and *Adra1b* expression, as well as 5-HT immunoreactivity, are virtually nonexistent in the raphe during adulthood in the absence of *Pet-1*, suggesting that the presence of *Pet-1* is necessary for the initial and continual expression of key serotonergic genes and the production of 5-HT[73]. In addition, they showed that in the absence of *Pet-1*, neurons in the DRN exhibit changes in their physiological properties. The physiology of these neurons appears to be stalled in an early developmental stage with increased excitability and a higher firing rate than neurons containing *Pet-1*[73].

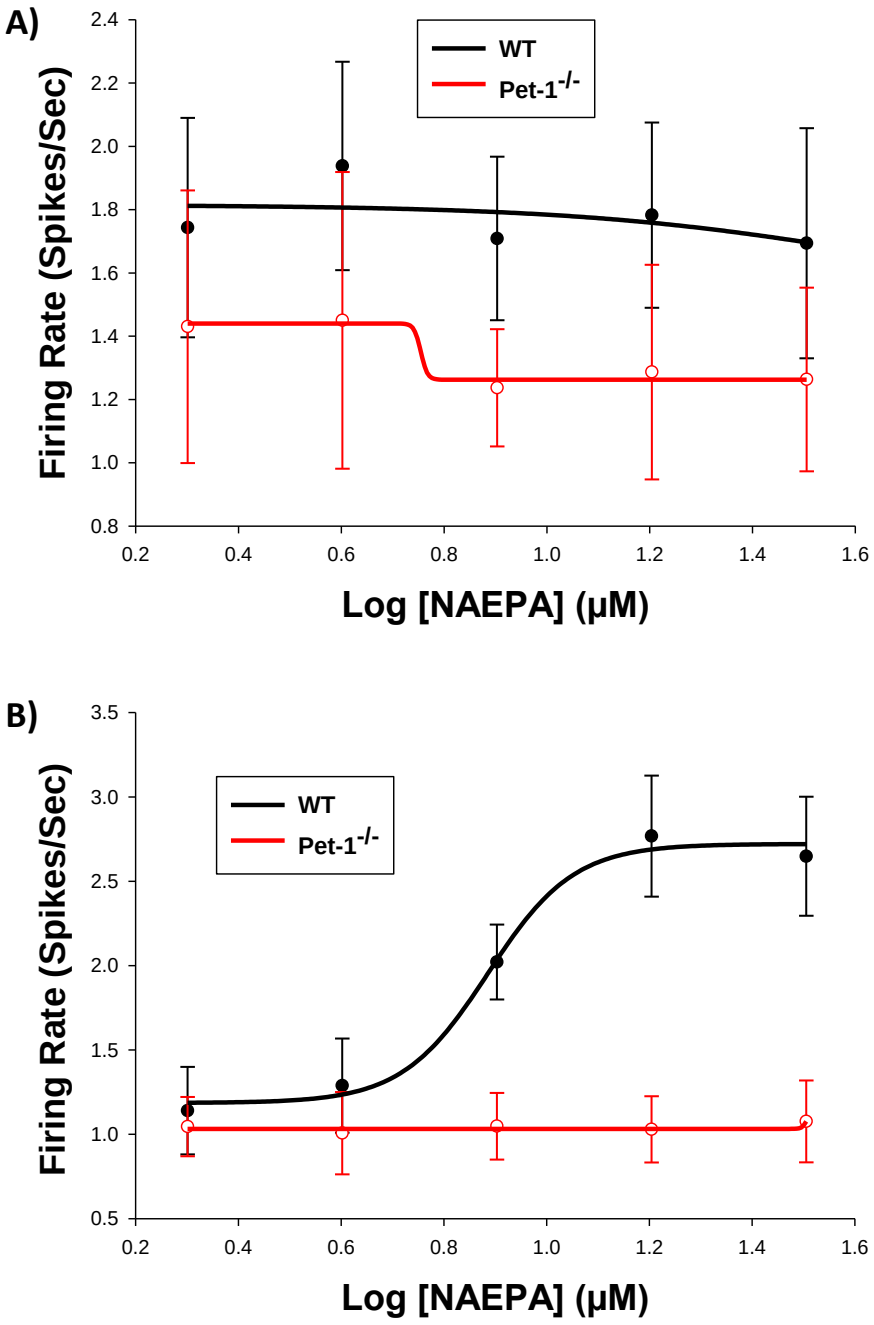
Finally, Deneris and colleagues have conducted RNAseq and identified a list of genes that are significantly altered in the absence of *Pet-1* (unpublished data). Two of these genes, *$Adra_{1b}$* and *Lpar1*, are of great interest. $ADRA_{1b}$ receptors are in the G_q coupled receptor family and are the main excitatory drive for 5-HT neurons in the raphe nuclei[178,179]. LPAR1 is a G_q coupled lysophosphatidic acid receptor that has been shown to modulate a variety of neuronal types but is heavily expressed in the DRN. We therefore decided to determine if the lack of expression of these two genes translated into a disruption of their function on 5-HT neurons in the DRN in the absence of *Pet-1*.

We used multielectrode array measurements to create a dose response curve to phenylephrine (PE), a selective $ADRA_{1b}$ receptor agonist, in mice lacking *Pet-1* and their WT littermates to determine if the absence of *Pet-1* disrupts $ADRA_{1b}$ function in the DRN . We were able to classify 5-HT neurons into two groups based on their waveform width and the coefficient of variance (COV) of their firing rate. One population had a large waveform and small COV and the other had a smaller waveform and a larger COV(see methods for details on the classification criterion). In WT animals, both populations of 5-HT cells display a characteristic excitatory dose response curve to PE, with firing rate increasing as the dose of PE increases (Large/Tonic:1μM- 0.44 $\pm$ 0.08, 3μM- 0.76 $\pm$ 0.11, 9μM- 1.29 $\pm$ 0.17, 27μM- 1.49 $\pm$ 0.17, 81μM- 1.48 $\pm$ 0.18, Small/Variable: 1μM- 0.76 $\pm$ 0.27, 3μM- 1.51 $\pm$ 0.35, 9μM- 1.65 $\pm$ 0.37, 27μM- 1.87 $\pm$ 0.41, 81μM- 1.99 $\pm$ 0.36, Fig. A5 A-B black lines). In mutant *Pet-1*$^{-/-}$ animals, however, both populations of 5-HT cells lose this dose response relationship with PE, with firing rates remaining constant across most doses of PE (Large/Tonic:1μM- 1.37 $\pm$ 0.53, 3μM- 1.57 $\pm$ 0.57, 9μM- 1.55 $\pm$ 0.51, 27μM- 1.55 $\pm$ 0.39, 81μM- 1.48 $\pm$ 0.42, Small/Variable: 1μM- 0.87 $\pm$ 0.15, 3μM- 1.32 $\pm$ 0.16, 9μM- 1.29 $\pm$ 0.14, 27μM- 1.17 $\pm$ 0.15, 81μM- 1.13 $\pm$ 0.12), Fig. A5 A-B red lines). This suggests that mutant 5-HT neurons no longer maintain their typical excitatory response to pharmacological stimulation of the $ADRA_{1b}$ receptor.

In addition, we investigated whether mutant 5-HT neurons lacked functional lysophosphatidic acid receptor 1 (LPAR1) responses using multielectrode array recordings. In WT animals, 5-HT cells (classified by their small waveform and high COV) display an excitatory dose response curve to (Z)-N-[2-(Phosphonooxy)ethyl]-9-octadecenamide (NAEPA), a selective LPAR1 receptor agonist, with firing rate increasing as the dose of NAEPA increases (Small/Variable: 2μM- 1.14 $\pm$ 0.26, 4μM- 1.29 $\pm$ 0.29, 8μM- 2.02 $\pm$ 0.22, 16μM- 2.77 $\pm$ 0.36, 32μM- 2.65 $\pm$ 0.35, Fig. A6 A black line). However, spikes classified by their large waveform and low COV did not exhibit this dose response (Large/Tonic: 2μM- 1.74 $\pm$ 0.35, 4μM- 1.94 $\pm$ 0.33, 8μM- 1.71 $\pm$ 0.26, 16μM- 1.78 $\pm$ 0.29, 32μM- 1.69 $\pm$ 0.36, Fig. A6 B black line). In mutant *Pet-1*$^{-/-}$



<u>Figure A5</u>. DRC to PE in 5-HT cells in the DRN of WT and *Pet-1*$^{-/-}$ mice. (**A**) In cells that displayed a large waveform and tonic firing pattern, WT mice display a characteristic excitatory dose response curve to PE while *Pet-1*$^{-/-}$ show no response to increasing doses of PE. (**B**) In cells that displayed a small waveform and variable firing pattern, WT mice display a characteristic excitatory dose response curve to PE while *Pet-1*$^{-/-}$ show no response to increasing doses of PE.



<u>Figure A6</u>. DRC to NAEPA in 5-HT cells in the DRN of WT and *Pet-1*$^{-/-}$ mice. (**A**) In cells that displayed a large waveform and tonic firing pattern, Both WT and *Pet-1*$^{-/-}$ mice do not display any response to increasing doses of NAEPA (**B**) In cells that displayed a small waveform and variable iring pattern, WT mice display a characteristic excitatory dose response curve to NAEPA while *Pet-1*$^{-/-}$ mice show no response to increasing doses of NAEPA.

animals, both populations of 5-HT cells do not exhibit the dose response relationship with NAEPA seen in the small WF /variable COV population of 5-HT cells of WT animals, with mutant neuronal firing rates remaining constant across all doses of NAEPA (Large/Tonic: 2μM- 1.43 ± 0.43, 4μM- 1.45 ± 0.47, 8μM- 1.24 ± 0.19, 16μM- 1.29 ± 0.34, 32μM- 1.26 ± 0.29, Small/Variable: 2μM- 1.05 ± 0.18, 4μM- 1.01 ± 0.24, 8μM- 1.05 ± 0.20, 16μM- 1.03 ± 0.20, 32μM- 1.08 ± 0.24, Fig. A6 A-B red lines). This result suggests that mutant 5-HT neurons with small waveform widths and large COVs no longer maintain their excitatory response to pharmacological stimulation of LPAR1.

