2015

G protein-coupled inwardly-rectifying potassium (GIRK) chanels mediate entrainment of circadian rhythms

Lauren Marie Hablitz
University of Alabama at Birmingham

G PROTEIN-COUPLED INWARDLY-RECTIFYING POTASSIUM (GIRK) CHANNELS MEDIATE ENTRAINMENT OF CIRCADIAN RHYTHMS

by

LAUREN M. HABLITZ

KAREN L. GAMBLE, COMMITTEE CHAIR
RITA M. COWELL
CATHERINE M. FULLER
LINDA OVERSTREET-WADICHE
MARTIN E. YOUNG

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2015

G PROTEIN-COUPLED INWARDLY-RECTIFYING POTASSIUM (GIRK) CHANNELS MEDIATE ENTRAINMENT OF CIRCADIAN RHYTHMS

LAUREN M. HABLITZ

CELL, MOLECULAR, AND DEVELOPMENTAL BIOLOGY;
GRADUATE BIOMEDICAL SCIENCES

ABSTRACT

Circadian rhythms are 24-hour cycles in biological and behavioral processes. These cycles enable an organism to predict changes in its environment, like changes in food availability and seasonality. Although endogenously driven, these rhythms can entrain or synchronize to daily changes in the environment, allowing the animal to adapt. One way entrainment occurs is shifts in circadian phase following the presentation of nonphotic, or non-light, stimuli, such as exercise, arousal, or stress at certain times of day. The molecular mechanisms underlying nonphotic entrainment are poorly understood - specifically, how nonphotic cues alter excitability within the suprachiasmatic nucleus (SCN) of the hypothalamus, the clock center of the mammalian brain, to change the timing of circadian rhythms. This dissertation tests the hypothesis that nonphotic stimuli activate G protein-coupled inwardly-rectifying potassium (GIRK) channels which decrease neuronal excitability, modulating the timing of circadian rhythms. We show that not only is GIRK channel protein and function regulated in a circadian manner within the SCN, but it is responsible for maintaining daytime resting membrane potential of these neurons even in the absence of a nonphotic signaling cue. Mice lacking the GIRK2 subunit have altered circadian entrainment, corresponding to decreased neuropeptide Y (NPY, a nonphotic neurotransmitter) signaling within SCN neurons. Loss of GIRK channel signaling also inhibits exogenous melatonin-induced phase-shifting of behavioral

rhythms and decreased firing of SCN neurons, indicating that GIRK channels are necessary for two different nonphotic neurotransmitters within the SCN - NPY and melatonin. Finally, activation of GIRK channels is sufficient to mimic a nonphotic phase shift, indicating that GIRK channel activation may be a conserved response within the SCN to nonphotic stimuli. This dissertation is the first to demonstrate a direct link between nonphotic neurotransmitters, a specific ion channel at the membrane, and subsequent regulation of circadian rhythm timing both within the SCN and in animal locomotor behavior.

ACKNOWLEDGEMENTS

I want to thank my wonderful lab mates and especially my mentor, Karen Gamble, for being the perfect kind of crazy. Knowing they were there made me excited to go to work. Without them my graduate school experience would have been immeasurably more difficult.

Thank you to Rita Cowell, Cathy Fuller, Linda Overstreet-Wadiche, and Martin Young for being on my committee and guiding me through graduate school. Most people don't look forward to four hour committee meetings. I did, thank you for that. Also, thank you to Robin Lester for introducing me to how fun electrophysiology and neuroscience research could be.

Thank you to my family. These past five years have been radically life changing for all of us, but we are on the path to happiness. I know I have their love and support in all that I do, even if they can't be here with me.

And finally, a special thank you to my sister, Michelle. She has had to listen, in detail, to every trial, tribulation, and success of my time at UAB. I'm grateful she has been there, and can never repay her for helping me keep my "muchness."

TABLE OF CONTENTS

LIST OF TABLES

SUPRACHIASMATIC NUCLEUS FUNCTION AND CIRCADIAN ENTRAINMENT ARE MODULATED BY G PROTEIN-COUPLED INWARDLY RECTIFYING (GIRK) CHANNELS

LIST OF FIGURES

INTRODUCTION

SUPRACHIASMATIC NUCLEUS FUNCTION AND CIRCADIAN ENTRAINMENT ARE MODULATED BY G-PROTEIN COUPLED INWARDLY-RECTIFYING POTASSIUM (GIRK) CHANNELS

INTRODUCTION

Circadian rhythms: an overview

We live in a complex environment. The sun drives critical dynamics such as day length, seasonality, temperature climates, resource competition, predator/prey dynamics, and ultimately, population growth. Most organisms on earth have developed 24-hour cycles in biological and behavioral processes that enable them to synchronize and predict daily changes in the environment. These cycles are called circadian rhythms. These rhythms include processes such as sleep/wake cycles, hormonal rhythms like cortisol and melatonin secretion, cognition, immune system function, and basic cellular metabolism (1, 2). Disruption of circadian rhythms has been linked to multiple diseases, such as depression (3, 4), epilepsy (5-7), cardiac arrhythmias (8-11), and many more.

At a cellular level, rhythms are controlled by a transcription/translation feedback loop commonly referred to as the "molecular clock." In brief, two basic helix-loop-helix transcription factors: circadian locomotor output cycles kaput (CLOCK) and brain-derived muscle arnt-like factor (BMAL) bind together and activate the promotor region of period (PER) and cryptochrome (CRY). PER and CRY then are produced, post-translationally modified, and are translocated back into the nucleus to inhibit the binding of the CLOCK/BMAL complex to their own promotors, creating a negative feedback loop and decreasing PER/CRY production (Figure 1). This feedback loop has multiple interconnecting circuits and influences rhythmic expression of clock-controlled genes governing a variety of cellular processes (12-14).

In mammals, circadian rhythms are orchestrated by the suprachiasmatic nucleus (SCN) of the hypothalamus within the brain. SCN lesions in several rodent models leads

to arrhythmic locomotor activity (15). Human patients with cancer-induced lesions of the SCN exhibit arrhythmic body temperature and disrupted sleep/wake rhythms (16, 17). Not only is the SCN necessary for rhythm maintenance, but several transplant studies have shown that implantation of a functioning SCN to the third ventricle in SCN-ablated rodents restores rhythmicity and is sufficient to determine free-running period (the intrinsic day-length of an organism in constant conditions without environmental resetting cues like light) (18, 19).

The SCN is unique in that each individual neuron regulates its excitability in a self-sustaining, oscillatory manner independently from other neurons in this area (20). During the day, action potential firing rates are higher within SCN neurons, corresponding to more depolarized resting membrane potentials. Conversely, at night these neurons are relatively silent and exhibit more hyperpolarized resting membrane potentials. These tightly regulated changes in electrical activity are due to highly coordinated expression and regulation of ion channels (21, 22). For example, slow-inactivating sodium channels regulate the initiation of SCN action potentials (23). Voltage-gated calcium channels co-modulate the initiation of the action potential (24), are sufficient to drive oscillations at more depolarized membrane potential (24, 25), and may play a key role in linking membrane excitability to intracellular signaling and the molecular clock (26-28). A wide variety of potassium channels modulate the firing rate differentially between the day and night (29-36). For example, large conductance potassium (BK) channels modulate spike timing by increasing the amplitude of the action potential after-hyperpolarization at night (33, 34). Finally, potassium leak channels may play a role in governing resting membrane potential (22, 37, 38). It is important to note

that although these ion channels have been implicated in regulating SCN excitability, the SCN is a heterologous structure (39, 40). Indeed, studies of dissociated SCN neurons show that while most individual neurons exhibit rhythmic firing, the amplitude of these rhythms differs greatly among the population, indicating that ion channel regulation may differ throughout the SCN (41, 42).

Ultimately, these neurons are synchronized within the SCN by a variety of factors including vasoactive intestinal peptide (43-45) to coordinate and convey time-of-day information. From there, the SCN projects directly to multiple areas of the brain including the amygdala, arcuate nucleus, and paraventricular nucleus, regulating multiple processes throughout the body (46).

Although surprising, the SCN keeps the same rhythmicity (high firing during the day, low firing at night) regardless of the temporal niche of the animal, indicating that diurnality or nocturnality is downstream of the core circadian oscillator (47). Previous reports comparing laboratory rats to diurnal grass rat (Arvicanthis niloticus) have identified the lateral subparaventricular zone (SPZ) as a potential regulator of diurnality. These studies show day-active grass rats have synchronized expression levels for c-Fos protein that are high during the day and low at night in both SCN and SPZ, whereas night-active laboratory rats exhibit c-Fos expression that is out of phase between the SCN and SPZ (48-50). Indeed, data from our lab shows that exclusively diurnal tree shrews have higher firing rates in both the SCN and SPZ during the day, corresponding with significantly higher cFOS positive neurons in the day compared to the night (Figure 2).

A key feature of the circadian clock is their ability to adapt and predict what behavioral patterns are necessary for day-to-day survival in the organism. The way

circadian rhythms entrain (synchronize) to environmental stimuli is by shifting the phase of the rhythm, or altering the time of sleep-wake behavior the next day so that it starts earlier (phase advance) or later (phase delay) depending on the timing of the input. Traditionally, entrainment is broken into two categories: photic (light-driven) and nonphotic (exercise, stress, arousal, etc.,) (1, 47, 51). Each cue has a different efficacy over the course of the day. For example, light exposure in the early night phase delays rhythms, shifting the phase to a later time on subsequent cycles, whereas light exposure in the late night phase advances rhythms so that the cycle begins earlier on subsequent days. The magnitude of these shifts can be plotted over time, defining a phase response curve (PRC) (52).

Nonphotic entrainment

The first "nonphotic" phase response curve was developed in response to novel wheel running in Syrian hamsters (Figure 3). These animals were restricted to a closed wheel for 2 hours at different times throughout the subjective day in constant darkness. The results showed that animals advanced their activity rhythms after running in the wheel during the middle of the day and delayed activity rhythms after wheel running during the late night, with the magnitudes of phase shifts directly proportional to the amount of wheel running (53). In a separate experiment, subcutaneous injections of saline induce phase advances during the day with minimal effect at night (Figure 3) (54, 55). These "nonphotic" effects are very different from those induced by light, indicating there are alternative entrainment pathways within the SCN.

Neuropeptide Y as a nonphotic entrainment neurotransmitter

The SCN receives light information from the retina through two different pathways. The direct pathway is through the retinohypothalamic tract, which travels from the retina, and directly innervates the SCN. The indirect pathway leads from the retina to the intergeniculate leaflet (IGL) of the thalamus, which extends neuropeptide Y (NPY) positive projections to the SCN (56, 57). Initially, the IGL was thought to only convey light information, but later studies showed that ablation of the IGL lengthens the free-running period of rodents and eliminates the effects of novel wheel on circadian phase, yet the animals have no difficulty entraining to light (58-61). These experiments demonstrated that the IGL projection to the SCN is not necessary for photic entrainment, though NPY does inhibit the effects of a light pulse in the early night (62-65), indicating that the IGL regulates some forms of photic signaling. Several experimental findings suggest that NPY release from the IGL is primarily involved in nonphotic neurotransmission to the SCN. First, wheel running activates the IGL, as measured by cFOS expression, yet not the SCN (55, 66). In addition, NPY injected into the third ventricle of hamsters produces a similar phase response curve as wheel running in rodents (67, 68). Also, when antisera to NPY is injected to the third ventricle, the effects of novel wheel are eliminated (69). Daytime microinjections to the SCN with NPY receptor Y_1 and Y_2 agonists induces phase advances of behavior in hamsters, with the Y_2 agonist-induced shifts greater than the Y_1 agonists (70). Finally, NPY knockout mice fail to alter free-running period in response to prolonged wheel-running activity (71).

Serotonin as a nonphotic entrainment neurotransmitter

The raphe nucleus is a cluster of serotonergic cells that projects directly to the SCN (72, 73). This area is mostly active when the animal is moving and awake. Initial studies depleting rats and hamsters of 5-HT positive neurons with 5,7-dihydroxytryptamine showed that animals were still rhythmic, but had an advanced phase angle in a light/dark (LD) cycle (activity onset started earlier with respect to lights-off than control animals) (74) as well as lengthened free-running period in constant light conditions (75). These studies demonstrate that serotonergic projections regulate circadian activity, but are not required to maintain an endogenous rhythm. It is important to note that ablation of serotonergic neurons does not eliminate the phase advancing effects of a novel wheel in hamsters (76), nor do serotonin agonists influence the magnitude of activity-induced phase shifts (77), which is why serotonin is thought to play a secondary role in the nonphotic pathways, such as the development of circadian rhythms (78-80) and mediating arousal and mood cues (81, 82) to the SCN. However, serotonin injections into the third ventricle do induce phase advances similar to that of NPY (83). Also, application of 5-HT and 5-HT1 receptor agonists phase advance SCN firing rhythms during the day (84, 85), with delays to no effect at night, similar to NPY. Thus, serotonin has a traditional nonphotic phase response curve (Figure 3).

Melatonin as a nonphotic entrainment neurotransmitter

Unlike NPY and serotonin, the pineal gland, the primary source of melatonin in the mammalian brain, does not directly innervate the SCN. Instead, the pineal gland is regulated through a multi-synaptic pathway (from the SCN to the paraventricular nucleus, the intermediolateral cell column of the thoracic spinal cord, the superior cervical

ganglion, which then innervates the pineal gland) (86), and regulates endogenous release of melatonin during the dark phase of the animal. As such, endogenous melatonin is not a typical nonphotic stimuli, but instead provides the organism with photoperiodic information encoding day length and acts as an anti-arousal cue (86-91). This being said, melatonin supplementation is an effective phase-shifting agent in humans and animal models. In rodents, melatonin application during the day phase advances both free-running locomotor behavior and SCN firing rate (92-95). The phase response curve is similar to melatonin administration in humans, with an advancing effect in the late day, and a delay during the night (96-98), indicating that melatonin, when given at different circadian phases, acts as a nonphotic stimuli (Figure 3).

A conserved nonphotic pathway?