Taken together, these results suggest that the $Adra_{1b}$ and *Lpar1* genes are expressed in the DRN at a much lower level in $Pet1^{-/-}$ mice than in WT. This level of expression does not appear to be sufficient to produce enough functional receptors to respond to *ex vivo* pharmacological stimulation, suggesting that *Pet-1*expression throughout life is critical for the function of both of these receptors.

Methods

Animals and Housing

All genotypes of mice were maintained on a 12/12 light cycle and provided with normal chow and water ad libitum. *Pet-1* KO mice and C57 littermates were generously provided by Dr. Evan Deneris at Case Western University. These animals were tested between 50 and 90 days old. Both genotypes were killed between the hours of 1100 and 1300. Experiments were performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Ex vivo culture

Mice were euthanized by cervical dislocation, brains were extracted and mounted in cold, oxygenated (95%O_2-5%CO_2) dissecting media (in mM: 114.5 NaCl, 3.5 KCL, 1 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 D(+)-glucose, and 35.7 NaCHO3), and 235 µm thick coronal slices were taken using a Vibroslicer (Campden Instruments). Dissecting media was frozen and chunks of dissecting media ice were added to the bath to maintain the low temperature of the bath while slicing. The dorsal raphe nuclei were isolated by removing the extraneous cortical tissue and placed sample in a slice chamber full of room temperature, oxygenated, extracellular recording media (in mM: 124 NaCl, 3.5 KCl, 1 NaH2PO4, 1.3MgSO4, 2.5 CaCl2,10 D(+)-glucose, and 20 NaHCO3).

Dose-Response Experiments

Dose Response Curve to PE- Dorsal raphe nucleus slices were placed on a perforated electrode arrays and immobilized with a harp for recording. No PE and 40 µM tryptophan was added to the ACSF perfused (1.3 mL/min) over the slice while settling. The first dose of PE (333 nM) was perfused over the slice for at least 3 minutes. The slice was then switched back to ACSF without PE until the firing rate silenced once more before perfusing subsequent doses of PE. This procedure was repeated for each subsequent dose.

Dose Response Curve to NAEPA- DRN slices were prepared and placed on the multielectrode array as previously described. ACSF containing 40 µM tryptophan and 3 µM PE was perfused over the slice for initial firing rate. The lowest dose of NAEPA (2 µM) was perfused over the slice after at least 4 minutes of recording the firing rate. Each subsequent does was added in a stepwise fashion and recordings were made after at least 2 minutes exposure to the desired dose.

Electrophysiological Recording and 5-HT Neuron Identification

As there are differences in the electrophysiological properties of neurons in the medial and lateral wing subfields of the DR, placement on the array and the dimensions of the electrode grid were used to ensure that only the ventromedial DR neurons (vmDR) were recorded from.

Mid-DRN slices of 280 micron thickness were taken between -4.5 mm and -4.75 mm back from bregma. We used a 6X10 perforated array with electrodes that have a diameter of 30 microns and 100 microns spacing between electrodes. The slice was placed so the electrodes cover an area spreading 1200 microns down from the cerebral aqueduct in the ventral direction and 340 microns laterally on either side of the midline (for a 680 micron total recording width).

Data files were saved as .mcd files and analyzed in offline sorter. For analysis a Besel filter with a 150 Hz frequency cut off was applied to the raw data traces. The threshold for detection was set manually to a level that will include all legitimate spikes with the least amount of unipolar noise spikes included (between 13 µV and 35 µV). Once spikes were detected they were sorted by a combination of a K means scan method and manual verification. The manual verification was conducted after the K means scan was run and divided spikes into groups based on criterion such as amplitude, power under the curve and spike duration (for full list of criterion see offline sorter V3 manual under the K means scan, Plexon Inc.). Once waveforms were sorted into groups and judged to be biologically relevant each spike was validated by eye and spikes that did not fit the average waveform shape were invalidated. Also all unsorted spikes were visualized manually and any spikes that matched the average waveform shape in the relevant group were added to that group.

Spikes were then sorted into two classes using mean spike width as well as coefficient of variance (COV= standard deviation/ mean) of their firing pattern to categorize each cell. Spikes were sorted into one group that had a large waveform (0.2 ms or longer starting from the initial depolarization to the end of recovery), as well as a low COV (below 0.9 arbitrary units) calculated using MATLAB by Mathworks:

```
load('D:\Noah MEA Data\11315\Pet-1 KO #165\matlab files\Data0877_85');
data= adc045; %enter channel number before running script
spiketimes = data(:,2);
pca1 = data(:,3);
pca2 = data(:,4);
pca3 = data(:,5);
```

```
isi = diff(spiketimes);
meanisi = mean(isi');
stdisi = std(isi');
cov = stdisi/meanisi;
```

The other population was defined by a smaller waveform (below 0.2 ms), and a more variable firing pattern (a COV above 0.9). These cutoff values were determined by trial and error with most cells that had a waveform width above 0.2 also displaying a COV below 0.9 and vice versa.

Although there is overlap in the electrophysiological characteristics of dorsal raphe serotonergic and non-serotonergic neuron[81] the placement of the array to record from the ventromedial DR region assured that a high proportion of cells in our recordings were serotonergic. The vmDR is highly enriched in serotonergic neurons. In the rat this has been carefully quantified with 75-95% of the neurons containing immunoreactivity to 5-HT, and 70-95% of neurons expressing the 5-HT_{1A} receptor also expressing 5-HT [82]. The mouse DRN is thought to be similarly highly enriched for serotonergic neurons in the vmDR region, and 84% of genetically identified 5-HT neurons respond with 5-HT_{1A} inhibition [83]. While we cannot be sure that each and every neuron we putatively identified as serotonergic indeed expressed serotonin, the combination of our anatomical recording site and physiological criteria assures that the overwhelming majority of neurons in our study were serotonergic. Therefore we have classified both of the populations identified in these recordings as serotonergic.

Data Analysis

Spike traces from multielectrode arrays were analyzed using offline sorting (Plexon) and spikes were sorted using a combination of manual identification and automatic K means based sorting software. All cells within each mouse were then averaged, and those values were used for ordinary One-Way ANOVA.

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Conflicts of Interests: None of the Authors have conflicts of interest pertaining to this work.

Appendix 5: Animal Breeding and Housing

$C3Hf^{+/+}$, MT1 KO and MT1/2 KO (on a $C3Hf^{+/+}$ background) mice were generously provided by Dr. Jean Luca Tossini's laboratory. Homozygote breeding pairs were placed in one of three housing conditions listed in the next paragraph. MT1 and MT1/2 KO mice were genotyped on arrival and periodically throughout the duration of all experiments (See table A1 for primers). Genomic DNA was run through 35 cycles of amplification at 94°C for 45s, 60°C for 45s, and 72°C for 3 min. Amplified DNA was separated on a 1.5% agarose gel.

Mice to be developed and maintained on an equinox photoperiod were left in our breeding room, which is maintained on a 12/12 light/dark cycle with a light intensity of ~300 lux. Mice to be developed on a long or short photoperiod were transferred to one of four 6'x22"x25" light-tight boxes with an interior light intensity of ~275 lux, each of which can house up to 14 cages (Fig. A7 A, B). No cages were stacked on top of one another in order to maintain consistent light exposure to all cages. Two of the four boxes maintained a long light cycle (16 hours of light and 8 hours of darkness) and two boxes were maintained on a short light cycle (8 hours of light and 16 hours of darkness). These boxes were not opened while the exterior room lights were turned on during the dark phase of each respective box. If a box had to be opened during the dark phase of the mice in that box, the exterior lights were shut off and night vision goggles were used to see the mice.

Experiments were not conducted on these mice until they had been maintained on their respective light cycles for at least two generations to avoid any biological complications due to the switching of the light cycle as well as to allow any changes that may occur *in utero* to take place in at least one parental generation before the pups were taken for experiments. To determine if the changes we observed were because of developmental photoperiod or proximal photoperiod (i.e., the photoperiod they were on at the time of testing), some animals were