As indicated above, NPY, serotonin, and melatonin all have a similar nonphotic phase response curve, phase advancing rhythms during the day with a minimal delay zone at night (Figure 3). Another facet of serotonin and NPY signaling is that they can block the phase advancing effects of light (62, 63, 99-102). Not only are the PRCs and ability to modulate light similar, but when applied to the SCN, both NPY and melatonin hyperpolarize the membrane of individual SCN neurons and reduce firing rate within the SCN (103-106). Finally, based on several studies applying agonists to the G protein-coupled receptors for NPY, melatonin, and 5-HT, the main response from these neurotransmitters is conveyed by $G_{i/o}$ heterotrimeric G protein signaling (76, 94, 107, 108). Indeed, the effects of melatonin within the SCN are pertussis toxin sensitive (109, 110). These attributes (PRCs, interaction with photic stimuli, influence on SCN

neurophysiology, and reliance upon G protein signaling) suggest there is a conserved response to nonphotic stimuli within the SCN to entrain circadian rhythms in a similar manner.

GIRK channels as a nonphotic convergence point

In other areas of the brain, NPY and serotonin have been shown to decrease neuronal excitability by activating G protein-coupled inwardly-rectifying potassium (GIRK) channels (111-114). Also, NPY, melatonin, and serotonin receptors are coupled with pertussis toxin-sensitive G proteins (110, 115-117), which can activate GIRK channels. GIRK channels are inwardly-rectifying and can be identified as an inward potassium current at greatly hyperpolarized potentials (beyond physiological resting membrane potential) (118). At more depolarized potentials in the physiological range of most neurons, GIRK channels pass an outward potassium current, causing membrane hyperpolarization and decreased neuronal excitability (118) similar to the effects of NPY, serotonin, and melatonin. Thus, GIRK channel activation is an ideal candidate mediator of nonphotic cues within the SCN. Additionally, GIRK channels may play a role in modulating day-night differences in SCN excitability, influencing photic entrainment as well.

GIRK channel physiology

GIRK channels are tetrameric channels that can be composed of four different subunits, GIRK1-4 (119). GIRK1 and GIRK2 are found primarily postsynaptically, and GIRK3 is normally found presynaptically. GIRK1-3 are present at similar levels

throughout the brain, and increase with age (120). The GIRK1 subunit lacks a membrane targeting motif and thus requires another subunit as a binding partner to be expressed, but increases single channel conductance and opening probability of the channel (118, 121). Although most research has focused on the postsynaptic mechanisms of regulation by GIRK1 and GIRK2, mRNA for all four GIRK subunits have been found in the supraoptic nucleus (122), and GIRK4 has been found in the arcuate nucleus and in lower levels throughout the brain (123, 124), indicating that future research should include GIRK3 and GIRK4.

GIRK channels are coupled to and activated by the βγ subunits of Gi/o heterotrimeric G proteins (118). These channels allow large inward potassium currents at negative potentials, with little outward current at more positive potentials. This inward rectification is caused by a cation block in the pore region of each GIRK subunit, where magnesium or large organic cations, like spermine or putrescine, bind and prevent outward current flow (125). The cation block in GIRK channels is not as efficient as other inward-rectifiers, and thus they are categorized as "moderate rectifiers (119)."

GIRK channels regulate intrinsic membrane properties and general firing characteristics of neurons throughout the brain. Within CA1, adenosine receptor A1-coupled GIRK channels hyperpolarize resting membrane potential by ~8 pA in dorsal neurons, with little to no effect on membrane properties of ventral neurons (126). Serotonin-induced depolarization of neurons in the lateral amygdala requires both TRPC activation and GIRK channel inhibition (127). In addition, the endogenous opioid receptor ligand dynorphan inhibits proopiomelanocortin cells within the arcuate nucleus by GIRK channel activation (128). These modulatory effects of GIRK channels on firing

rate and resting membrane potential can be modulated by different firing patterns of neurons within the ventral-tegmental area (129). Thus, GIRK channels can have cell autonomous, neuron-to-neuron, and network regulatory roles (118). To date, little is known about whether GIRK channels are expressed in the SCN and how/if they regulate neuronal firing.

Hypothesis and major objectives

Based on what is known about nonphotic signaling within the SCN and GIRK channel physiology, the main hypothesis of this dissertation is: **nonphotic stimuli activate GIRK channels which decrease neuronal excitability, modulating the timing of circadian rhythms.** Three main questions will be discussed in the subsequent two chapters including: 1) are GIRK channels present within the SCN, and if so are they regulated in either a light-driven or circadian manner, 2) how does endogenous GIRK channel activation alter SCN excitability (such as spontaneous firing rate, and intrinsic membrane properties such as input resistance and resting membrane potential of individual SCN neurons), 3) can GIRK channels mediate multiple nonphotic cues, specifically NPY and melatonin (Figure 4), and finally, 4) is GIRK channel activation sufficient to cause a nonphotic phase shift. These questions will be answered with a variety of techniques including western blotting protein analysis, whole cell patch clamp electrophysiology, real time bioluminescence monitoring of the molecular clock, and circadian behavioral analysis.

This dissertation is broken into two chapters, the first investigating the role of GIRK channels in SCN physiology, and ultimately nonphotic entrainment. The second

chapter investigates how GIRK channels mediate the phase shifting and neuronal silencing effects of G protein-coupled melatonin signaling within the SCN. Ultimately, the goal of this dissertation is to have a better understanding of the fundamental mechanisms behind nonphotic entrainment, and how regulation of excitability within the SCN influences circadian phase of the SCN and the animal.

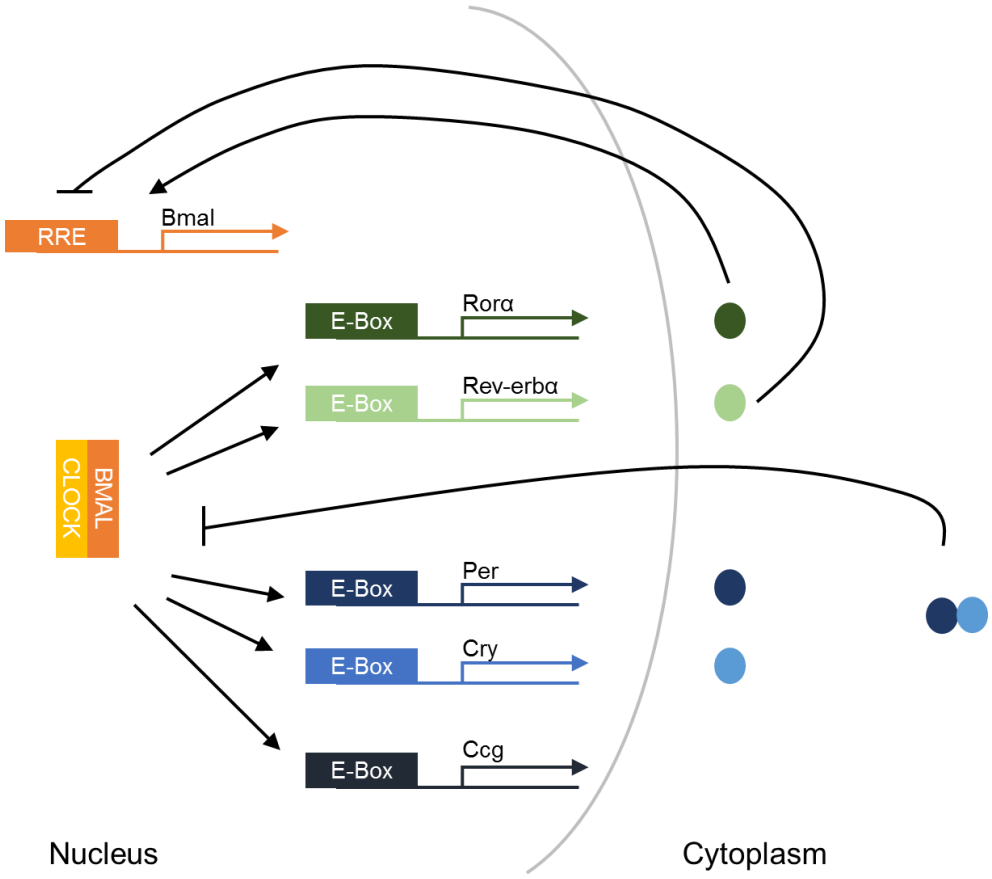

Figure 1: A basic schematic of the molecular clock. This diagram includes the negative feedback loop of Per and Cry inhibiting their own transcription, and the positive feedback loop of Rorα and Rev-erbα directly modulating BMAL expression.

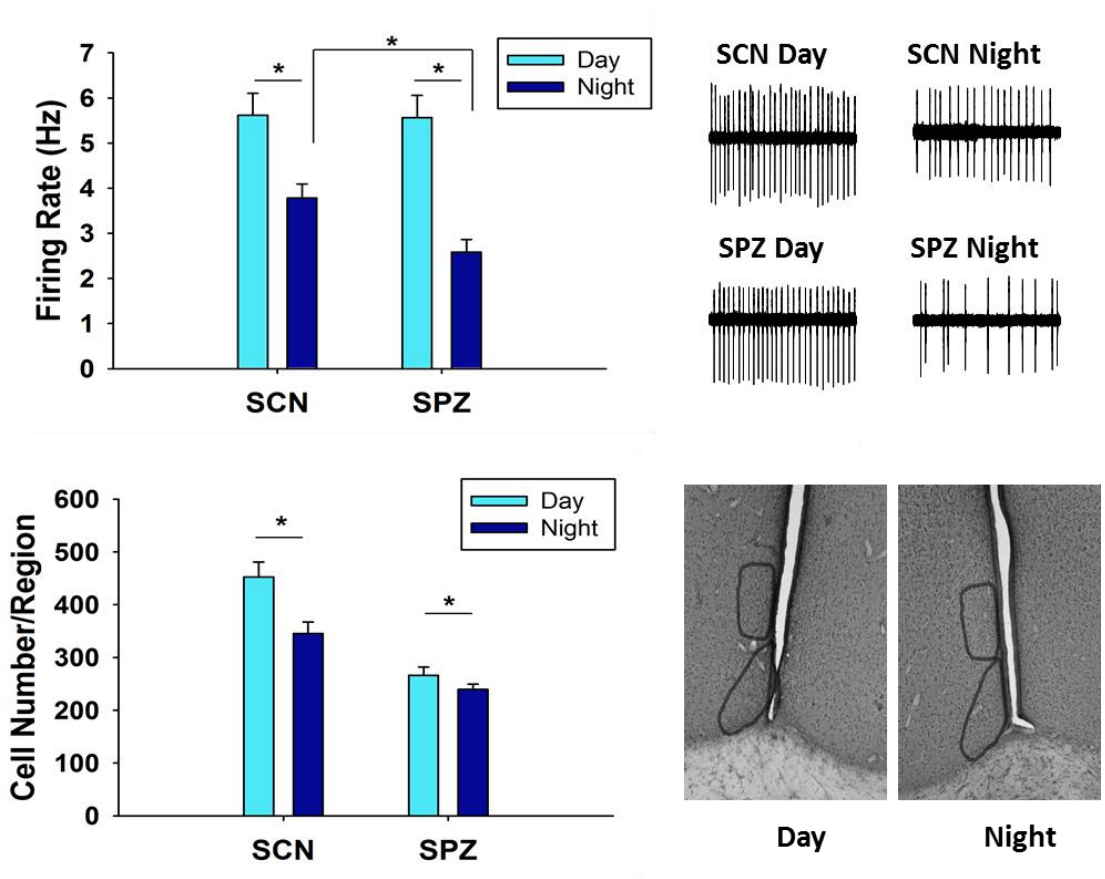

Figure 2: The SCN and SPZ exhibit increased spontaneous firing rate and cFOS positive cells during the day in the diurnal tree shrew. Top: Spontaneous firing rates (mean ± SEM) for SCN and SPZ neurons during the day and night (left). Representative loose-patch traces (right, 5s). *$p < 0.05$, n = 3 slices, >75 cells/group. Bottom: Number of cFOS positive cells (mean ± SEM) for SCN and SPZ regions of interest during the day and night (left). Representative IHC during the day and night (right). *$p < 0.05$, n = at least 2 animals, ≥3 slices/group.

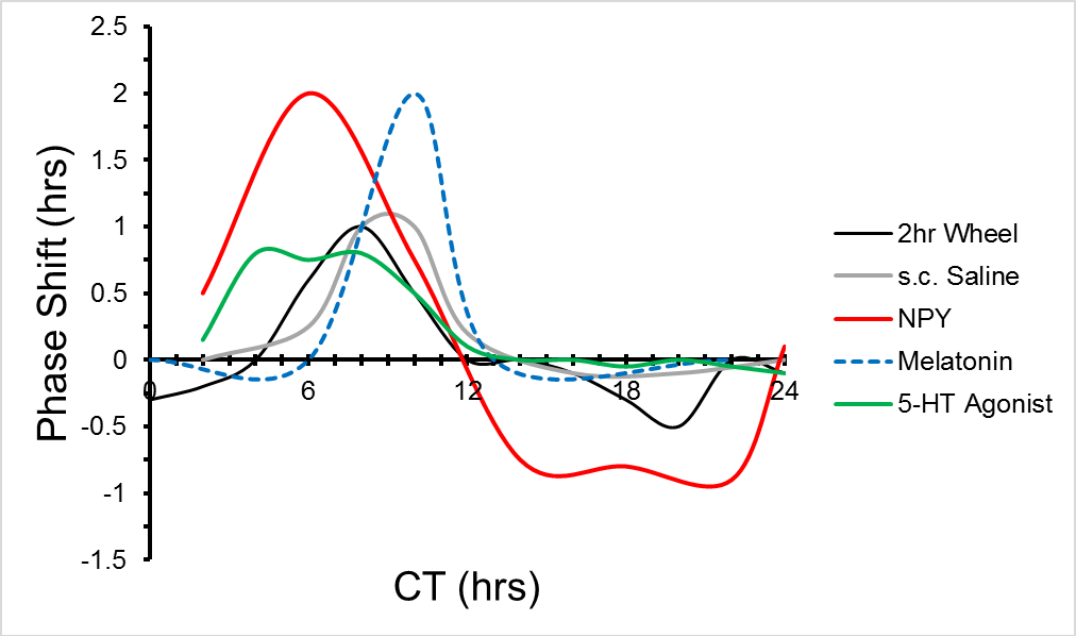

Figure 3: Cumulative nonphotic phase response curves. Phase response curves to 2hr novel wheel (53), sub cutaneous saline injections (54), microinjections of NPY into the third ventricle (67), I.P. injections of melatonin (dotted line indicates predicted curve based on 2 time points) (130), and microinjections of serotonin agonists (83) were given to hamsters in constant darkness at different CTs. Notice most stimuli induce phase advances during the subjective day with minimal delays during subjective night.

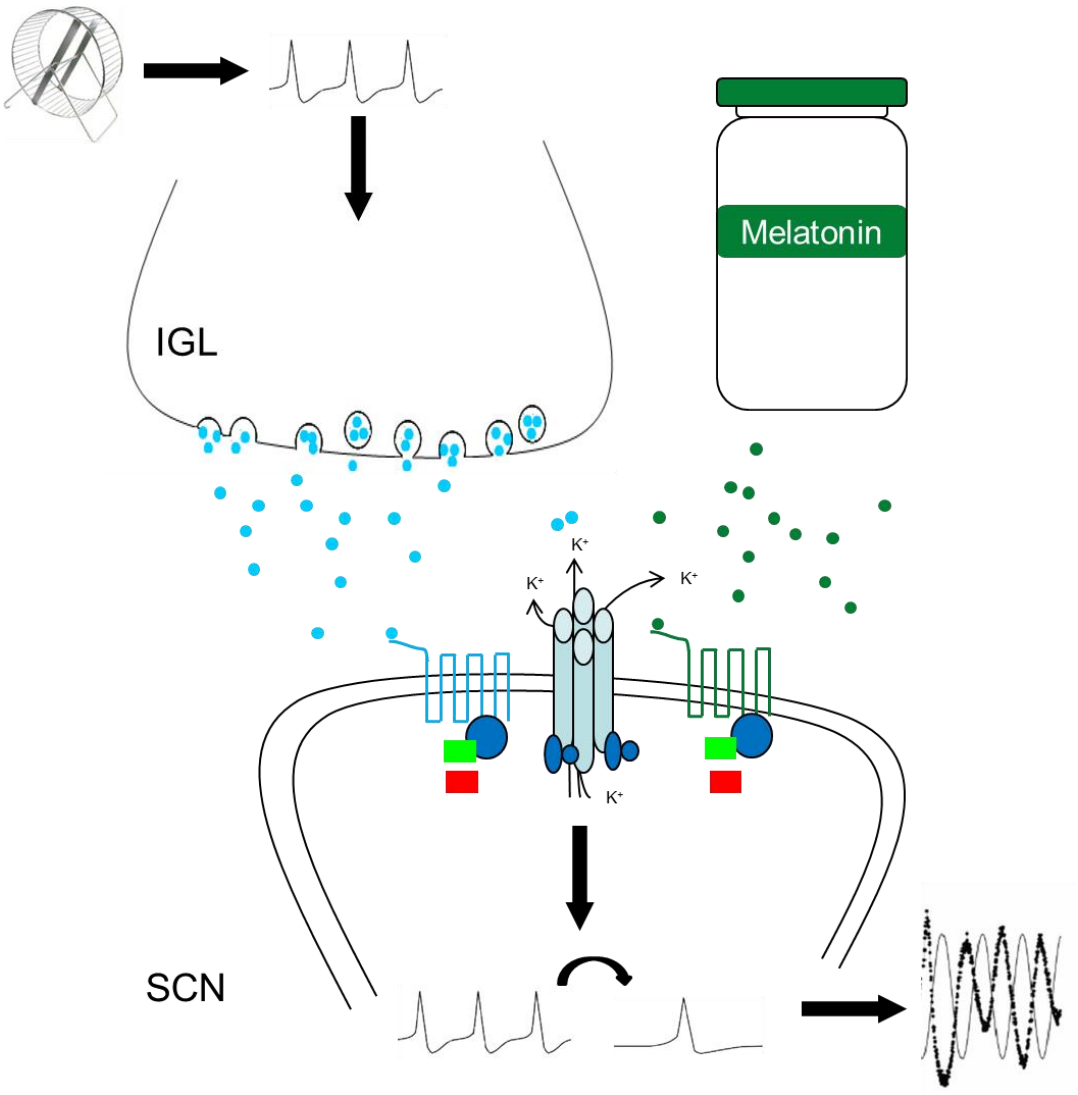

Figure 4: A Model of GIRK mediated nonphotic entrainment. During the day both nonphotic stimuli-induced activation of the IGL releases NPY onto SCN neurons, and exogenous application of melatonin activates Gi/o coupled GPCRs, opening GIRK channels. Efflux of K+ ions hyperpolarizes the membrane and decreases firing rate of action potentials. This causes a phase advance of the molecular clock and wheel running activity.

SUPRACHIASMATIC NUCLEUS FUNCTION AND CIRCADIAN ENTRAINMENT ARE MODULATED BY G-PROTEIN COUPLED INWARDLY-RECTIFYING POTASSIUM (GIRK) CHANNELS

by

LAUREN M. HABLITZ, HYLTON E. MOLZOF, JODI R. PAUL, RUSSELL L. JOHNSON, AND KAREN L. GAMBLE

Journal of Physiology 2014 Nov 15; 592(Pt 22):5079-92

CHAPTER 1

SUPRACHIASMATIC NUCLEUS FUNCTION AND CIRCADIAN ENTRAINMENT ARE MODULATED BY G-PROTEIN COUPLED INWARDLY-RECTIFYING POTASSIUM (GIRK) CHANNELS

Abstract

G protein signaling within the central circadian oscillator, the suprachiasmatic nucleus (SCN), is essential for conveying time-of-day information. We sought to determine whether G protein-coupled inwardly-rectifying potassium channels (GIRKs) modulate SCN physiology and circadian behavior. We show that GIRK current and GIRK2 protein expression are greater during the day. Pharmacological inhibition of GIRKs and genetic loss of GIRK2 depolarized the day-time resting membrane potential of SCN neurons compared to controls. Behaviorally, GIRK2 KO mice failed to shorten free running period in response to wheel access in constant darkness and entrained more rapidly to a 6-hr advance of a 12:12LD cycle than WT littermate controls. We next examined whether these effects were due to disrupted signaling of neuropeptide Y (NPY), which is known to mediate nonphotic phase shifts, attenuate photic phase shifts and activate GIRKs. Indeed, GIRK2 KO SCN slices had significantly fewer silent cells in response to NPY. This deficit likely contributed to the absence of NPY-induced phase-advances of *Per2*$^{Luc+/-}$ rhythms in organotypic SCN cultures from GIRK2 KO mice. Finally, GIRK channel activation is sufficient to cause a nonphotic-like phase advance of PER2::LUC rhythms. These results suggest that rhythmic regulation of GIRK2 protein and channel

function in the SCN contributes to day-time resting membrane potential, providing a mechanism for the fine tuning responses to nonphotic and photic stimuli. Further investigation could provide insight into disorders with circadian disruption comorbidities such as epilepsy and addiction, in which GIRK channels have been implicated.

Introduction

The central circadian oscillator in the suprachiasmatic nucleus (SCN) of the hypothalamus contains ~20,000 coupled neurons (Welsh et al., 2010). A transcription/translation feedback loop called the "molecular clock" drives rhythmic gene expression in individual SCN neurons and regulates daily oscillations in action potential firing and excitability, with increased neuronal firing during the day and relative quiescence during the night (Kuhlman & McMahon, 2006). This day/night variation in electrical output from the SCN drives circadian rhythms in other brain areas and the body. The timing of these molecular and neurophysiological rhythms can be altered by environmental cues including photic (light) and nonphotic (stress, exercise, etc.) stimuli (Albrecht, 2012). These alterations or phase shifts occur daily, entraining the animal to its environment. Although much is known about the neurotransmitter systems that underlie these entraining pathways, the molecular mechanisms that couple receptor activation to the changes in SCN neuronal output that ultimately shift circadian phase and drive entrainment are not fully understood.

One mediator of intracellular phase shifting signals is G protein signaling, and many of these second messenger pathways are critical for maintaining SCN rhythmicity and enabling photic and nonphotic entrainment (Cheng et al., 2004; Aton et al., 2006;

Dahdal et al., 2010; Doi et al., 2011; Brancaccio et al., 2013). We hypothesize that this abundance of crucial G protein signaling in SCN neurons may activate G protein-coupled inwardly-rectifying potassium (GIRK) channels, which have been implicated in diseases such as epilepsy and addiction (Hibino et al., 2010; Luscher & Slesinger, 2010). Furthermore, GIRK channel activation within the SCN may alter neurophysiological function and the ability of environmental stimuli to reset circadian clock phase. We use a variety of electrophysiological, behavioral, and molecular assays to determine: when GIRK channel protein levels and activation are highest within the SCN, the effect of GIRK channel activation on SCN neuronal function, and the necessity of GIRK channels for circadian entrainment.

Methods

Ethical approval

All animal care, handling, and housing were in compliance with the University of Alabama at Birmingham's Institutional Animal Care and Use Committee guidelines.

Animals and housing

All mice in these experiments were 2-4 months of age to reduce developmental or aging phenotypes (Turek et al., 1995; Biello, 2009). Only male mice were used for western blotting and behavioral experiments (Ruiz de Elvira et al., 1992; Vyazovskiy et al., 2006). Three separate mouse lines were used: 1) C57/BL/6 mice (western blotting experiments); 2) GIRK2 knockout animals on a C57/BL6 background (Signorini et al., 1997) (electrophysiology and circadian behavior); and 3) Per2Luc+/- mice (Yoo et al.,

2004) on a C57/BL6 background crossed with the GIRK2 line for at least 2 generations before use in experiments (bioluminescence assays of molecular clock function). Separate cohorts of mice were used for each different type of experiment.

Western blotting

For light/dark (LD) experiments (Fig. 1AB), mice were group housed with food and water ad libitum in a 12:12 LD cycle. Mice were killed every 4 hours over a 24-hour period (Zeitgeber Time 0 or ZT0, defined as lights on). For constant darkness (DD) experiments (Fig. 1CD), mice were single-housed on running wheels in DD for two weeks. Animals were killed during either the subjective day at circadian time 4 (CT4; CT 12 defined activity onset) or subjective night (CT 16). Activity onset was predicted using linear regression analysis for the preceding seven activity onsets. For all experiments during the dark, mice were killed by cervical dislocation followed by enucleation with the aid of night vision goggles in order to prevent photic signaling during brain extraction. Hypothalamic slices (600 μm) were prepared using a vibroslicer (7000smz, Campden Instruments Ltd., Lafayette, IN), followed by dissection of the SCN under a Zeiss dissection microscope. Protein lysates were prepared, and immunoblotting for GIRK1 (1:500, Alamone Labs, Israel) and GIRK2 (1:750, Millipore, MA, USA) was performed by loading 10μg of protein per sample as analyzed by a BCA protein assay. Previous studies have shown that GIRK1 and GIRK2 both exhibit multiple bands in western blots within the brain. For GIRK1, the three bands indicate different glycosylation states (Koyrakh et al., 2005; Aguado et al., 2008). GIRK2 exhibits multiple splice variants (Lesage et al., 1995; Koyrakh et al., 2005; Aguado et al., 2008). For densitometry

analysis, all bands for each protein were quantified together for assessment of total protein levels, and the uppermost heavily glycosylated band of GIRK1 (75 kDa, Fig. 1) was analyzed for rhythmicity of GIRK1 glycosylation. For the LD analysis, each blot was normalized to the mean ZT5 time point as a positive control. β-Actin (1:40,000, Millipore, MA, USA) was used as a loading control.

Electrophysiology

Mice were killed either at ZT 2 or ZT10.5 (day and night recordings respectively) by cervical dislocation. For whole-cell electrophysiology, all recordings were made between projected ZT 3-9 (day) or ZT 12-17 (night). For loose-patch electrophysiology, all recordings were made between projected ZT 4-6 in the presence of 2.35 μM NPY (American Peptide, Sunnyvale, CA) or vehicle (water). Brains were harvested, sectioned at 200 μm on a vibroslicer (7000smz, Campden Instruments Ltd., Lafayette, IN), and transferred to an open recording chamber (Warner Instruments, Hamden, CT) that was continuously perfused at a rate of 2.0 ml/min with extracellular solution consisting of (in mM) NaCl (124), NaHCO3 (20), Na2HPO4 (1), MgSO4 (1.3), glucose (10), KCl (3.5), CaCl2 (2.5; added the day of the experiment) with osmolality adjusted to 300-305 mOsm), bubbled with 5% CO2 / 95% O2, and heated to 34 ± 0.5 ºC. Neurons were visualized with an Olympus BX51WI (Olympus America Inc., Center Valley, PA) using infrared-differential interference contrast optics. Electrodes with a pipette resistance of ~4-6 MΩ were filled with filtered, potassium gluconate solution consisting of (in mM): K-gluconate (135), KCl (10), HEPES (10), EGTA (0.5), then adjusted to pH 7.4 with KOH (as in Kuhlman & McMahon, 2004). Electrophysiological signals were processed

and controlled by a Multiclamp 700B amplifier, and pClamp 10.02 software (Axon Instruments, Union City, CA). Recordings were sampled at 20 kHz and filtered at 10 kHz. In order to block synaptic transmission (as in Fig. 2): bicuculline (30µM) and CdCl2 (200µM) (Sigma-Aldritch, St. Louis, MO), D-AP5 (50µM) and CNQX (10µM) (Abcam, Cambrige, MA), and TTX (1µM) (Tocris Biosciences, Minneanapolis, MN) were added to the bath solution. To isolate GIRK currents (Fig. 2) the concentration of KCl was increased from 3.5 mM to 30 mM in order to increase potassium conductance across the membrane (refer to (Fu et al., 2004)). The GIRK channel antagonist Tertiapin-Q (0.2 µM) (Alamone Labs, Israel) was used for experiments in Fig. 3. Input resistance and resting membrane potential were calculated as specified in (Kuhlman et al., 2003). Resting membrane potential, action potential amplitude, and firing rate were calculated from 30-s gap-free current clamp recordings. All cells included in these analyses had, in voltage-clamp mode, ≤35 pA holding current to clamp membrane potential at −65 mV and an action potential amplitude of greater than 10pA during the current clamp step protocol. All data were collected within 6 min of membrane rupture to minimize any potential washout effects from the whole-cell recording (Schaap et al., 1999). Loose patch recordings were obtained in gap-free mode with an average seal resistance of 39 MΩ. Average spike rate was calculated from at least one minute of the two minute trace. Induced currents for ML297 and NPY application were determined by holding the cell at -80mV in gap-free voltage clamp mode in a high potassium (30mM) solution (as in (Hamasaki et al., 2013)). Either ML297 (10µM) or NPY (2.35µM) were bath applied for at least 1 minute. The change in current following drug application was compared to that of high potassium alone within the same cell. For all electrophysiological experiments, at

least 3 biological replicates with at least 4 cells per animal were used, unless otherwise indicated. There was no specific regional bias when recording within the SCN.