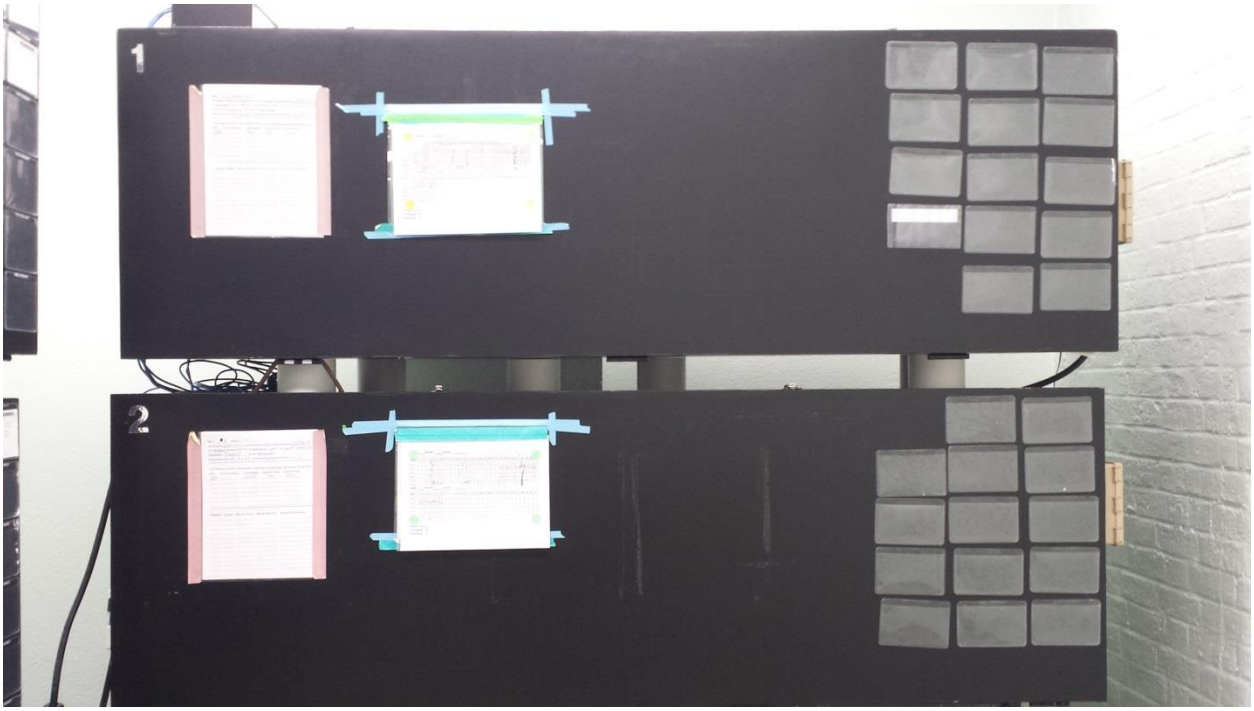
switched to the opposite light cycle at P30. P30 was chosen because this is slightly after weaning and the retina has fully developed at this point.

For critical period experiments, breeders were taken from the equinox light cycle and placed into a long light cycle and the first generation of offspring was used in order to avoid any genetic or epigenetic programming of the parents of the experimental mice. We were therefore able to isolate the light cycle on which the mice were gestated and developed as the only dependent variable in establishing the critical period. On the day they were born the pups and parents were transferred to a short light cycle and one experimental cohort was transferred back to long at 30 days old, to isolate the prenatal, postnatal/pre-weaning, and postnatal/post-weaning developmental periods. Another cohort was left to remain in a short light cycle until experimentation after being switched at birth to isolate the *in utero* developmental period from all development after birth.

Genotype and primer Direction	Sequence	Band Size
MT1 Receptor		366 bp
Forward	5'-GAGTCCAAGTTGCTGGGCAGTGGA-3'	
Reverse	5'-GAAGTTTTCTCAGTGTCCCGCAATG-3	
MT2 Receptor		367 bp
Forward	5'-CTCAGTGCTCAGGAACCGCAAGCT-3'	
Reverse	5'-CCTAGTATGAGATTTCTGGGGTGT-3'	

Table A1. Primers for genotyping of MT1 KO and MT1/2 KO mice.

A)



B)

<u>Figure A7</u>. Light tight boxes in which animals were housed in order to control their light cycle. (**A**) Exterior. (**B**) Interior.

Appendix 6: Multielectrode Array Recording Preparation

The dissecting area was prepared as shown in Figure A7. Mice were sacrificed by cervical dislocation and then decapitated. The skin was removed and the brain was extracted by making a lateral cut in the skull between the eyes and a medial cut down the center line of the skull starting at the foramen magnum and continuing rostrally until the initial lateral incision between the eyes was reached. The two halves of the skull were peeled off using forceps exposing the intact brain. Brains were then mounted in cold, oxygenated (95%O_2-5%CO_2) dissecting media (in mM: 114.5 NaCl, 3.5 KCL, 1 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 D(+)-glucose, and 35.7 NaCHO3- see Table A2) for at least one minute. This process should, ideally, take under 30 seconds but never more than one minute. The brain was then placed onto a piece of cold filter paper mounted on the bottom of a Petri dish and sliced midway between the olfactory bulbs and the cerebellum. The caudal portion of the brain was then mounted on the slicing chuck using super glue with the rostral portion face down and the cerebellum face up. The chuck was then inserted into the slicer as soon as possible to avoid cell death through the lack of nutrients and oxygen that occurs when the brain is not in solution. The slicing dish was also filled with dissecting media. The first slice removed the cerebellum and then 235-μm slices were taken through the raphe.

Slices were then placed into a Petri dish containing oxygenated extracellular recording media (in mM: 124 NaCl, 3.5 KCl, 1 NaH2PO4, 1.3MgSO4, 2.5 CaCl2,10 D(+)-glucose, and 20 NaHCO3- see Table A3, Fig. A8). The lateral and dorsal edges were removed with a scalpel leaving the center of the brain slice containing the DRN (Fig. A9). The raphe sections were then placed into a slice tray (Fig. A8) containing oxygenated recording solution and allowed to sit for at least 30 minutes before mounting on the MEA. While the slice recovers, 40μM tryptophan and 3μM phenylephrine were added to the recording solution. The raphe sections were then

Reagent	Concentration	Molecular Weight	Actual Amount
NaCl	114.5 mM	58.44 g/mol	6.69138 g
KCl	3.5 mM	74.55 g/mol	0.260925 g
NaH2PO4-2H2O	1 mM	156.01 g/mol	0.15601 g
MgSO4-7H2O	1.3 mM	246.47 g/mol	0.320411 g
CaCl2-2H2O	2.5 mM	147.01 g/mol	0.367525 g
D(+)-glucose	10 mM	180.16 g/mol	1.8016 g
NaHCO3	35.7 mM	84.01 g/mol	2.999157 g

Table A2. Composition of dissecting solution. Reagents were dissolved in 1L of filtered water.

Reagent	Concentration	Molecular Weight	Actual Amount
NaCl	124 mM	58.44 g/mol	7.24656 g
KCl	3.5 mM	74.55 g/mol	0.260925 g
NaH2PO4-2H2O	1 mM	156.01 g/mol	0.15601 g
MgSO4-7H2O	1.3 mM	246.47 g/mol	0.320411 g
CaCl2-2H2O	2.5 mM	147.01 g/mol	0.367525 g
D(+)-glucose	10 mM	180.16 g/mol	1.8016 g
NaHCO3	26 mM	84.01 g/mol	2.18426 g

Table A3. Composition of extracellular recording solution. Reagents were dissolved in 1L of filtered water.

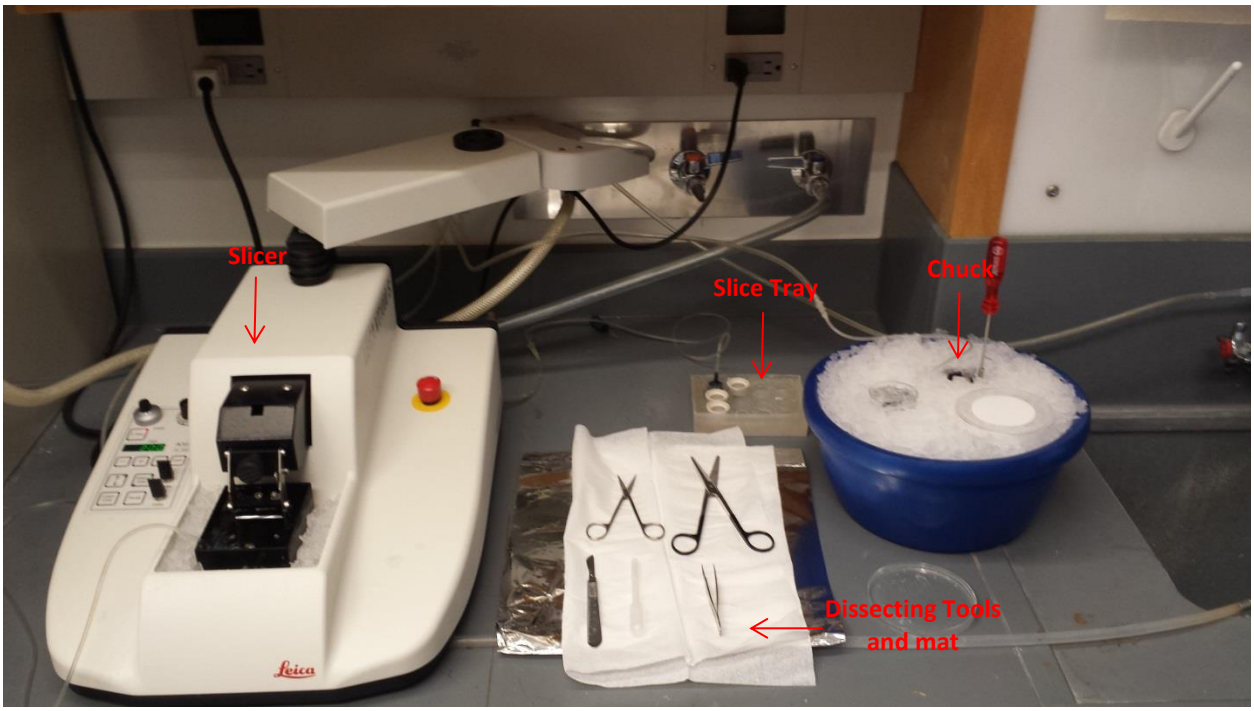
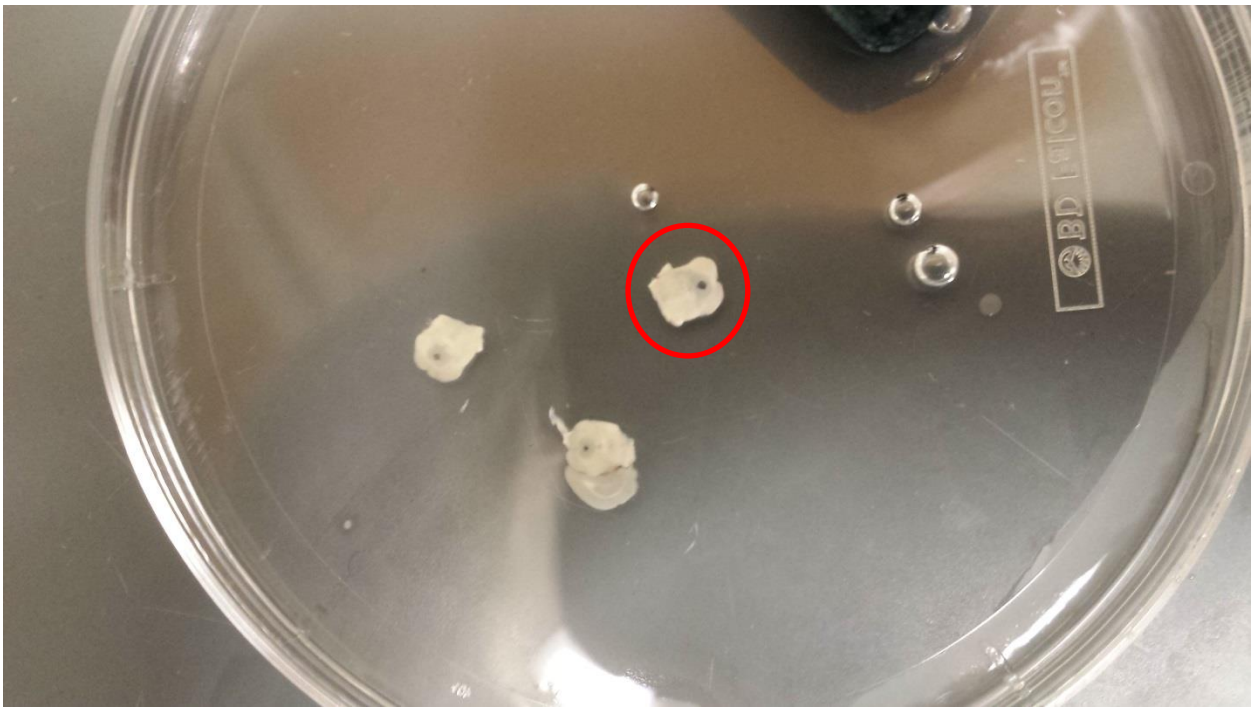
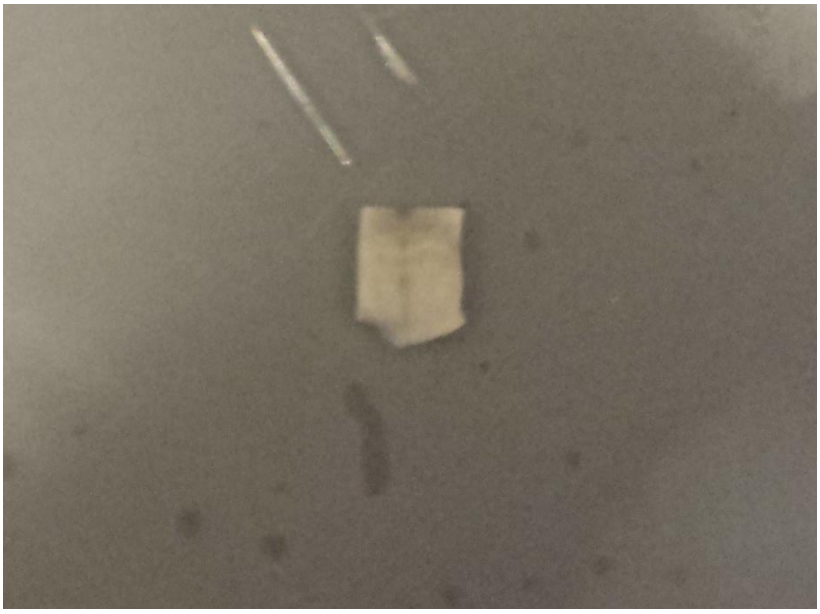


Figure A8. Brain extraction and slicing set up.





<u>Figure A9</u>. Slices through the raphe nuclei. (**A**) Raphe slices before extraneous tissue is removed. Red circle indicates the slice used for recording. (**B**) Raphe slice that is ready for recording after extraneous tissue is removed.

mounted on the MEA using a fine-bristle paint brush to position the dorsomedial portion of the raphe (directly below the cerebral aqueduct) over the array. The slice was then immobilized by a harp and the recording dish was filled with oxygenated recording solution. The array was then placed on the baseplate and the amplifier was attached (Fig. A10). The perfusion inflow and outflow were connected to the same peristaltic pump to ensure equivalent amounts of solution were being added and removed simultaneously. However, a safety suction tube was placed at the top of the array recording dish to prevent any excess fluid from overflowing and damaging the array or amplifier. Finally the slice was allowed to settle for at least 45 minutes before recording.

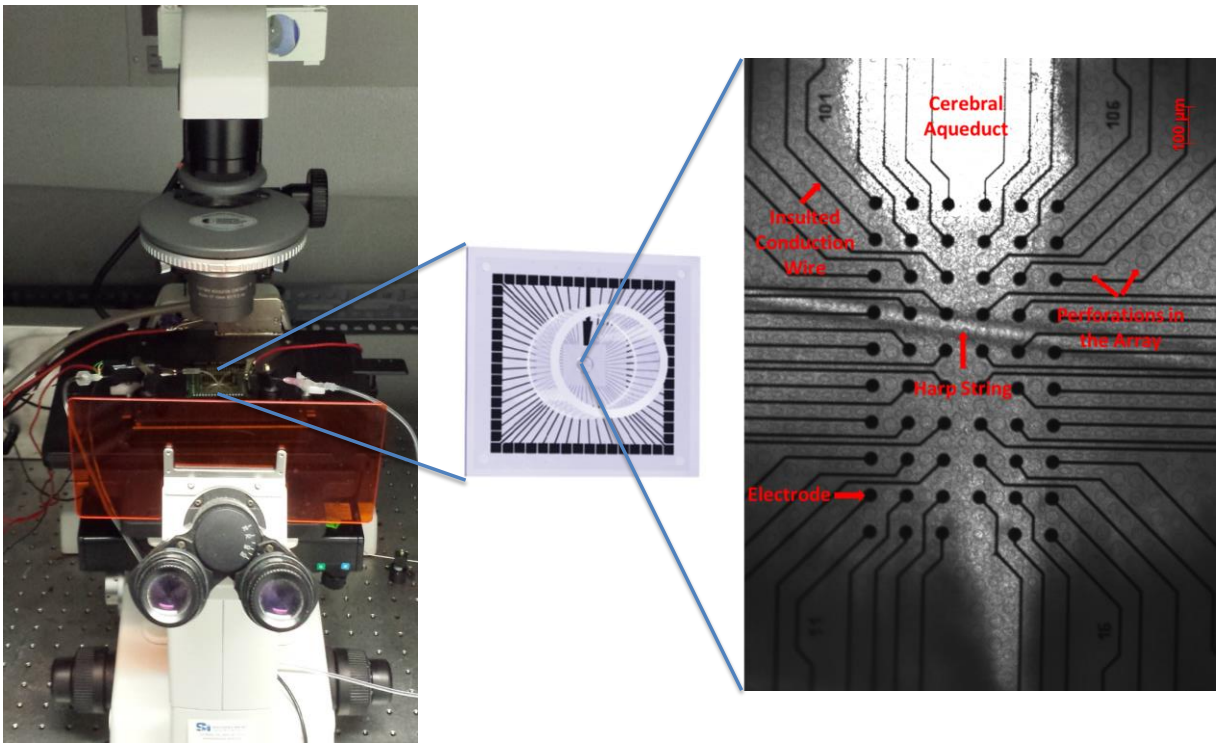


Figure A10. MEA rig, MEA and slice sitting on the array itself.

References

1 Khavrus, V. a. S., I. Introduction to solar motion geometry on the basis of a simple model. *Physics Education* **45**, 641-653 (2010).

2 Khavrus, V. a. S., I. Geometry and physics of seasons. *Physics Education* **47**, 680-692 (2012).

3 Briggs, D. a. S., P. *Fundamentals of physical geography*. Vol. 1 Chapter 6 (Rowan and Littlefield, 1986).

4 Hartman, W. K. a. D., D.R. . Satellite-Sized Planetesimals and Lunar Origin. *Icarus* **24**, 504-515 (1975).

5 Belbruno, E. a. G., J.R. Where did the moon come from. *Astronomical Journal* **129**, 1724-1745 (2005).

6 Bertolucci, C. & Foa, A. Seasonality and role of SCN in entrainment of lizard circadian rhythms to daily melatonin injections. *Am J Physiol* **274**, R1004-1014 (1998).

7 Dawson, A., King, V. M., Bentley, G. E. & Ball, G. F. Photoperiodic control of seasonality in birds. *J Biol Rhythms* **16**, 365-380 (2001).

8 Shaw, D. & Goldman, B. D. Influence of prenatal and postnatal photoperiods on postnatal testis development in the Siberian hamster (Phodopus sungorus). *Biol Reprod* **52**, 833-838 (1995).

9 Maywood, E. S., Buttery, R. C., Vance, G. H., Herbert, J. & Hastings, M. H. Gonadal responses of the male Syrian hamster to programmed infusions of melatonin are sensitive to signal duration and frequency but not to signal phase nor to lesions of the suprachiasmatic nuclei. *Biol Reprod* **43**, 174-182 (1990).

10 Arendt, J., Symons, A. M., Laud, C. A. & Pryde, S. J. Melatonin can induce early onset of the breeding season in ewes. *J Endocrinol* **97**, 395-400 (1983).