Behavioral Analysis

All mice were housed in individual wheel cages. Wheel-running activity or general cage activity (via infrared motion-sensors from Spy Town, Melville, NY; as in Fig. 4AB) was recorded and analyzed using Clocklab software (Actimetrics, Wilmette, IL). For behavioral analysis, one WT animal was excluded due to low activity/equipment failure. Free-running period was measured by chi-square periodogram analysis of 7-10 days in constant dark conditions (Fig. 4). One KO mouse (out of 8) had a free running period of 23.3h, which was 6.5 SD's from the mean and was therefore excluded from behavioral analysis. Activity profiles from mice housed on wheels in constant darkness were acquired using Clocklab software. Each profile was aligned to CT 12, activity onset. Then, the average counts of WT and KO mice during the subjective day and subjective night were compared. Entrainment was defined as the first day when both the activity onset and alpha length (activity period) were within twenty minutes of those predicted 4 days prior to the light change. Clocklab software was used to determine activity onset. Offset was defined as the last activity bout where three out of six of the previous bouts were above 3 revolutions per minute. Alpha length was calculated by subtracting onset time from offset time.

Bioluminescence Assays

Organotypic SCN cultures from GIRK2 KO or WT Per2Luc+/- mice were prepared and treated within the first 7 days for one hour beginning at CT 3-5 (where CT 12 is defined as peak bioluminescence, drug timing specified for each experiment) with 2.35 µM NPY (American Peptide, Sunnyvale, CA), 10µM ML297 (Days et al., 2010) or vehicle (either water or 0.02% DMSO respectively), using identical culture media and methods described in (Besing et al., 2012). Data were acquired and analyzed with Lumicycle Analysis software (Actimetrics, Inc, Wilmette, IL), and recordings with a goodness of fit greater than 85% were used for analysis. Phase shifts were calculated by comparing two predictions for the time of the first peak post-treatment: one prediction determined from at least three cycles before treatment and a second prediction determined from at least three cycles after treatment. The difference between these two predictions indicated the size of the phase shift.

Statistical Analysis

All statistical analysis was performed with PASW Statistics 18. GIRK channel protein expression was analyzed for rhythmicity using a cosinor analysis (as in (Bray et al., 2013)). For comparisons of means, an independent samples t-test or ANOVA was used for comparisons between two means or two or more means, respectively. Two factor designs were analyzed with a two-way ANOVA with repeated measures when appropriate. In cases of a non-normal distribution, a nonparametric Kruskal-Wallis test followed by median test post hoc analyses. Finally, to assess whether the number of silent cells changed in response to NPY treatment, a G likelihood-ratio test was used. Post hoc

analyses employed a Fisher's exact test, with a Bonferroni-corrected alpha of 0.025. For all other tests, significance was ascribed at $P < 0.05$.

Results

GIRK channel protein and function is regulated in a circadian manner within the SCN, influencing day-time neurophysiology.

To determine if GIRK1 and GIRK2 channel subunits were present within the SCN, and if levels of these proteins changed over the course of the day, we analyzed SCN samples from mice at 4-h time points across a 12:12 Light/Dark (LD) cycle. Using western blot analysis, GIRK1 and GIRK2 protein was present within the SCN, and GIRK2 (but not GIRK1) expression patterns significantly fit a 24-h rhythm (cosinor analysis; GIRK1: $R^2= 0.114$, $P > 0.05$; GIRK2: $R2= 0.184$, $P < 0.05$; n=3-5/time point) with peak GIRK2 protein levels occurring at ZT 6.47 ± 0.3h (amplitude, -0.25 ± 0.1; mesor, 0.59 ± 0.1; Fig. 1AB). Because GIRK1 shows variation in the heavily glycosylated state, the glycosylated state was analyzed for rhythmicity and showed diurnal variation but failed to reach statistical significance ($R^2= 0.195$, $P = 0.06$). Although GIRK2 expression exhibited a 24-h rhythm in LD, it was necessary to examine protein levels in the SCN from animals housed in constant darkness (DD) to assess whether this rhythm was endogenously generated rather than driven by the light cycle. GIRK2 protein expression from animals housed for at least 14 days in DD had significantly higher protein levels during the subjective day (CT4 based on activity onset) compared to night (CT 16; independent samples t-test, $t(5) = 2.83$, $P< 0.05$; n= 3-4/time

point). As expected, levels of both the heavily glycosylated form and total GIRK1 did not vary between time points (Fig. 1CD).

In order to determine whether higher levels of GIRK2 protein during the day could contribute to a change in basal GIRK activation over the course of the day, we used whole-cell, voltage-clamp electrophysiology and pharmacological inhibition of synaptic transmission (TTX (1µM), bicuculline (30µM), D-AP5 (50µM), CNQX (10µM), and CdCl2 (200µM)) along with increased KCl (30mM) for increased potassium conductance, in order to isolate currents from SCN neurons from wild-type and GIRK2 knockout animals in response to a slow ramp (2.5 s) from -140 mV to -20 mV. Peak inward current was significantly greater during the day in WT neurons (-88.1 ± 5.7 pA) compared to night (-61.3 ± 4.6 pA), and this difference was not seen in KO neurons (day: -64.4 ± 5.4pA; night:-67.9 ± 6.3pA) (two-way ANOVA, genotype by time interaction: $F(1,122) = 6.53$, $P < 0.01$; Tukey HSD post hoc comparison, $P < 0.05$ Fig. 2; n= ≥ 20 cells/group), showing that day-night differences in this current is specific to increased levels of GIRK2 (Fig. 2).

GIRK channels are known to decrease cellular excitability by hyperpolarizing the membrane (Luscher & Slesinger, 2010). To determine whether loss of GIRK2 plays a role in regulating SCN neuronal membrane properties, we measured resting membrane potential (RMP) of WT and KO SCN neurons during the day and night (n = ≥25 cells/group). Consistent with previous results (Kuhlman & McMahon, 2004), there was an overall day/night difference in RMP (Kruskal Wallis test, $H(3) = 37.403$, $P < 0.01$; median post hoc test, $P < 0.05$ for both WT day compared to night, and KO day compared to night; Fig. 3AB). As predicted, KO neurons were significantly more

depolarized than WT neurons during the day only (WT day: -41.6 ± 1.0 mV; KO day: -36.5 ± 1.1 mV; median post hoc test, $P < 0.05$). To eliminate the possibility that the depolarized RMP in KO mice was driven by altered regulation of ion channel expression to compensate for loss of GIRK2, we applied a GIRK channel antagonist, Tertiapin-Q (TPQ) (0.2 μM), to WT slices during the day (Fig. 3B). We found that TPQ caused a similar magnitude of depolarization (-36.9 ± 1.0 mV, n = 38 cells), demonstrating that GIRK channels are necessary for maintaining normal day-time resting membrane potential. There were no significant differences in either input resistance (Kruskal Wallis test, $H(3) = 2.662$, $P > 0.05$), action potential firing rate (Kruskal Wallis test, $H(3) = 4.879$, $P > 0.05$), or peak current amplitude of action potential firing (Kruskal Wallis test, $H(3) = 2.744$, $P > 0.05$) among groups (Fig. 3CDE), indicating that GIRK channels primarily regulate resting membrane potential.

Loss of GIRK2 alters the behavioral response to nonphotic and photic cues.

After defining the temporal pattern of GIRK subunit expression and describing a role for GIRK channels in setting day-time resting membrane potential, we next evaluated the functional role of GIRK2 in the entrainment of behavioral locomotor rhythms. There were no behavioral differences between heterozygous and WT animals (data not shown), and therefore, the following results are from WT-KO comparisons only. First, we examined general cage activity of mice single-housed with the running wheel locked because several studies have shown that wheel running (a nonphotic entraining stimulus) shortens the period of behavioral locomotor rhythms in DD (Edgar et al., 1991a; Edgar et al., 1991b; Kuroda et al., 1997; Deboer & Tobler, 2000; Harrington

et al., 2007). Activity monitored with infrared motion sensors revealed no significant differences between genotypes in terms of average activity counts or circadian rhythmic amplitude in LD (Fig. 4AB; Table 1). In addition, WT and KO mice had very similar period lengths (or tau) when placed into DD (Table 1, n =15-16/group). To test whether loss of GIRK2 alters the period shortening response to the nonphotic stimulus of wheel running (Harrington et al., 2007), we placed WT and KO animals on running wheels and assessed tau in DD (Fig. 4BC). In an LD cycle, there was no difference between KO and WT average activity counts (Table 1). In DD, KO animals had higher daily activity in general (Table 1); however, this effect was mostly driven by increased activity of KO mice during the subjective night (mean ± SEM counts, WT night: 2,867.5 ± 303.0, KO night: 3,996.6 ± 252.7) but not during the day (mean ± SEM counts, WT day: 287.2 ± 37.9, KO day: 296.0 ± 60.5) consistent with reports of hyperactivity in these mice (Blednov et al., 2001). A repeated measures ANOVA revealed a significant time-of-day by genotype interaction ($F(2,21) = 4.906$, $P < 0.05$) with KO night activity significantly greater than WT night activity (Tukey HSD post hoc comparison, $P <0.05$; Fig. 4BC). In contrast to general cage activity, KO mice with access to a running wheel did not exhibit the shorter free running period observed in WT mice (mean ± SEM, WT: 23.80 ± 0.04 h, KO: 23.95 ± 0.04 h; $t(13) = 2.46$, $P < 0.05$, n=7-8/group; $\eta^2 = 0.32$) indicating that GIRK2 is necessary for proper nonphotic signaling in response to wheel running.

Because the behavioral response to nonphotic cues was altered upon loss of GIRK2, we examined whether GIRK2 contributes to photic entrainment as well. Mice housed in a 12:12 LD cycle were subjected to a 6-h phase advance of the LD cycle (Fig. 5), and the number of days required for re-entrainment was determined. Entrainment was

defined as the first day in which the activity length returned to the same length observed before the light shift (see Materials and Methods). Mice lacking GIRK2 required approximately half the number of days to re-entrain to the new light cycle compared to WT (mean ± SEM days to entrain, WT: 7.2 ± 0.5, KO: 3.5 ± 0.8; $t(10) = 4.07$, $P < 0.01$; n = 6/group). However, KO mice entrained to a 6-h phase delay at the same rate as WT (mean ± SEM days to entrain, WT: 4.7 ± 0.7, KO: 4.7 ± 0.5; $t(10) = -0.23$, $P > 0.05$; n = 6/group). These results indicate that GIRK2 signaling slows the rate of re-entrainment to photic phase advances, but not delays.

Neuropeptide Y signaling in the SCN requires GIRK2.

In rodents, disruption of the nonphotic neurotransmitter NPY abolishes the period shortening effect of wheel access (Pickard et al., 1987; Pickard, 1994; Kuroda et al., 1997; Lewandowski & Usarek, 2002; Harrington et al., 2007). Because NPY signaling also antagonizes phase advances to light (Yannielli & Harrington, 2000; Lall & Biello, 2003; Yannielli et al., 2004), disruption of the NPY-dependent nonphotic signaling pathway may explain the loss of wheel running-induced period shortening and the enhanced photic entrainment of KO mice. Specifically, we hypothesized that NPY-induced phase shifts within the SCN requires GIRK2 activation because application of NPY to the SCN in vivo or in vitro during the day induces phase advances in behavior and neuronal activity rhythms (Yannielli & Harrington, 2000; Maywood et al., 2002; Lall & Biello, 2003; Yannielli et al., 2004; Besing et al., 2012) and NPY-induced effects in other brain regions require GIRK activation (Paredes et al., 2003; Fu et al., 2004).

First, we investigated whether the suppressive effects of NPY on SCN neuron firing rate (van den Pol et al., 1996; Besing et al., 2012) were lost in GIRK2 KO mice using loose patch electrophysiology with and without 2.35 μM NPY in the bath ($n = \geq 45$ cells/group). Spontaneous firing rate was significantly different among the four groups (Kruskal Wallis test, $H(3) = 79.271$, $P < 0.01$). Surprisingly, firing rates of WT and KO neurons did not differ (median post hoc test, $P > 0.05$; Fig. 6A); however, KO neurons exhibited significantly higher firing rates than WT neurons in response to NPY (mean ± SEM, WT: 0.8 ± 0.2 Hz, KO: 2.2 ± 0.3 Hz; median post hoc test, $P < 0.01$; Fig. 6AB). Contingency analysis revealed a significant effect of genotype and treatment on the percentage of silent cells ($\chi^2(3) = 53.193$, $P < 0.01$). Specifically, in vehicle-treated slices, the number of silent cells was not significantly different between KO and WT (Fisher's exact test, $P > 0.05$); however, WT slices treated with NPY had significantly more silent cells than did NPY-treated KO slices (Fisher's exact test, $P < 0.01$; Fig. 6BC), suggesting that NPY failed to silent many of the SCN neurons in absence of GIRK2. In order to determine whether GIRK channels directly mediate NPY-induced current, gap-free, whole-cell voltage clamp was used to hold the cells at -80mV in high potassium (30mM) in order to readily measure the GIRK-mediated current (as in (Hamasaki et al., 2013)). Upon application of NPY (2.35 μM), 4 out of 5 cells responded to NPY with inward current (range, 15 - 119 pA; mean ± SEM, 44.5 ± 23.2 pA , n = 4 cells from two animals). Of these, 3 out of 4 cells had reduced inward current by ~50% in response to TPQ (0.2 μM). TPQ alone had a net inward current of 3.1 ± 4.4 pA (n = 4 cells from two animals). These results indicate that GIRK channels mediate at least part of the NPY-induced current within SCN neurons, consistent with the partial effect of NPY in the

GIRK2 KO animals (Fig. 6). This failure to significantly reduce excitability in GIRK2 KO animals may impede NPY-dependent phase shifts to the circadian clock.

To test this hypothesis, we crossed GIRK2 WT and KO animals onto the Per2Luc+/- reporter line (see Materials and Methods) and measured the phase shifting effect of NPY on the molecular clock. Because these animals showed no difference in behavioral circadian rhythmicity (Fig. 4), it was not surprising that the PER2::LUC rhythms showed no difference in period between genotypes pre-treatment (mean period ± SEM, WT: 24.42 ± 0.12h, KO: 24.38 ± 0.16h; $t(28) = 0.99$, $P > 0.05$). As has been previously published by Besing et al. (2012) and more recently by Belle et al. (2014), 1-h treatment with 2.35 µM NPY during the early day (CT 4-5) produced ~3-h phase advances in PER2::LUC rhythms in WT mice; however, phase advances in KO animals were reduced to the level of controls (two-way ANOVA, genotype by treatment interaction: $F(1,26) = 4.77$, $P < 0.05$; Tukey HSD post hoc, $P < 0.05$, Fig. 7; n= 6-8 cultures/group). Thus, these results suggest that GIRK2 is necessary for NPY-induced phase advances within the SCN, and that loss of NPY signaling may be an underlying cause for the circadian entrainment alterations observed in GIRK2 KO mice.

GIRK channel activation is sufficient to cause a nonphotic-like phase shift.

We have shown that GIRK channels partially mediate NPY-induced silencing of SCN neurons as well as NPY-induced phase advances of the molecular clock. However, GIRK channels can be fully-opened by a variety of neurotransmitter signaling (Luscher & Slesinger, 2010), including other nonphotic signals. To test the hypothesis that GIRK channel activation was sufficient to mimic nonphotic signals, we applied 10µM ML297,

a GIRK channel agonist (Days et al., 2010; Kaufmann et al., 2013), for one hour to PER2::LUC SCN cultures starting between CT3-4. This concentration was sufficient to induce GIRK currents in SCN neurons (-60.3 ± 9.7, n= 3 cells), consistent with recent papers demonstrating ML297 specificity in cultured hippocampal neurons (Wydeven et al., 2014). Activation of GIRK channels with ML297 significantly phase advanced PER2::LUC rhythms compared to vehicle-treated controls (mean ± SEM, ML297: 3.6 ± 1.1, vehicle: 0.3 ± 0.2; $t(5.23) = -3.10$, $P < 0.05$; Fig. 8; n= 6 cultures/group), suggesting a broader role for GIRK channels in mediating nonphotic signals.

Discussion

Although G protein signaling is critical for circadian rhythmicity (Cheng et al., 2004; Aton et al., 2006; Zuberi et al., 2008; Doi et al., 2011; Brancaccio et al., 2013), the role of G protein-coupled potassium channels, and specifically GIRK channels, in modulating time-of-day cues has not been studied. This paper is the first to show that a single, circadian-regulated GIRK channel subunit can modulate SCN neurophysiology, mediate the effects of daytime NPY, and alter behavior in response to both photic and nonphotic cues. Specifically, we found that GIRK2 protein and function exhibited an endogenous rhythm within the SCN with a peak around midday. This increase during the day was necessary for proper maintenance of day-time resting membrane potential within SCN neurons since GIRK channel inhibition or GIRK2 knockout further depolarized resting membrane potential. Furthermore, animals with genetic loss of GIRK2 showed altered entrainment properties, such that the GIRK2 KO animals (compared to WT mice) re-entrained to a 6-h phase advance of the light-dark cycle more rapidly and did not

exhibit period-shortening with wheel access. These behavioral phenotypes may be due, in part, to a loss of NPY signaling within the SCN as indicated by the reduced effects of NPY on firing rate of SCN neurons in GIRK2 KO animals as well as impaired NPY-induced phase advances in GIRK2 KO organotypic cultures of the SCN. Finally, GIRK channel activation was sufficient to cause a daytime phase advance in PER2::LUC rhythms. Taken together, the results of our study indicate a role for circadian regulation of GIRK channels in modulation of neurophysiological rhythms and establishing proper responses to time-of-day stimuli.

The circadian regulation of neural activity in SCN neurons has been well-documented with peak excitation occurring during the day (Kuhlman & McMahon, 2006). GIRK channels are inwardly-rectifying and can be identified as an inward potassium current at greatly hyperpolarized potentials (beyond physiological resting membrane potential). At more depolarized potentials in the physiological range of most neurons, GIRK channels pass an outward potassium current, causing membrane hyperpolarization and decreased neuronal excitability (Luscher & Slesinger, 2010). In the present study, GIRK2 channel protein and current was highest during the day (Fig. 1 and 2). Therefore, we hypothesized that GIRK channel activation during the day may act as a stop-gate on excitability. Indeed, we found that loss of GIRK2 channels or pharmacological GIRK channel inhibition resulted in depolarization of the resting membrane potential by ~5 mV (Fig. 3). This basal GIRK-mediated hyperpolarization is consistent with the effects of GIRK channel activation by neurotransmitters such as NPY, serotonin, melatonin, glutamate, acetylcholine, and GABA in a variety of central and peripheral areas (Krapivinsky et al., 1995; Nelson et al., 1996; Luscher et al., 1997; Fu et

al., 2004; Acuna-Goycolea et al., 2005; Luscher & Slesinger, 2010). For example, GIRK channels have been shown to couple directly with GABAB receptors (Luscher et al., 1997; Arora et al., 2011), and GABA signaling within the SCN is dependent upon Gi/o G proteins (Aton et al., 2006) which are necessary for GIRK channel opening.

The neurotransmitters listed above also play key roles in regulating the timing of circadian rhythms (Albrecht, 2012). Underlying mechanisms of nonphotic entrainment, or synchronization of rhythms to cues like exercise or stress, remain largely understudied even though they block the effects of light and reset clock phase both in rodents and humans (Hastings et al., 1998; Mistlberger & Skene, 2005). For example, both melatonin and NPY induce large phase advances of locomotor behavior and spike rate rhythms during the day and hyperpolarize the resting membrane potential in a potassium-sensitive manner (Jiang et al., 1995; Hall et al., 1999; Scott et al., 2010). Our results showed that GIRK2 activation was necessary and sufficient to induce these large nonphotic-like, phase advances of the molecular clock (Fig. 6 and 8). It is interesting to note that both NPY and melatonin hyperpolarize the membrane and reduce spontaneous firing rate of SCN neurons when treated during the subjective day (Jiang et al., 1995; Hall et al., 1999; Scott et al., 2010). Based on these observations, it can be speculated that these channels mediate multiple phase-resetting signals and may act as a convergence point for nonphotic signaling, resulting in similar phase response curves for serotonin, NPY, and melatonin (Yannielli & Harrington, 2004). An important function of increased GIRK current during the day could be to allow for daytime environmental cues to phase shift the SCN through hyperpolarization. This hypothesis is supported by the fact that activation of GIRK channels with ML297 during the early day is sufficient to cause a

phase-advance of molecular clock rhythms (Fig. 8). However, GIRK channel activation does not preclude possible co-activation of second messenger signaling cascades by nonphotic cues such as PKC activation in response to NPY (Biello et al., 1997). Indeed, NPY still has a partial effect on spike rate in GIRK2 KO animals (Fig. 6).

Photic and nonphotic entraining stimuli can interact and in general, are mutually inhibitory. For example, NPY injection into the SCN region of light-exposed hamsters in the late night attenuates the characteristically ensuing phase advance and up-regulation of clock gene expression (Yannielli et al., 2004; Gamble et al., 2006). Conversely, NPY blockade of NPY Y5 receptors enhance light-induced phase advances (Yannielli et al., 2004). Similar experiments have been done with light mimicked by the glutamate receptor agonist NMDA, such that phase advances in SCN firing or wheel running behavior were diminished by NPY and NPY antagonists (Gamble et al., 2004; Soscia & Harrington, 2004; Yannielli & Harrington, 2004; Soscia & Harrington, 2005). In addition to acute phase shifts, NPY is also critical for other nonphotic behavioral effects, such as period shortening in response to wheel running. Elimination of the primary source of NPY to the SCN through lesions of the intergeniculate leaflet blocks period shortening induced by wheel access (Pickard et al., 1987; Pickard, 1994; Kuroda et al., 1997; Lewandowski & Usarek, 2002). This result is consistent with more recent evidence that NPY-deficient mice also fail to shorten free running period in response to wheel access (Harrington et al., 2007). The present data suggests that GIRK channel activation may be involved in these phenotypes. Specifically, loss of GIRK2 channels resulted in a lack of period shortening typically observed in response to nonphotic wheel access (Fig. 5) as well as enhanced phase advances in the light-dark cycle (Fig. 4). It appears that in

absence of GIRK signaling, entrainment to photic phase advances are functionally enhanced, while the nonphotic pathway is suppressed. Because there was no change in entrainment to photic delays, future studies should determine whether this photic phenotype is a broad strengthening of photic entrainment pathway, or simply a removal of nonphotic inhibition to photic phase advances in behavior. In addition, future studies should examine the role of GIRK in retinal ganglion cells and within IGL given its wide-spread expression in neurons (Signorini et al., 1997; Luscher & Slesinger, 2010). A better understanding of the nonphotic/photic interaction is an important area for future research given that an animal is unlikely to encounter a photic or nonphotic stimulus in isolation.

In the present study, we found that both GIRK2 protein expression and current amplitude were regulated over the day-night cycle. This result is consistent with the finding that GIRK2 mRNA expression in microarrays of SCN tissue is rhythmic (Pizarro et al., 2013), indicating that the gene transcribing GIRK2 mRNA (KCNJ6) may be under direct clock-control. In general, our understanding of the mechanisms underlying circadian regulation of ion channels is limited (Colwell, 2011), but some studies suggest epigenetic mechanisms or microRNA modifications may determine transcript stability (Wang, 2013), while other studies have shown that free radical homeostasis can also regulate ion channel gating (Wang et al., 2012). Determining clock control of ionic channel regulation in the SCN could provide insight into how ion channels are regulated in a circadian manner in other brain areas and tissues. GIRK channels have been implicated in regulating dopamine signaling within the VTA (Lomazzi et al., 2008; Arora et al., 2011). If GIRK channels are regulated in a time-of-day-sensitive manner in the

VTA (as in the SCN) then the present results may have broader implications for addiction and withdrawal and the circadian regulation of this disease.

GIRK channels have also been associated in diseases of hyper-excitability such as epilepsy (Loddenkemper et al., 2011; Zarowski et al., 2011), chronic atrial fibrillation (Capucci et al., 2012; Shusterman et al., 2012), and long QT syndrome (Jeyaraj et al., 2012; Takigawa et al., 2012). A key feature these disorders is that seizure or fibrillation onset is more likely to occur during the day in humans, suggesting that circadian control of GIRK channel function may underlie risk dependence on time-of-day. Indeed, microarray studies have shown that GIRK1 and GIRK4 transcripts are rhythmic in mouse whole-heart homogenate (Pizarro et al., 2013). Perhaps by understanding the interplay between GIRK channel dysfunction and circadian regulation of excitability throughout the body, novel time-of-day-sensitive therapeutics could be developed with fewer off-target effects.

Acknowledgements

L.M.H. and K.L.G. were responsible for conception and design of the experiments, collection, analysis and interpretation of the data, and drafting the article. H.E.M., J.R.P., and R.L.J. contributed to collection, analysis, and interpretation of the data. We thank Rita M. Cowell and Rachel C. Besing for technical assistance and Robin Lester for editing the manuscript

Additional information

No authors have any conflicts of interest. This work was supported by the National Institutes of Health Grants F31NS084683 to L.M.H, R00GM086683, and R01NS082413 to K.L.G.

References

Acuna-Goycolea C, Tamamaki N, Yanagawa Y, Obata K & van den Pol AN. (2005). Mechanisms of neuropeptide Y, peptide YY, and pancreatic polypeptide inhibition of identified green fluorescent protein-expressing GABA neurons in the hypothalamic neuroendocrine arcuate nucleus. *J Neurosci* 25, 7406-7419.

Aguado C, Colon J, Ciruela F, Schlaudraff F, Cabanero MJ, Perry C, Watanabe M, Liss B, Wickman K & Lujan R. (2008). Cell type-specific subunit composition of G protein-gated potassium channels in. *J Neurochem* 105, 497-511.

Albrecht U. (2012). Timing to perfection: the biology of central and peripheral circadian clocks. *Neuron* 74, 246-260.

Arora D, Hearing M, Haluk DM, Mirkovic K, Fajardo-Serrano A, Wessendorf MW, Watanabe M, Lujan R & Wickman K. (2011). Acute cocaine exposure weakens GABA(B) receptor-dependent G-protein-gated inwardly rectifying K+ signaling in dopamine neurons of the ventral tegmental area. *J Neurosci* 31, 12251-12257.

Aton SJ, Huettner JE, Straume M & Herzog ED. (2006). GABA and Gi/o differentially control circadian rhythms and synchrony in clock neurons. *Proc Natl Acad Sci U S A* 103, 19188-19193.

Belle MD, Hughes AT, Bechtold DA, Cunningham P, Pierucci M, Burdakov D & Piggins HD. (2014). Acute suppressive and long-term phase modulation actions of orexin on the mammalian circadian clock. *J Neurosci* 34, 3607-3621.

Besing RC, Hablitz LM, Paul JR, Johnson RL, Prosser RA & Gamble KL. (2012). Neuropeptide Y-induced phase shifts of PER2::LUC rhythms are mediated by long-term suppression of neuronal excitability in a phase-specific manner. *Chronobiol Int* 29, 91-102.

Biello SM. (2009). Circadian clock resetting in the mouse changes with age. *Age (Dordr)* 31, 293-303.

Biello SM, Golombek DA, Schak KM & Harrington ME. (1997). Circadian phase shifts to neuropeptide Y In vitro: cellular communication and signal transduction. *J Neurosci* 17, 8468-8475.

Blednov YA, Stoffel M, Chang SR & Harris RA. (2001). GIRK2 deficient mice. Evidence for hyperactivity and reduced anxiety. *Physiol Behav* 74, 109-117.

Brancaccio M, Maywood ES, Chesham JE, Loudon AS & Hastings MH. (2013). A Gq-Ca2+ axis controls circuit-level encoding of circadian time in the suprachiasmatic nucleus. *Neuron* 78, 714-728.