11 Meijer, J. H., Thio, B., Albus, H., Schaap, J. & Ruijs, A. C. Functional absence of extraocular photoreception in hamster circadian rhythm entrainment. *Brain Res* **831**, 337-339 (1999).

12 Eastman, C. I., Martin, S. K. & Hebert, M. Failure of extraocular light to facilitate circadian rhythm reentrainment in humans. *Chronobiol Int* **17**, 807-826 (2000).

13 Lockley, S. W. *et al.* Extraocular light exposure does not suppress plasma melatonin in humans. *J Clin Endocrinol Metab* **83**, 3369-3372, doi:10.1210/jcem.83.9.5244 (1998).

14 Panda, S., Hogenesch, J. B. & Kay, S. A. Circadian light input in plants, flies and mammals. *Novartis Found Symp* **253**, 73-82; discussion 82-78, 102-109, 281-104 (2003).

15 Panda, S., Hogenesch, J. B. & Kay, S. A. Circadian rhythms from flies to human. *Nature* **417**, 329-335 (2002).

16 Ralph, M. R., Foster, R. G., Davis, F. C. & Menaker, M. Transplanted suprachiasmatic nucleus determines circadian period. *Science* **247**, 975-978 (1990).

17 Panda, S. *et al.* Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. *Science* **298**, 2213-2216 (2002).

18 Provencio, I., Jiang, G., De Grip, W. J., Hayes, W. P. & Rollag, M. D. Melanopsin: An opsin in melanophores, brain, and eye. *Proc Natl Acad Sci U S A* **95**, 340-345 (1998).

19 Rao, S. *et al.* A direct and melanopsin-dependent fetal light response regulates mouse eye development. *Nature* **494**, 243-246, doi:10.1038/nature11823 (2013).

20 Ciarleglio, C. M., Axley, J. C., Strauss, B. R., Gamble, K. L. & McMahon, D. G. Perinatal photoperiod imprints the circadian clock. *Nat Neurosci* **14**, 25-27, doi:10.1038/nn.2699 (2010).

21 Ebihara, S., Marks, T., Hudson, D. J. & Menaker, M. Genetic control of melatonin synthesis in the pineal gland of the mouse. *Science* **231**, 491-493 (1986).

22 Binkley, S. Circadian rhythm in pineal N-acetyltransferase activity: phase shifting by light pulses (II). *J Neurochem* **41**, 273-276 (1983).
23 Goldman, B. D. Pattern of melatonin secretion mediates transfer of photoperiod information from mother to fetus in mammals. *Science's STKE* **2003**, PE29, doi:10.1126/stke.2003.192.pe29 (2003).
24 Weaver, D. R., Keohan, J. T. & Reppert, S. M. Definition of a prenatal sensitive period for maternal-fetal communication of day length. *Am J Physiol* **253**, E701-704 (1987).
25 Dubocovich, M. L., Benloucif, S. & Masana, M. I. Melatonin receptors in the mammalian suprachiasmatic nucleus. *Behav Brain Res* **73**, 141-147 (1996).
26 Ciarleglio, C. M., Resuehr, H. E. S. & McMahon, D. G. Interactions of the serotonin and circadian systems: nature and nurture in rhythms and blues. *Neuroscience*, 1-9, doi:10.1016/j.neuroscience.2011.09.036 (2011).
27 Wirz-Justice, A. Theoretical and therapeutic potential of indolamine precursors in affective disorders. *Neuropsychobiology* **3**, 199-233 (1977).
28 Paresh D Patela, C. P., Sharon Burkea. Robust and tissue-specific expression of TPH2 versus TPH1 in rat raphe and pineal gland. *Biological Psychiatry*, 428-433 (2004).
29 Barassin, S. *et al.* Circadian tryptophan hydroxylase levels and serotonin release in the suprachiasmatic nucleus of the rat. *Eur J Neurosci* **15**, 833-840 (2002).
30 Liang, J., Wessel, J. H., 3rd, Iuvone, P. M., Tosini, G. & Fukuhara, C. Diurnal rhythms of tryptophan hydroxylase 1 and 2 mRNA expression in the rat retina. *Neuroreport* **15**, 1497-1500 (2004).
31 Malek, Z. S., Pevet, P. & Raison, S. Circadian change in tryptophan hydroxylase protein levels within the rat intergeniculate leaflets and raphe nuclei. *Neuroscience* **125**, 749-758 (2004).
32 Nexon, L., Sage, D., Pévet, P. & Raison, S. Glucocorticoid-mediated nycthemeral and photoperiodic regulation of tph2 expression. *Eur J Neurosci* **33**, 1308-1317, doi:10.1111/j.1460-9568.2010.07586.x (2011).
33 Scheving, L. E., Harrsion W.H., Gordon P., Pauly J. E., . Daily fluctuations (circadian and ultradian) in biogenic amines of the rat brain. *American Journal of physiology* **214**, 166-173 (1968).
34 Meera Vaswani, Farzana Kadar Linda, Subramanyam Ramesh. Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **27**, 85-102 (2003).
35 Kan, J. P. *et al.* Daily variations of various parameters of serotonin metabolism in the rat brain. I. Circadian variations of tryptophan-5-hydroxylase in the raphe nuclei and the striatum. *Brain Res* **123**, 125-136 (1977).
36 Steininger, T. L., Wainer, B. H., Blakely, R. D. & Rye, D. B. Serotonergic dorsal raphe nucleus projections to the cholinergic and noncholinergic neurons of the pedunculopontine tegmental region: a light and electron microscopic anterograde tracing and immunohistochemical study. *J Comp Neurol* **382**, 302-322 (1997).
37 Efrain C. Azmitia1, a. S., ‡. An autoradiographic analysis of the differential ascending projections of the dorsal and median raphe nuclei in the rat. *Comparative Neurology* **129**, 641-667 (2004).
38 Vertes*, D. R. P. A PHA-L analysis of ascending projections of the dorsal raphe nucleus in the rat. *Comparative Neurology* **313**, 643-668 (1991).
39 C Peyron, J.-M. P., C Rampon, M Jouvet, P.-H Luppi. Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. *Neuroscience* **82**, 443-468 (1997).
40 Hay-Schmidt, A., Vrang, N., Larsen, P. J. & Mikkelsen, J. D. Projections from the raphe nuclei to the suprachiasmatic nucleus of the rat. *J Chem Neuroanat* **25**, 293-310 (2003).

41 Meyer-Bernstein, E. L., Blanchard, J. H. & Morin, L. P. The serotonergic projection from the median raphe nucleus to the suprachiasmatic nucleus modulates activity phase onset, but not other circadian rhythm parameters. *Brain Res* **755**, 112-120 (1997).
42 Moore, R. Y. & Speh, J. C. Serotonin innervation of the primate suprachiasmatic nucleus. *Brain Res* **1010**, 169-173 (2004).
43 Morin, L. P. Serotonin and the regulation of mammalian circadian rhythmicity. *Ann Med* **31**, 12-33 (1999).
44 Pickard, G. E. The afferent connections of the suprachiasmatic nucleus of the golden hamster with emphasis on the retinohypothalamic projection. *J Comp Neurol* **211**, 65-83 (1982).
45 Deurveilher, S. & Semba, K. Indirect projections from the suprachiasmatic nucleus to major arousal-promoting cell groups in rat: implications for the circadian control of behavioural state. *Neuroscience* **130**, 165-183 (2005).
46 Kawano, H., Decker, K. & Reuss, S. Is there a direct retina-raphe-suprachiasmatic nucleus pathway in the rat? *Neurosci Lett* **212**, 143-146 (1996).
47 Fite, K. V. & Janusonis, S. Retinal projection to the dorsal raphe nucleus in the Chilean degus (Octodon degus). *Brain Res* **895**, 139-145 (2001).
48 Bobrzynska, K. J., Godfrey, M. H. & Mrosovsky, N. Serotonergic stimulation and nonphotic phase-shifting in hamsters. *Physiol Behav* **59**, 221-230 (1996).
49 Glass, J. D., Grossman, G. H., Farnbauch, L. & DiNardo, L. Midbrain raphe modulation of nonphotic circadian clock resetting and 5-HT release in the mammalian suprachiasmatic nucleus. *J Neurosci* **23**, 7451-7460 (2003).
50 Ying, S. W. & Rusak, B. Effects of serotonergic agonists on firing rates of photically responsive cells in the hamster suprachiasmatic nucleus. *Brain Res* **651**, 37-46 (1994).
51 Domínguez-López, S. *et al.* Short term effects of melatonin and pinealectomy on serotonergic neuronal activity across the light-dark cycle. *Journal of Psychopharmacology*, doi:10.1177/0269881111408460 (2011).
52 Miguez, J. M., F.J. Martin, M. Aldegunde. Melatonin effects on serotonin synthesis and metabolism in the striatum, nucleus accumbens, and dorsal and medial raphe nuclei of rats. *Neurochemical Research* **22**, 87-92 (1997).
53 Foster, R. G. & Roenneberg, T. Human responses to the geophysical daily, annual and lunar cycles. *Curr Biol* **18**, R784-R794 (2008).
54 Disanto, G. *et al.* Seasonal Distribution of Psychiatric Births in England. *PLoS ONE* **7**, e34866, doi:10.1371/journal.pone.0034866.t001 (2012).
55 Chotai, J. & Adolfsson, R. Converging evidence suggests that monoamine neurotransmitter turnover in human adults is associated with their season of birth. *Eur Arch Psychiatry Clin Neurosci* **252**, 130-134, doi:10.1007/s00406-002-0372-7 (2002).
56 Chotai, J., Serretti, A., Lattuada, E., Lorenzi, C. & Lilli, R. Gene-environment interaction in psychiatric disorders as indicated by season of birth variations in tryptophan hydroxylase (TPH), serotonin transporter (5-HTTLPR) and dopamine receptor (DRD4) gene polymorphisms. *Psychiatry Res* **119**, 99-111 (2003).
57 Péter Dömea, B. K., Györgyi Ignitse, Zoltán Rihmera. Season of Birth Is Significantly Associated with the Risk of Completed Suicide. *Biological Psychiatry* **68**, 148-155 (2010).
58 Jayanti Chotai, E. S. R., Lars Jacobsson. Season of birth associated with the age and method of suicide. *Archives of Suicide Research* **5**, 245-254 (1999).
59 Natale, V., Adan, A. & Chotai, J. Further results on the association between morningness-eveningness preference and the season of birth in human adults. *Neuropsychobiology* **46**, 209-214, doi:10.1159/000067803 (2002).

60 K.J. Brookes1, B. N., X. Xu A. Thapar, M. Gill, K. Langley, Z. Hawi, J. Mill, E. Taylor, B. Franke, W. Chen, R. Ebstein, J. Buitelaar, T. Banaschewski, E. Sonuga-Barke, J. Eisenberg, I. Manor, A. Miranda, R.D. Oades, H. Roeyers, A. Rothenberger, J. Sergeant, H.C. Steinhausen, S.V. Faraone and P. Asherson. Differential dopamine receptor D4 allele association with ADHD dependent of proband season of birth. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics* **147B**, 94-99 (2008).

61 RICHARD LIVINGSTON, B. S. A., H. STEFAN BRACHA,. Season of Birth and Neurodevelopmental Disorders: Summer Birth is Associated with Dyslexia. *Journal of the American Academy of Child & Adolescent Psychiatry* **32**, 612-616 (1993).

62 Michael C. Stevens, D. F. L. H. W. Season of Birth Effects in Autism. *Journal of Clinical and Experimental Neuropsychology* **22**, 399-407 (2000).

63 Barak, Y. R., Aliza; Sulkes, Juliet; Gabbay, Uri. Season of birth and autistic disorder in Israel. *The American Journal of Psychiatry* **152**, 798-800 (1995).

64 Roseboom, P. H., M.A.A. Namboodiri, D.B. Zimonjic, N.C. Popescu, I.R. Rodrigeuz, J.A. Gastel, D.C. & Klein, D. Natural melatonin knock down in C57BL/6J mice: Rare mechanism truncates serotonin Nacetyltransferase. *Molecular Brain Research* **63**, 189-197 (1998).

65 Lolley, S. Y. S. a. R. N. CYCLIC-NUCLEOTIDE PHOSPHODIESTERASE An Early Defect in Inherited Retinal Degeneration of C3H Mice. *JCB* **57**, 117-123 (1973).

66 Susana Contreras-Alcantara, K. B. a. T. Removal of Melatonin Receptor Type 1 Induces Insulin Resistance in the Mouse. *Obesity* **18**, 1861-1863 (2010).

67 WIESEL, T. N. & HUBEL, D. H. SINGLE-CELL RESPONSES IN STRIATE CORTEX OF KITTENS DEPRIVED OF VISION IN ONE EYE. *J Neurophysiol* **26**, 1003-1017 (1963).

68 WIESEL, T. N. & HUBEL, D. H. EFFECTS OF VISUAL DEPRIVATION ON MORPHOLOGY AND PHYSIOLOGY OF CELLS IN THE CATS LATERAL GENICULATE BODY. *J Neurophysiol* **26**, 978-993 (1963).

69 Morin, L. P. Neuroanatomy of the extended circadian rhythm system. *Experimental Neurology* **243**, 4-20, doi:10.1016/j.expneurol.2012.06.026 (2013).

70 Gooley, J. J., Lu, J., Fischer, D. & Saper, C. B. A broad role for melanopsin in nonvisual photoreception. *J Neurosci* **23**, 7093-7106 (2003).

71 Anguelova, M., Benkelfat, C. & Turecki, G. A systematic review of association studies investigating genes coding for serotonin receptors and the serotonin transporter: I. Affective disorders. *Mol Psychiatry* **8**, 574-591, doi:10.1038/sj.mp.4001328 (2003).

72 Cook, E. H. & Leventhal, B. L. The serotonin system in autism. *Current opinion in pediatrics* **8**, 348-354 (1996).

73 Hendricks, T. J. *et al.* Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. *Neuron* **37**, 233-247 (2003).

74 Ruby, N. F. *et al.* Role of melanopsin in circadian responses to light. *Science* **298**, 2211-2213 (2002).

75 Ciarleglio, C. M., Axley, J. C., Strauss, B. R., Gamble, K. L. & McMahon, D. G. Perinatal photoperiod imprints the circadian clock. *Nat Neurosci* **14**, 25-27, doi:10.1038/nn.2699 (2011).

76 Dulcis, D., Jamshidi, P., Leutgeb, S. & Spitzer, N. C. Neurotransmitter switching in the adult brain regulates behavior. *Science* **340**, 449-453, doi:10.1126/science.1234152 (2013).

77 Pyter, L. M. & Nelson, R. J. Enduring effects of photoperiod on affective behaviors in Siberian hamsters (Phodopus sungorus). *Behavioral Neuroscience* **120**, 125-134, doi:10.1037/0735-7044.120.1.125 (2006).

78 Baba, K. *et al.* Melatonin modulates visual function and cell viability in the mouse retina via the MT1 melatonin receptor. *Proc Natl Acad Sci USA* **106**, 15043-15048, doi:10.1073/pnas.0904400106 (2009).

79 Thompson, B. J. *et al.* Transgenic elimination of high-affinity antidepressant and cocaine sensitivity in the presynaptic serotonin transporter. *Proc Natl Acad Sci USA* **108**, 3785-3790, doi:10.1073/pnas.1011920108 (2011).
80 Veenstra-VanderWeele, J. *et al.* Autism gene variant causes hyperserotonemia, serotonin receptor hypersensitivity, social impairment and repetitive behavior. *Proc Natl Acad Sci USA* **109**, 5469-5474, doi:10.1073/pnas.1112345109 (2012).
81 Beck, S. G., Pan, Y.-Z., Akanwa, A. C. & Kirby, L. G. Median and dorsal raphe neurons are not electrophysiologically identical. *Journal of Neurophysiology* **91**, 994-1005, doi:10.1152/jn.00744.2003 (2004).
82 Kirby, L. G., Pernar, L., Valentino, R. J. & Beck, S. G. Distinguishing characteristics of serotonin and non-serotonin-containing cells in the dorsal raphe nucleus: electrophysiological and immunohistochemical studies. *Neuroscience* **116**, 669-683, doi:10.1016/S0306-4522(02)00584-5 (2003).
83 Kiyasova, V. *et al.* A genetically defined morphologically and functionally unique subset of 5-HT neurons in the mouse raphe nuclei. *Journal of Neuroscience* **31**, 2756-2768, doi:10.1523/JNEUROSCI.4080-10.2011 (2011).
84 Howell, D. *Statistical Methods for Psychology*. Vol. 7 (2010).
85 Levine, E. S. & Jacobs, B. L. Neurochemical afferents controlling the activity of serotonergic neurons in the dorsal raphe nucleus: microiontophoretic studies in the awake cat. *J Neurosci* **12**, 4037-4044 (1992).
86 Rood, B. D. *et al.* Dorsal raphe serotonin neurons in mice: immature hyperexcitability transitions to adult state during first three postnatal weeks suggesting sensitive period for environmental perturbation. *Journal of Neuroscience* **34**, 4809-4821, doi:10.1523/JNEUROSCI.1498-13.2014 (2014).
87 Prendergast, B. J. & Kay, L. M. Affective and adrenocorticotrophic responses to photoperiod in Wistar rats. *J Neuroendocrinol* **20**, 261-267, doi:10.1111/j.1365-2826.2007.01633.x (2008).
88 Prendergast, B. J. & Nelson, R. J. Affective responses to changes in day length in Siberian hamsters (Phodopus sungorus). *Psychoneuroendocrinology* **30**, 438-452, doi:10.1016/j.psyneuen.2004.08.008 (2005).
89 Einat, H., Kronfeld-Schor, N. & Eilam, D. Sand rats see the light: short photoperiod induces a depression-like response in a diurnal rodent. *Behav Brain Res* **173**, 153-157, doi:10.1016/j.bbr.2006.06.006 (2006).
90 Torres-Farfan, C. *et al.* Maternal melatonin selectively inhibits cortisol production in the primate fetal adrenal gland. *The Journal of Physiology* **554**, 841-856, doi:10.1113/jphysiol.2003.056465 (2004).
91 Sengupta, A. *et al.* Localization of melatonin receptor 1 in mouse retina and its role in the circadian regulation of the electroretinogram and dopamine levels. *PLoS ONE* **6**, e24483, doi:10.1371/journal.pone.0024483 (2011).
92 Magnusson, A. & Boivin, D. Seasonal affective disorder: an overview. *Chronobiol Int* **20**, 189-207 (2003).
93 Chotai, J. & Asberg, M. Variations in CSF monoamine metabolites according to the season of birth. *Neuropsychobiology* **39**, 57-62, doi:26561 (1999).
94 Luykx, J. J. *et al.* Season of sampling and season of birth influence serotonin metabolite levels in human cerebrospinal fluid. *PLoS One* **7**, e30497, doi:10.1371/journal.pone.0030497 (2012).
95 Lowry, C. A. *et al.* Serotonergic systems, anxiety, and affective disorder: focus on the dorsomedial part of the dorsal raphe nucleus. *Annals of the New York Academy of Sciences* **1148**, 86-94, doi:10.1196/annals.1410.004 (2008).