Bray MS, Ratcliffe WF, Grenett MH, Brewer RA, Gamble KL & Young ME. (2013). Quantitative analysis of light-phase restricted feeding reveals metabolic dyssynchrony in mice. *Int J Obes (Lond)* 37, 843-852.

Capucci A, Calcagnini G, Mattei E, Triventi M, Bartolini P, Biancalana G, Gargaro A, Puglisi A & Censi F. (2012). Daily distribution of atrial arrhythmic episodes in sick sinus syndrome patients: implications for atrial arrhythmia monitoring. *Europace* 14, 1117-1124.

Cheng HY, Obrietan K, Cain SW, Lee BY, Agostino PV, Joza NA, Harrington ME, Ralph MR & Penninger JM. (2004). Dexras1 potentiates photic and suppresses nonphotic responses of the circadian clock. *Neuron* 43, 715-728.

Colwell CS. (2011). Linking neural activity and molecular oscillations in the SCN. *Nat Rev Neurosci* 12, 553-569.

Dahdal D, Reeves DC, Ruben M, Akabas MH & Blau J. (2010). Drosophila pacemaker neurons require g protein signaling and GABAergic inputs to generate twenty-four hour behavioral rhythms. *Neuron* 68, 964-977.

Days E, Kaufmann K, Romaine I, Niswender C, Lewis M, Utley T, Du Y, Sliwoski G, Morrison R, Dawson ES, Engers JL, Denton J, Daniels JS, Sulikowski GA, Lindsley CW & Weaver CD. (2010). Discovery and Characterization of a Selective Activator of the G-Protein Activated Inward-Rectifying Potassium (GIRK) Channel. In *Probe Reports from the NIH Molecular Libraries Program*. National Center for Biotechnology Information (US), Bethesda (MD).

Deboer T & Tobler I. (2000). Running wheel size influences circadian rhythm period and its phase shift in mice. *J Comp Physiol A* 186, 969-973.

Doi M, Ishida A, Miyake A, Sato M, Komatsu R, Yamazaki F, Kimura I, Tsuchiya S, Kori H, Seo K, Yamaguchi Y, Matsuo M, Fustin JM, Tanaka R, Santo Y, Yamada H, Takahashi Y, Araki M, Nakao K, Aizawa S, Kobayashi M, Obrietan K, Tsujimoto G & Okamura H. (2011). Circadian regulation of intracellular G-protein signalling mediates intercellular synchrony and rhythmicity in the suprachiasmatic nucleus. *Nat Commun* 2, 327.

Edgar DM, Kilduff TS, Martin CE & Dement WC. (1991a). Influence of running wheel activity on free-running sleep/wake and drinking circadian rhythms in mice. *Physiol Behav* 50, 373-378.

Edgar DM, Martin CE & Dement WC. (1991b). Activity feedback to the mammalian circadian pacemaker: influence on observed measures of rhythm period length. *J Biol Rhythms* 6, 185-199.

Fu LY, Acuna-Goycolea C & van den Pol AN. (2004). Neuropeptide Y inhibits hypocretin/orexin neurons by multiple presynaptic and postsynaptic mechanisms: tonic depression of the hypothalamic arousal system. *J Neurosci* 24, 8741-8751.

Gamble KL, Novak CM & Albers HE. (2004). Neuropeptide Y and N-methyl-D-aspartic acid interact within the suprachiasmatic nuclei to alter circadian phase. *Neuroscience* 126, 559-565.

Gamble KL, Paul KN, Karom MC, Tosini G & Albers HE. (2006). Paradoxical effects of NPY in the suprachiasmatic nucleus. *Eur J Neurosci* 23, 2488-2494.

Hall AC, Earle-Cruikshanks G & Harrington ME. (1999). Role of membrane conductances and protein synthesis in subjective day phase advances of the hamster circadian clock by neuropeptide Y. *Eur J Neurosci* 11, 3424-3432.

Hamasaki R, Shirasaki T, Soeda F & Takahama K. (2013). Tipepidine activates VTA dopamine neuron via inhibiting dopamine D(2) receptor-mediated inward rectifying K(+) current. *Neuroscience* 252, 24-34.

Harrington M, Molyneux P, Soscia S, Prabakar C, McKinley-Brewer J & Lall G. (2007). Behavioral and neurochemical sources of variability of circadian period and phase: studies of circadian rhythms of npy-/- mice. *Am J Physiol Regul Integr Comp Physiol* 292, R1306-1314.

Hastings MH, Duffield GE, Smith EJ, Maywood ES & Ebling FJ. (1998). Entrainment of the circadian system of mammals by nonphotic cues. *Chronobiol Int* 15, 425-445.

Hibino H, Inanobe A, Furutani K, Murakami S, Findlay I & Kurachi Y. (2010). Inwardly rectifying potassium channels: their structure, function, and physiological roles. *Physiol Rev* 90, 291-366.

Jeyaraj D, Haldar SM, Wan X, McCauley MD, Ripperger JA, Hu K, Lu Y, Eapen BL, Sharma N, Ficker E, Cutler MJ, Gulick J, Sanbe A, Robbins J, Demolombe S, Kondratov RV, Shea SA, Albrecht U, Wehrens XH, Rosenbaum DS & Jain MK. (2012). Circadian rhythms govern cardiac repolarization and arrhythmogenesis. *Nature* 483, 96-99.

Jiang ZG, Nelson CS & Allen CN. (1995). Melatonin activates an outward current and inhibits Ih in rat suprachiasmatic nucleus neurons. *Brain Res* 687, 125-132.

Kaufmann K, Romaine I, Days E, Pascual C, Malik A, Yang L, Zou B, Du Y, Sliwoski G, Morrison RD, Denton J, Niswender CM, Daniels JS, Sulikowski GA, Xie XS, Lindsley CW & Weaver CD. (2013). ML297 (VU0456810), the First Potent and Selective Activator of the GIRK Potassium Channel, Displays Antiepileptic Properties in Mice. *ACS Chem Neurosci* 4, 1278-1286.

Koyrakh L, Lujan R, Colon J, Karschin C, Kurachi Y, Karschin A & Wickman K. (2005). Molecular and cellular diversity of neuronal G-protein-gated potassium channels. *J Neurosci* 25, 11468-11478.

Krapivinsky G, Gordon EA, Wickman K, Velimirovic B, Krapivinsky L & Clapham DE. (1995). The G-protein-gated atrial K+ channel IKACh is a heteromultimer of two inwardly rectifying K(+)-channel proteins. *Nature* 374, 135-141.

Kuhlman SJ & McMahon DG. (2004). Rhythmic regulation of membrane potential and potassium current persists in SCN neurons in the absence of environmental input. *Eur J Neurosci* 20, 1113-1117.

Kuhlman SJ & McMahon DG. (2006). Encoding the ins and outs of circadian pacemaking. *J Biol Rhythms* 21, 470-481.

Kuhlman SJ, Silver R, Le Sauter J, Bult-Ito A & McMahon DG. (2003). Phase resetting light pulses induce Per1 and persistent spike activity in a subpopulation of biological clock neurons. *J Neurosci* 23, 1441-1450.

Kuroda H, Fukushima M, Nakai M, Katayama T & Murakami N. (1997). Daily wheel running activity modifies the period of free-running rhythm in rats via intergeniculate leaflet. *Physiol Behav* 61, 633-637.

Lall GS & Biello SM. (2003). Attenuation of circadian light induced phase advances and delays by neuropeptide Y and a neuropeptide Y Y1/Y5 receptor agonist. *Neuroscience* 119, 611-618.

Lesage F, Guillemare E, Fink M, Duprat F, Heurteaux C, Fosset M, Romey G, Barhanin J & Lazdunski M. (1995). Molecular properties of neuronal G-protein-activated inwardly rectifying K+ channels. *J Biol Chem* 270, 28660-28667.

Lewandowski MH & Usarek A. (2002). Effects of intergeniculate leaflet lesions on circadian rhythms in the mouse. *Behav Brain Res* 128, 13-17.

Loddenkemper T, Vendrame M, Zarowski M, Gregas M, Alexopoulos AV, Wyllie E & Kothare SV. (2011). Circadian patterns of pediatric seizures. *Neurology* 76, 145-153.

Lomazzi M, Slesinger PA & Luscher C. (2008). Addictive drugs modulate GIRK-channel signaling by regulating RGS proteins. *Trends Pharmacol Sci* 29, 544-549.

Luscher C, Jan LY, Stoffel M, Malenka RC & Nicoll RA. (1997). G protein-coupled inwardly rectifying K+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. *Neuron* 19, 687-695.

Luscher C & Slesinger PA. (2010). Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. *Nat Rev Neurosci* 11, 301-315.

Maywood ES, Okamura H & Hastings MH. (2002). Opposing actions of neuropeptide Y and light on the expression of circadian clock genes in the mouse suprachiasmatic nuclei. *Eur J Neurosci* 15, 216-220.

Mistlberger RE & Skene DJ. (2005). Nonphotic entrainment in humans? *J Biol Rhythms* 20, 339-352.

Nelson CS, Marino JL & Allen CN. (1996). Melatonin receptors activate heteromeric G-protein coupled Kir3 channels. *Neuroreport* 7, 717-720.

Paredes MF, Greenwood J & Baraban SC. (2003). Neuropeptide Y modulates a G protein-coupled inwardly rectifying potassium current in the mouse hippocampus. *Neurosci Lett* 340, 9-12.

Pickard GE. (1994). Intergeniculate leaflet ablation alters circadian rhythms in the mouse. *Neuroreport* 5, 2186-2188.

Pickard GE, Ralph MR & Menaker M. (1987). The intergeniculate leaflet partially mediates effects of light on circadian rhythms. *J Biol Rhythms* 2, 35-56.

Pizarro A, Hayer K, Lahens NF & Hogenesch JB. (2013). CircaDB: a database of mammalian circadian gene expression profiles. *Nucleic Acids Res* 41, D1009-1013.

Ruiz de Elvira MC, Persaud R & Coen CW. (1992). Use of running wheels regulates the effects of the ovaries on circadian rhythms. *Physiol Behav* 52, 277-284.

Schaap J, Bos NP, de Jeu MT, Geurtsen AM, Meijer JH & Pennartz CM. (1999). Neurons of the rat suprachiasmatic nucleus show a circadian rhythm in membrane properties that is lost during prolonged whole-cell recording. *Brain Res* 815, 154-166.

Scott FF, Belle MD, Delagrange P & Piggins HD. (2010). Electrophysiological effects of melatonin on mouse Per1 and non-Per1 suprachiasmatic nuclei neurones in vitro. *J Neuroendocrinol* 22, 1148-1156.

Shusterman V, Warman E, London B & Schwartzman D. (2012). Nocturnal peak in atrial tachyarrhythmia occurrence as a function of arrhythmia burden. *J Cardiovasc Electrophysiol* 23, 604-611.

Signorini S, Liao YJ, Duncan SA, Jan LY & Stoffel M. (1997). Normal cerebellar development but susceptibility to seizures in mice lacking G protein-coupled, inwardly rectifying K+ channel GIRK2. *Proc Natl Acad Sci U S A* 94, 923-927.

Soscia SJ & Harrington ME. (2004). Neuropeptide Y attenuates NMDA-induced phase shifts in the SCN of NPY Y1 receptor knockout mice in vitro. *Brain Res* 1023, 148-153.

Soscia SJ & Harrington ME. (2005). Neuropeptide Y does not reset the circadian clock in NPY Y2-/- mice. *Neurosci Lett* 373, 175-178.

Takigawa M, Kawamura M, Noda T, Yamada Y, Miyamoto K, Okamura H, Satomi K, Aiba T, Kamakura S, Sakaguchi T, Mizusawa Y, Itoh H, Horie M & Shimizu W. (2012). Seasonal and circadian distributions of cardiac events in genotyped patients with congenital long QT syndrome. *Circ J* 76, 2112-2118.

Turek FW, Penev P, Zhang Y, van Reeth O & Zee P. (1995). Effects of age on the circadian system. *Neurosci Biobehav Rev* 19, 53-58.

van den Pol AN, Obrietan K, Chen G & Belousov AB. (1996). Neuropeptide Y-mediated long-term depression of excitatory activity in suprachiasmatic nucleus neurons. *J Neurosci* 16, 5883-5895.

Vyazovskiy VV, Kopp C, Wigger E, Jones ME, Simpson ER & Tobler I. (2006). Sleep and rest regulation in young and old oestrogen-deficient female mice. *J Neuroendocrinol* 18, 567-576.

Wang TA, Yu YV, Govindaiah G, Ye X, Artinian L, Coleman TP, Sweedler JV, Cox CL & Gillette MU. (2012). Circadian rhythm of redox state regulates excitability in suprachiasmatic nucleus neurons. *Science* 337, 839-842.

Wang Z. (2013). miRNA in the regulation of ion channel/transporter expression. *Compr Physiol* 3, 599-653.

Welsh DK, Takahashi JS & Kay SA. (2010). Suprachiasmatic nucleus: cell autonomy and network properties. *Annu Rev Physiol* 72, 551-577.

Wydeven N, Marron Fernandez de Velasco E, Du Y, Benneyworth MA, Hearing MC, Fischer RA, Thomas MJ, Weaver CD & Wickman K. (2014). Mechanisms underlying the activation of G-protein-gated inwardly rectifying K+ (GIRK) channels by the novel anxiolytic drug, ML297. *Proc Natl Acad Sci U S A* 111, 10755-10760.

Yannielli P & Harrington ME. (2004). Let there be "more" light: enhancement of light actions on the circadian system. *Prog Neurobiol* 74, 59-76.

Yannielli PC, Brewer JM & Harrington ME. (2004). Blockade of the NPY Y5 receptor potentiates circadian responses to light: complementary in vivo and in vitro studies. *Eur J Neurosci* 19, 891-897.

Yannielli PC & Harrington ME. (2000). Neuropeptide Y applied in vitro can block the phase shifts induced by light in vivo. *Neuroreport* 11, 1587-1591.

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M & Takahashi JS. (2004). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent. *Proc Natl Acad Sci U S A* 101, 5339-5346.

Zarowski M, Loddenkemper T, Vendrame M, Alexopoulos AV, Wyllie E & Kothare SV. (2011). Circadian distribution and sleep/wake patterns of generalized seizures in. *Epilepsia* 52, 1076-1083.