96 Lira, A. *et al.* Altered depression-related behaviors and functional changes in the dorsal raphe nucleus of serotonin transporter-deficient mice. *Biol Psychiatry* **54**, 960-971 (2003).
97 Kelsoe, J. R. *et al.* Genetic linkage study of bipolar disorder and the serotonin transporter. *Am J Med Genet* **67**, 215-217 (1996).
98 Iqbal, N., Asnis, G. M., Wetzler, S., Kay, S. R. & van Praag, H. M. The role of serotonin in schizophrenia. New findings. *Schizophr Res* **5**, 181-182 (1991).
99 Elizabeth E Steinberg, D. J. C., Karl Deisseroth, Robert C Malenka,. Illuminating circuitry relevant to psychiatric disorders with optogenetics. *Current Opinion in Neurobiology* **30**, 9-16 (2015).
100 Waider, J., Araragi, N., Gutknecht, L. & Lesch, K.-P. Tryptophan hydroxylase-2 (TPH2) in disorders of cognitive control and emotion regulation: a perspective. *Psychoneuroendocrinology* **36**, 393-405, doi:10.1016/j.psyneuen.2010.12.012 (2011).
101 Clark, J. A., Pai, L.-Y., Flick, R. B. & Rohrer, S. P. Differential hormonal regulation of tryptophan hydroxylase-2 mRNA in the murine dorsal raphe nucleus. *Biol Psychiatry* **57**, 943-946, doi:10.1016/j.biopsych.2005.01.013 (2005).
102 Liu, C. *et al.* Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* **19**, 91-102 (1997).
103 Davis, F. C. Melatonin: Role in Development. *J. of Biological Rhythms* **12**, 498-508 (1997).
104 Meaney, M. J. Epigenetics and the biological definition of gene x environment interactions. *Child Dev* **81**, 41-79, doi:10.1111/j.1467-8624.2009.01381.x (2010).
105 Fyodorov, D., Nelson, T. & Deneris, E. Pet-1, a novel ETS domain factor that can activate neuronal nAchR gene transcription. *J Neurobiol* **34**, 151-163 (1998).
106 Hendricks, T., Francis, N., Fyodorov, D. & Deneris, E. S. The ETS domain factor Pet-1 is an early and precise marker of central serotonin neurons and interacts with a conserved element in serotonergic genes. *J Neurosci* **19**, 10348-10356 (1999).
107 Scott, M. M., Krueger, K. C. & Deneris, E. S. A differentially autoregulated Pet-1 enhancer region is a critical target of the transcriptional cascade that governs serotonin neuron development. *J Neurosci* **25**, 2628-2636, doi:10.1523/JNEUROSCI.4979-04.2005 (2005).
108 Liu, C. *et al.* Pet-1 is required across different stages of life to regulate serotonergic function. *Nat Neurosci* **13**, 1190-1198, doi:10.1038/nn.2623 (2010).
109 Green, Noah H., Jackson, Chad R., Iwamoto, H., Tackenberg, Michael C. & McMahon, Douglas G. Photoperiod Programs Dorsal Raphe Serotonergic Neurons and Affective Behaviors. *Current Biology*, doi:10.1016/j.cub.2015.03.050.
110 Kayoko W. Miyazaki, K. M., Kenji F. Tanaka, Akihiro Yamanaka, Aki Takahashi, Sawako Tabuchi, Kenji Doya. Optogenetic Activation of Dorsal Raphe Serotonin Neurons Enhances Patience for Future Rewards. *Current Biology* **24**, 2033-2040 (2014).
111 Lam, R. W. & Levitan, R. D. Pathophysiology of seasonal affective disorder: a review. *J Psychiatry Neurosci* **25**, 469-480 (2000).
112 Thorne, H. C., Jones, K. H., Peters, S. P., Archer, S. N. & Dijk, D.-J. Daily and seasonal variation in the spectral composition of light exposure in humans. *Chronobiol Int* **26**, 854-866, doi:10.1080/07420520903044315 (2009).
113 Cahill, G. M., Parsons, S. E. & Besharse, J. C. Spectral sensitivity of melatonin synthesis suppression in Xenopus eyecups. *Vis Neurosci* **15**, 499-502 (1998).
114 Foster, R. G. Neurobiology: bright blue times. *Nature* **433**, 698-699 (2005).
115 Gehring, W. & Rosbash, M. The coevolution of blue-light photoreception and circadian rhythms. *J Mol Evol* **57 Suppl 1**, S286-289 (2003).
116 Van Gelder, R. N. Blue light and the circadian clock. *Br J Ophthalmol* **88**, 1353; author reply 1353 (2004).

117 Hankins, M. W., Peirson, S. N. & Foster, R. G. Melanopsin: an exciting photopigment. *Trends Neurosci* **31**, 27-36 (2008).
118 Kennaway, D. J. Light, neurotransmitters and the suprachiasmatic nucleus control of pineal melatonin production in the rat. *Biol Signals* **6**, 247-254 (1997).
119 Khalsa, S. B. S., Jewett, M. E., Cajochen, C. & Czeisler, C. A. A phase response curve to single bright light pulses in human subjects. *J Physiol (Lond)* **549**, 945-952, doi:10.1113/jphysiol.2003.040477 (2003).
120 Avery, D. H. A turning point for seasonal affective disorder and light therapy research? *Arch Gen Psychiatry* **55**, 863-864 (1998).
121 Burgess, H. J., Fogg, L. F., Young, M. A. & Eastman, C. I. Bright light therapy for winter depression--is phase advancing beneficial? *Chronobiol Int* **21**, 759-775 (2004).
122 Eastman, C. I., Young, M. A., Fogg, L. F., Liu, L. & Meaden, P. M. Bright light treatment of winter depression: a placebo-controlled trial. *Arch Gen Psychiatry* **55**, 883-889 (1998).
123 Gagné, A.-M., Lévesque, F., Gagné, P. & Hébert, M. Impact of blue vs red light on retinal response of patients with seasonal affective disorder and healthy controls. *Prog Neuropsychopharmacol Biol Psychiatry* **35**, 227-231, doi:10.1016/j.pnpbp.2010.11.009 (2011).
124 Glickman, G., Byrne, B., Pineda, C., Hauck, W. W. & Brainard, G. C. Light Therapy for Seasonal Affective Disorder with Blue Narrow-Band Light-Emitting Diodes (LEDs). *Biol Psychiatry* (2005).
125 Caspi, A., Hariri, A. R., Holmes, A., Uher, R. & Moffitt, T. E. Genetic sensitivity to the environment: the case of the serotonin transporter gene and its implications for studying complex diseases and traits. *Am J Psychiatry* **167**, 509-527, doi:10.1176/appi.ajp.2010.09101452 (2010).
126 Caspi, A. *et al.* Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science* **301**, 386-389, doi:10.1126/science.1083968 (2003).
127 Pjrek, E. *et al.* Seasonality of birth in seasonal affective disorder. *J Clin Psychiatry* **65**, 1389-1393 (2004).
128 Doyle, S. & Menaker, M. Circadian photoreception in vertebrates. *Cold Spring Harb Symp Quant Biol* **72**, 499-508 (2007).
129 Altimus, C. M. *et al.* Rods-cones and melanopsin detect light and dark to modulate sleep independent of image formation. *Proc Natl Acad Sci U S A* **105**, 19998-20003 (2008).
130 de Vries, M. J., Treep, J. A., de Pauw, E. S. & Meijer, J. H. The effects of electrical stimulation of the optic nerves and anterior optic chiasm on the circadian activity rhythm of the Syrian hamster: involvement of excitatory amino acids. *Brain Res* **642**, 206-212 (1994).
131 Levine, J. D., Zhao, X. S. & Miselis, R. R. Direct and indirect retinohypothalamic projections to the supraoptic nucleus in the female albino rat. *J Comp Neurol* **341**, 214-224 (1994).
132 Brammer, G. L., Morley, J. E., Geller, E., Yuwiler, A. & Hershman, J. M. Hypothalamus-pituitary-thyroid axis interactions with pineal gland in the rat. *Am J Physiol* **236**, E416-420 (1979).
133 Gauer, F. & Craft, C. M. Circadian regulation of hydroxyindole-O-methyltransferase mRNA levels in rat pineal and retina. *Brain Res* **737**, 99-109 (1996).
134 Pevet, P. & Challet, E. Melatonin: both master clock output and internal time-giver in the circadian clocks network. *J Physiol Paris* **105**, 170-182, doi:10.1016/j.jphysparis.2011.07.001 (2011).
135 Gupta, B. B., Spessert, R. & Vollrath, L. Molecular components and mechanism of adrenergic signal transduction in mammalian pineal gland: regulation of melatonin synthesis. *Indian J Exp Biol* **43**, 115-149 (2005).
136 Kopp, M., Meissl, H. & Korf, H. W. The pituitary adenylate cyclase-activating polypeptide-induced phosphorylation of the transcription factor CREB (cAMP response element binding

protein) in the rat suprachiasmatic nucleus is inhibited by melatonin. *Neurosci Lett* **227**, 145-148 (1997).

137 Belle, M. D. *et al.* Daily electrical silencing in the mammalian circadian clockElectrophysiological effects of melatonin on mouse Per1 and non-Per1 suprachiasmatic nuclei neurones in vitro Spatiotemporal heterogeneity in the electrical activity of suprachiasmatic nuclei neurons and their response to photoperiod. *Science* **326**, 281-284 (2009).

138 Cassone, V. M. Melatonin's role in vertebrate circadian rhythms. *Chronobiol Int* **15**, 457-473 (1998).

139 Johnston, J. D., Messager, S., Barrett, P. & Hazlerigg, D. G. Melatonin action in the pituitary: neuroendocrine synchronizer and developmental modulator? *J Neuroendocrinol* **15**, 405-408 (2003).

140 Shibata, S. & Moore, R. Y. Development of neuronal activity in the rat suprachiasmatic nucleus. *Brain Res* **431**, 311-315 (1987).