Zuberi Z, Birnbaumer L & Tinker A. (2008). The role of inhibitory heterotrimeric G proteins in the control of in vivo heart rate dynamics. *Am J Physiol Regul Integr Comp Physiol* 295, R1822-1830.

Table 1. Circadian behavioral analysis of GIRK2 KO mice

		WT		KO		Independent samples t-test		
No Wheel		Avg	SE	Avg	SE	t	df	Sig. (2-tailed)
LD	Power	705.21	54.07	646.28	42.49	-0.86	24	0.400
	Avg Counts (counts/min)	3.02	0.26	2.56	0.18	-1.44	24	0.164
DD	Tau	23.92	0.04	23.85	0.04	-1.08	24	0.292
	Power	539.24	2.79	534.05	28.64	-0.15	24	0.885
	Avg Counts (counts/min)	2.91	0.20	2.87	0.20	-0.11	24	0.913
Wheel								
LD	Power	1099.63	59.83	1170.28	118.43	0.55	13	0.589
	Avg Counts (counts/min)	14.51	1.98	16.13	1.87	0.59	13	0.566
DD	Tau	23.80	0.04	23.95	0.04	2.46	13	0.029
	Power	1173.99	86.84	1430.68	86.55	2.08	13	0.058
	Avg Counts (counts/min)	11.85	1.65	18.72	1.95	0.76	13	0.018

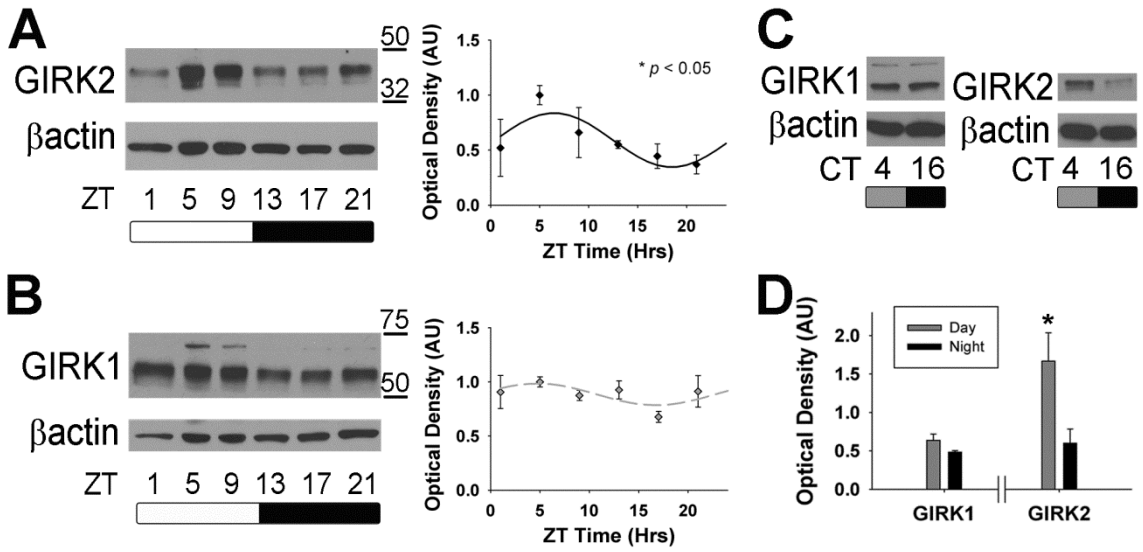

Figure 1: GIRK2, but not GIRK1, protein levels are regulated in a circadian manner. A) Representative immunoblots for GIRK2 from mouse SCN out of an LD cycle (left). Relative optical density (mean ± SEM) for GIRK2 throughout the day with lines indicating the predicted cosinor curve (*$p < 0.05$, right). n = 5-6 per time point. B) Representative immunoblot for GIRK1 in mouse SCN across an LD cycle (left). Relative optical density (mean ± SEM) for GIRK1 at each time point throughout the day with predicted cosinor curve (right). n = 3-5 per time point. C) Representative GIRK1 (left) and GIRK2 (right) blots from mouse SCN out of DD at CT 4 (subjective day) and CT 16 (subjective night). D) Relative optical density (mean ± SEM) between subjective day and night for GIRK1 and GIRK2. *$p < 0.05$, n = 3-4 per time point.

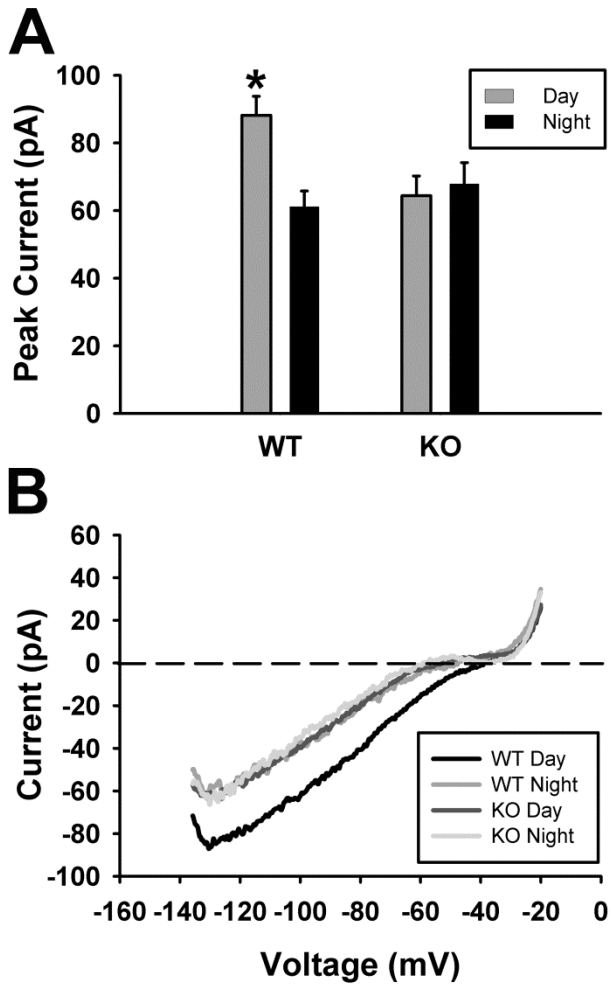

Figure 2: GIRK currents are increased during the day in SCN neurons. A) Peak inward current (mean ± SEM) during the day vs. night (*$p < 0.05$) for GIRK2 knockout (KO) and wild-type (WT) mice. B) Representative voltage-clamp ramp traces. Recordings were done in the presence of TTX (1µM), bicuculline (30µM), D-AP5 (50µM), CNQX (10µM), and CdCl2 (200µM) in order to block synaptic transmission. All recordings were done in 30 mM KCl to increase potassium conductance. n = at least 3 animals and 20 cells per group.

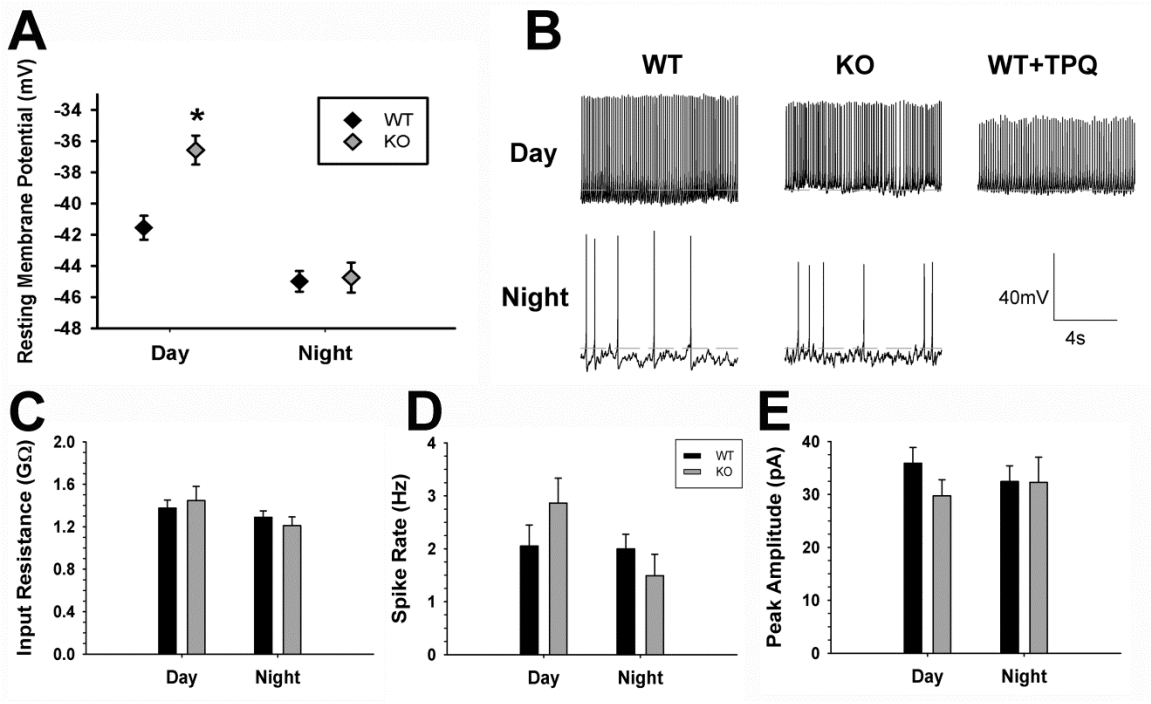

Figure 3: GIRK2 knockout SCN neurons exhibit more depolarized resting membrane potential compared to wild-type controls. A) Resting membrane potential of WT and KO SCN neurons during the day and night. B) Representative gap-free current clamp of KO and WT SCN neurons during the day and at night. A representative trace from WT slices treated with the GIRK2 antagonist Tertiapin-Q (0.2μM) during the day is also included. Dotted line indicates -40 mV. C, D, E) Input resistance, action potential frequency from gap-free current clamp recordings, and action potential amplitudes from gap-free current clamp recordings, respectively, from WT and KO neurons during the day and night. n = 3-5 animals and at least 25 cells per group, * $p < 0.05$.

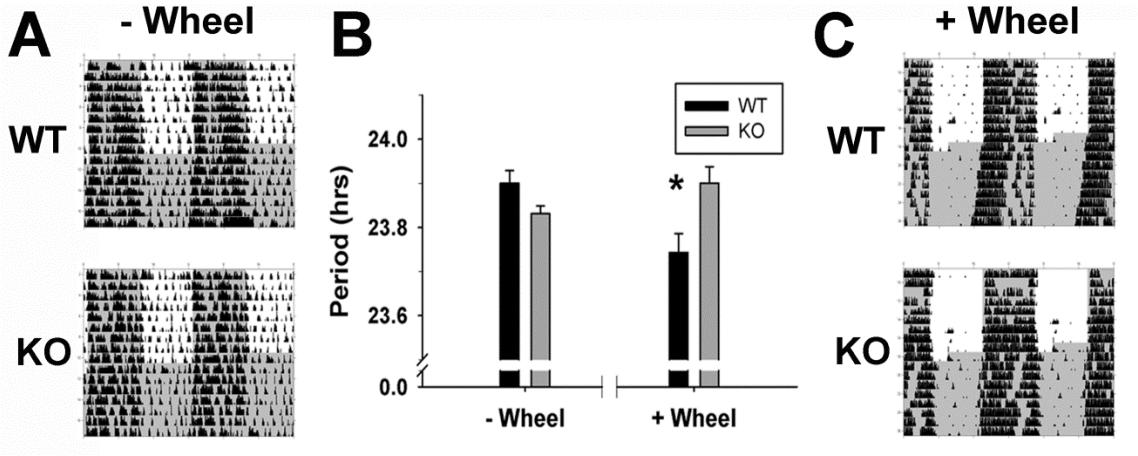

Figure 4: GIRK2 knockout mice fail to shorten free-running period in response to wheel-running activity. A) Representative, double-plotted actograms of WT (top) and KO (bottom) mice without wheel access (activity measured by infrared motion sensors), n =14-15 per group. B) Wheel-locked infrared free-running period (mean ± SEM), and wheel-running activity-based free-running period (mean ± SEM) for KO and WT mice. *$p < 0.05$. C) Representative actograms for WT and KO mice with wheel access (right, activity measured by wheel revolutions); n = 7-8 animals per each group. Time of lights off is indicated by dark gray; black tick marks indicate activity counts.

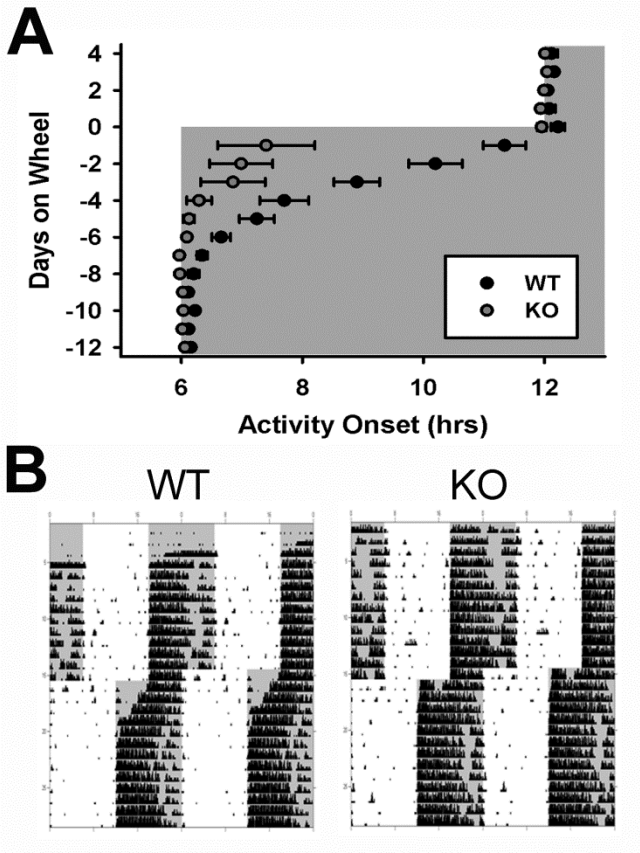

Figure 5: GIRK2 knockout mice entrain more rapidly to a 6-hour light cycle advance. A) Activity onset (mean ± SEM) in days prior and following a 6-hour advance of an LD cycle (day 0) for WT and KO mice. Lights off represented by gray shading. B) Representative actograms of WT (top) and KO (bottom) mice housed on wheels in a 12:12 LD cycle. Lights off represented by gray shading; black tick marks indicate activity counts. n = 6 animals per group.