141 Fuchs, J. L. & Moore, R. Y. Development of circadian rhythmicity and light responsiveness in the rat suprachiasmatic nucleus: a study using the 2-deoxy[1-14C]glucose method. *Proc Natl Acad Sci U S A* **77**, 1204-1208 (1980).

142 Aguilar-Roblero, R., Shibata, S., Speh, J. C., Drucker-Colin, R. & Moore, R. Y. Morphological and functional development of the suprachiasmatic nucleus in transplanted fetal hypothalamus. *Brain Res* **580**, 288-296 (1992).

143 Malek, Z. S., Dardente, H., Pevet, P. & Raison, S. Tissue-specific expression of tryptophan hydroxylase mRNAs in the rat midbrain: anatomical evidence and daily profiles. *Eur J Neurosci* **22**, 895-901 (2005).

144 Malek, Z. S., Sage, D., Pevet, P. & Raison, S. Daily Rhythm of Tryptophan Hydroxylase-2 Messenger Ribonucleic Acid within Raphe Neurons Is Induced by Corticoid Daily Surge and Modulated by Enhanced Locomotor Activity. *Endocrinology* **148**, 5165-5172, doi:10.1210/en.2007-0526 (2007).

145 Poirel, V. J. *et al.* MT1 melatonin receptor mRNA tissular localization by PCR amplification. *Neuro Endocrinol Lett* **24**, 33-38 (2003).

146 Savaskan, E. *et al.* Distribution of melatonin MT1 receptor immunoreactivity in human retina. *J Histochem Cytochem* **50**, 519-526 (2002).

147 Wu, Y.-H. *et al.* Distribution of MT1 melatonin receptor immunoreactivity in the human hypothalamus and pituitary gland: colocalization of MT1 with vasopressin, oxytocin, and corticotropin-releasing hormone. *J. Comp. Neurol.* **499**, 897-910, doi:10.1002/cne.21152 (2006).

148 Baraban, J. M. & Aghajanian, G. K. Suppression of firing activity of 5-HT neurons in the dorsal raphe by alpha-adrenoceptor antagonists. *Neuropharmacology* **19**, 355-363 (1980).

149 Simonneaux, V. & Ribelayga, C. Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacological reviews* **55**, 325-395, doi:10.1124/pr.55.2.2 (2003).

150 Fuller, P. M., Gooley, J. J. & Saper, C. B. Neurobiology of the sleep-wake cycle: sleep architecture, circadian regulation, and regulatory feedback. *J Biol Rhythms* **21**, 482-493, doi:10.1177/0748730406294627 (2006).

151 Levine, S. & Treiman, D. M. Differential Plasma Corticosterone Response to Stress in Four Inbred Strains of Mice. *Endocrinology* **75**, 142-144, doi:10.1210/endo-75-1-142 (1964).

152 Armario, A., Castellanos, J. M. & Balasch, J. Chronic and acute stress interrelationship: corticosterone response. *Horm Metab Res* **13**, 413-414, doi:10.1055/s-2007-1019286 (1981).

153 Armario, A., Garcia-Marquez, C. & Jolin, T. The effects of chronic stress on corticosterone, GH and TSH response to morphine administration. *Brain Res* **401**, 200-203 (1987).

154 Torres-Farfan, C. *et al.* mt1 Melatonin receptor in the primate adrenal gland: inhibition of adrenocorticotropin-stimulated cortisol production by melatonin. *J Clin Endocrinol Metab* **88**, 450-458, doi:10.1210/jc.2002-021048 (2003).
155 Raghavendra, V. & Kulkarni, S. K. Melatonin reversal of DOI-induced hypophagia in rats; possible mechanism by suppressing 5-HT(2A) receptor-mediated activation of HPA axis. *Brain Res* **860**, 112-118 (2000).
156 Swaab, D. F., Bao, A. M. & Lucassen, P. J. The stress system in the human brain in depression and neurodegeneration. *Ageing Res Rev* **4**, 141-194 (2005).
157 Judge, S. J., Ingram, C. D. & Gartside, S. E. GABA receptor modulation of 5-HT neuronal firing: characterization and effect of moderate in vivo variations in glucocorticoid levels. *Neurochem Int* **45**, 1057-1065 (2004).
158 Judge, S. J., Ingram, C. D. & Gartside, S. E. Moderate differences in circulating corticosterone alter receptor-mediated regulation of 5-hydroxytryptamine neuronal activity. *J Psychopharmacol* **18**, 475-483 (2004).
159 Clark, J. A. *et al.* Glucocorticoid modulation of tryptophan hydroxylase-2 protein in raphe nuclei and 5-hydroxytryptophan concentrations in frontal cortex of C57/Bl6 mice. *Mol Psychiatry* **13**, 498-506, doi:10.1038/sj.mp.4002041 (2008).
160 Espallergues, J. *et al.* HDAC6 regulates glucocorticoid receptor signaling in serotonin pathways with critical impact on stress resilience. *Journal of Neuroscience* **32**, 4400-4416, doi:10.1523/JNEUROSCI.5634-11.2012 (2012).
161 Laaris, N., Haj-Dahmane, S., Hamon, M. & Lanfumey, L. Glucocorticoid receptor-mediated inhibition by corticosterone of 5-HT1A autoreceptor functioning in the rat dorsal raphe nucleus. *Neuropharmacology* **34**, 1201-1210 (1995).
162 Crino, O. L., Driscoll, S. C. & Breuner, C. W. Corticosterone exposure during development has sustained but not lifelong effects on body size and total and free corticosterone responses in the zebra finch. *Gen Comp Endocrinol* **196**, 123-129, doi:10.1016/j.ygcen.2013.10.006 (2014).
163 Csaba, G. & Fulop, A. K. Localisation of 3H-corticosterone inside thymocytes at different stages of ontogenetic development. *J Submicrosc Cytol* **19**, 567-571 (1987).
164 Diez, J. A., Sze, P. Y. & Ginsburg, B. E. Postnatal development of mouse plasma and brain corticosterone levels: new findings contingent upon the use of a competitive protein-binding assay. *Endocrinology* **98**, 1434-1442, doi:10.1210/endo-98-6-1434 (1976).
165 Henning, S. J. Plasma concentrations of total and free corticosterone during development in the rat. *Am J Physiol* **235**, E451-456 (1978).
166 Su-Ying Tsai, C.-Y. C., Wen-Ming Hsu, Tung-Ping Tom Su, Jorn-Hon Liu & Chou, a. P. ASSOCIATION BETWEEN VISUAL IMPAIRMENT AND DEPRESSION IN THE ELDERLY. *J Formos Med Assoc* **102**, 86-90 (2003).

167 Albrecht, U. Invited review: regulation of mammalian circadian clock genes. *J Appl Physiol* **92**, 1348-1355 (2002).
168 Yan, L., Takekida, S., Shigeyoshi, Y. & Okamura, H. Per1 and Per2 gene expression in the rat suprachiasmatic nucleus: circadian profile and the compartment-specific response to light. *Neuroscience* **94**, 141-150 (1999).
169 Jones, J. R., Tackenberg, M. C. & McMahon, D. G. Manipulating circadian clock neuron firing rate resets molecular circadian rhythms and behavior. *Nat Neurosci* **18**, 373-375, doi:10.1038/nn.3937 (2015).
170 Rao, A., Harms, K. J. & Craig, A. M. Neuroligation: building synapses around the neurexin-neuroligin link. *Nat Neurosci* **3**, 747-749, doi:10.1038/77636 (2000).

171 Craig, A. M. & Kang, Y. Neurexin-neuroligin signaling in synapse development. *Curr Opin Neurobiol* **17**, 43-52, doi:10.1016/j.conb.2007.01.011 (2007).
172 Varoqueaux, F., Jamain, S. & Brose, N. Neuroligin 2 is exclusively localized to inhibitory synapses. *European journal of cell biology* **83**, 449-456, doi:10.1078/0171-9335-00410 (2004).
173 Talebizadeh, Z. *et al.* Do known mutations in neuroligin genes (NLGN3 and NLGN4) cause autism? *J Autism Dev Disord* **34**, 735-736 (2004).
174 Kohl, C. *et al.* Hippocampal neuroligin-2 links early-life stress with impaired social recognition and increased aggression in adult mice. *Psychoneuroendocrinology* **55**, 128-143, doi:10.1016/j.psyneuen.2015.02.016 (2015).
175 Kohl, C. *et al.* Hippocampal neuroligin-2 overexpression leads to reduced aggression and inhibited novelty reactivity in rats. *PLoS One* **8**, e56871, doi:10.1371/journal.pone.0056871 (2013).
176 van der Kooij, M. A. *et al.* Impaired hippocampal neuroligin-2 function by chronic stress or synthetic peptide treatment is linked to social deficits and increased aggression. *Neuropsychopharmacology* **39**, 1148-1158, doi:10.1038/npp.2013.315 (2014).
177 Ciau-Uitz, A., Wang, L., Patient, R. & Liu, F. ETS transcription factors in hematopoietic stem cell development. *Blood cells, molecules & diseases* **51**, 248-255, doi:10.1016/j.bcmd.2013.07.010 (2013).
178 Plaznik, A., Danysz, W., Kostowski, W., Bidzinski, A. & Hauptmann, M. Interaction between noradrenergic and serotonergic brain systems as evidenced by behavioral and biochemical effects of microinjections of adrenergic agonists and antagonists into the median raphe nucleus. *Pharmacol Biochem Behav* **19**, 27-32 (1983).
179 Heym, J., Trulson, M. E. & Jacobs, B. L. Effects of adrenergic drugs on raphe unit activity in freely moving cats. *Eur J Pharmacol* **74**, 117-125 (1981).