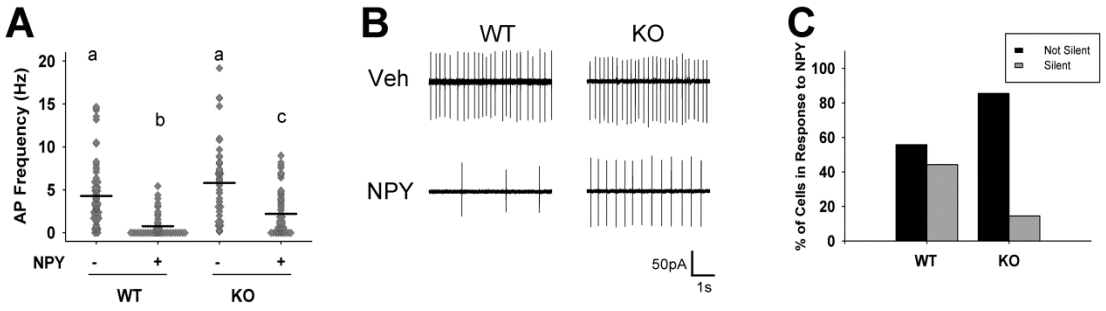

Figure 6: Loss of GIRK2 reduces effect of NPY on SCN neuron spontaneous firing rate. A) Frequency plot of individual WT and KO SCN neurons treated with 2.35 µM NPY or vehicle from ZT 4 to 6. Mean value indicated by black solid line. Lowercase letters (a, b, c) indicate groups that are significantly different (*p<0.05). B) Representative loose patch traces (5 sec) of neurons in (A). C) Percentage of silent cells vs. non-silent cells with NPY treatment. n = 3 animals and ≥45 cells per group.

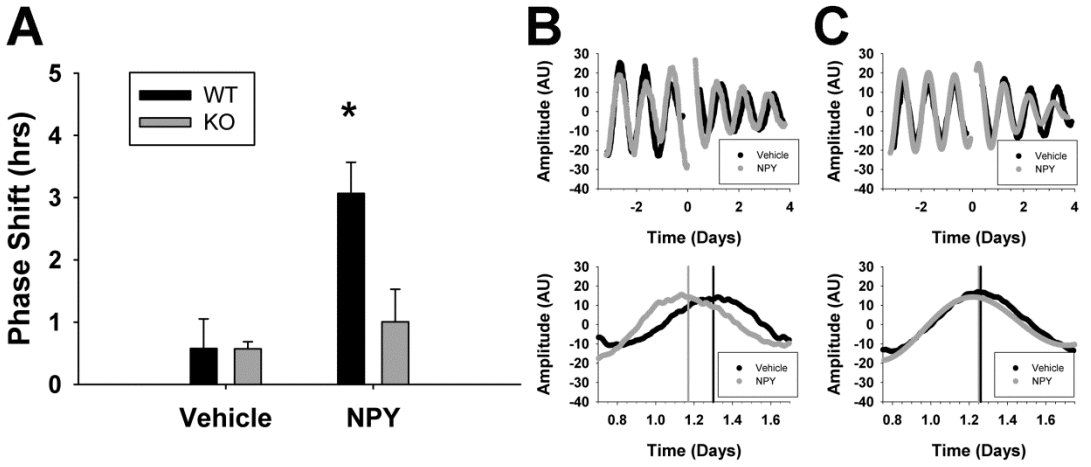

Figure 7: GIRK2 is necessary for NPY-induced phase shifts in the molecular clock. A) Phase shifts of PER2::LUC rhythms in response to a 1-hr treatment of 2.35 µM NPY or vehicle at CT4 in WT and KO mice. *p < 0.05. B,C) Representative bioluminescence traces from two WT (B) or two KO (C) SCN cultures treated with vehicle (black) or NPY (gray) for three cycles before and after treatment (top) or day 1 after treatment (bottom). Time 0: onset of NPY application. Predicted peak time, determined from 3 cycles post treatment, is indicated by vertical bars for each group. n = 6-8 cultures per group.

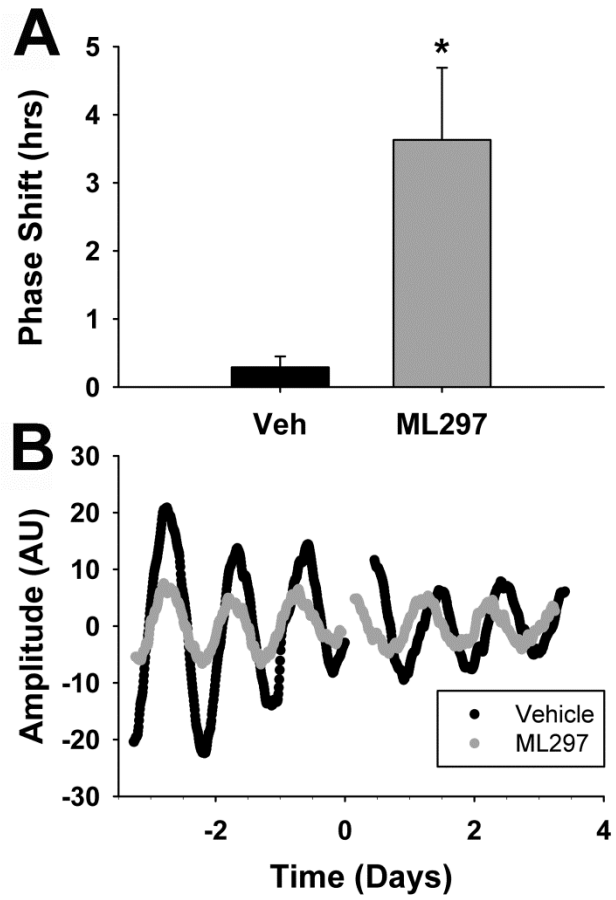

Figure 8: Activation of GIRK channels induces nonphotic-like phase advances. A) Phase shifts of PER2::LUC rhythms in response to a 1-hr treatment of 10 µM ML297 or vehicle starting between CT3-4.5. B) Representative bioluminescence traces from SCN cultures treated with vehicle (black) or ML297 (gray) for three cycles before and after treatment. n = 6 cultures per group.

GIRK CHANNELS MEDIATE THE NONPHOTIC EFFECTS OF EXOGENOUS MELATONIN

by

LAUREN M. HABLITZ, HYLTON E. MOLZOF, AND KAREN L. GAMBLE

Submitted to *The Journal of Neuroscience*

Format adapted for dissertation

CHAPTER 2

GIRK CHANNELS MEDIATE THE NONPHOTIC EFFECTS OF EXOGENOUS MELATONIN

Abstract

Melatonin supplementation has been used as a therapeutic agent for several diseases, yet little is known about the underlying molecular mechanisms by which melatonin acts within the brain to synchronize circadian rhythms. G protein signaling plays a large role in melatonin-induced phase shifts of locomotor behavior. Melatonin receptors have been shown to activate G protein-coupled inwardly-rectifying potassium (GIRK) channels in Xenopus oocytes. The present study tested the hypothesis that melatonin influences circadian phase and electrical activity within the clock center of the brain, the suprachiasmatic nucleus (SCN), through GIRK channel activation. The results showed that, unlike wild-type littermates, GIRK2 knockout mice failed to phase advance wheel-running behavior in response to 3-day subcutaneous injections of melatonin in the late day. Loose patch electrophysiological recordings of SCN neurons revealed a significant reduction in the average intrinsic action potential rate in response to melatonin. This effect was lost in the presence of a GIRK antagonist, tertiapin-q (TPQ), and in SCN neurons from GIRK2 knockout mice. The melatonin-induced suppression of firing rate corresponded with an increased inward current that was blocked by TPQ. Finally, application of ramelteon, a potent melatonin receptor agonist, also significantly decreased

firing rate and increased inward current within SCN neurons in a GIRK-dependent manner. These results are the first to show that GIRK channels are necessary for the effects of melatonin and ramelteon within the SCN. This study suggests that GIRK channels may be an alternative therapeutic target for diseases with evidence of circadian disruption, including aberrant melatonin signaling.

Introduction

The hormone melatonin, produced by the pineal gland, is a potent regulator of circadian rhythms or 24-hour cycles in behavior and biological processes (Dubocovich, 2007). Exogenous melatonin has been used to treat a variety of diseases that exhibit circadian rhythm comorbidities such as epilepsy (Banach et al., 2011; Jain and Besag, 2013), delayed sleep phase syndrome (Mundey et al., 2005), cardiometabolic diseases (Paulis et al., 2012), and mood disorders such as depression (Racagni et al., 2007; Campos Costa et al., 2013; Comai and Gobbi, 2014; Laudon and Frydman-Marom, 2014). However, the molecular mechanisms linking melatonin-induced changes in neuronal activity to regulating the timing of circadian rhythms is poorly understood.

In both humans and rodents, exogenous melatonin administered during the late day advances the phase of circadian cycle, shifting activity onset to an earlier time (McArthur et al., 1991; Benloucif and Dubocovich, 1996; Hunt et al., 2001; Dubocovich et al., 2005; Mundey et al., 2005). Within the primary clock center of the brain, the suprachiasmatic nucleus (SCN) of the hypothalamus, melatonin application hyperpolarizes the resting membrane potential and suppresses spontaneous action potential rate in neurons within acute SCN slices from mice and rats (Jiang et al., 1995; van den Top et al., 2001; Scott et

al., 2010). These effects on the circadian system are thought to be mediated through G protein-coupled signaling. G protein-coupled melatonin receptors mediate melatonin-induced phase shifts in behavior and changes in SCN firing rate (Hunt et al., 2001; Dubocovich and Markowska, 2005). In addition, melatonin-induced hyperpolarization of SCN neurons has been shown to be pertussis toxin-sensitive (van den Top et al., 2001), indicating Gi/o heterotrimeric G protein signaling is critical for the acute electrophysiological effects of melatonin. G protein-coupled inwardly-rectifying potassium (GIRK) channels are potential candidate mediators of this inhibitory effect of melatonin, given that GIRK currents are increased by melatonin receptor activation in a Xenopus oocyte expression system (Nelson et al., 1996). Recently, we have shown that GIRK channel activation varies over the day-night cycle and that day-time activation is sufficient to induce phase advances of the molecular clock within the SCN (Hablitz et al., 2014). We hypothesize that GIRK channels mediate the phase advancing effects of exogenous melatonin. Here, we use behavioral and electrophysiological techniques to ascertain: whether GIRK channels are necessary for the inhibitory and phase synchronizing effects of melatonin on SCN neurons and wheel-running behavior, and if ramelteon, a potent clinically relevant melatonin receptor agonist (Kato et al., 2005), requires GIRK channels to alter SCN electrophysiology.

Materials and Methods

Ethical approval

All animal care, handling, and housing were in compliance with the University of Alabama at Birmingham's Institutional Animal Care and Use Committee guidelines.

Animals and housing

All mice in these experiments were 2-4 months of age to reduce developmental or aging phenotypes (Turek et al., 1995; Biello, 2009). Only male mice were used for behavioral experiments (Ruiz de Elvira et al., 1992; Vyazovskiy et al., 2006). GIRK2 knockout (KO) animals on a C57/BL6 background (Signorini et al., 1997) and wild-type (WT) littermate controls were used for electrophysiology and circadian behavioral analysis. Although C57/BL6 mice are melatonin deficient, studies have confirmed that melatonin binding and phase shifting effects of melatonin are still intact and comparable to other mouse strains (Siuciak et al., 1990; Liu et al., 1997). Separate cohorts of mice were used for each different experiment. Unless otherwise stated, mice were group housed on a 12:12 light/dark (LD) cycle with food and water ad libitum.

Behavioral Analysis

All mice were housed in individual wheel cages. Wheel-running activity was recorded and analyzed using Clocklab software (Actimetrics, Wilmette, IL). After entrainment to a 12:12 LD cycle, mice were released into constant darkness (DD) for at least 10 days prior to treatment. Mice were then treated at approximately CT10 (circadian time 10; CT12 is defined as activity onset) with either subcutaneous injections of vehicle (3% ethanol/saline) or melatonin (90μg/mouse) for three consecutive days (as in (Dubocovich et al., 2005)). Phase shifts were measured as the difference between two predictions for the time of activity onset the day after treatment (using linear regression for each time period pre and post treatment for each animal). The first prediction was based on the 7 onsets prior to the first injection and the second prediction was based on

the 7 onsets after three stabilization days post injections. Mice that were injected before CT 9.5 were excluded from analysis. When possible, mice were treated with vehicle and melatonin using a crossover design. During the experiment, 9 of 13 WT and 6 of 7 KO mice received both treatments with at least 2 weeks between trials.

Electrophysiology

Mice were killed either at ZT8.5 (Zeitgeber time 8.5; ZT12 defined as lights off) by cervical dislocation. All recordings were made between projected ZT 10-12. Brains were harvested, sectioned at 200 µm on a vibroslicer (7000smz, Campden Instruments Ltd., Lafayette, IN), and transferred to an open recording chamber (Warner Instruments, Hamden, CT) that was continuously perfused at a rate of 2.0 ml/min with extracellular solution consisting of (in mM), NaCl 124, NaHCO3 20, Na2HPO4 1, MgSO4 1.3, glucose 10, KCl 3.5, CaCl2 2.5 (added the day of experiment). Osmolality was adjusted to 300-305 mOsm), bubbled with 5% CO2 / 95% O2 and heated to 34 ± 0.5 ºC. Neurons were visualized with an Olympus BX51WI (Olympus America Inc., Center Valley, PA) using infrared-differential interference contrast optics. Electrodes with a pipette resistance of ~4-6 MΩ were filled with filtered, potassium gluconate solution consisting of (in mM): K-gluconate 135, KCl 10, HEPES 10, EGTA 0.5, then adjusted to pH 7.4 with KOH (Kuhlman and McMahon, 2004). Electrophysiological signals were processed and controlled by a Multiclamp 700B amplifier, and pClamp 10.02 software (Axon Instruments, Union City, CA). Recordings were sampled at 20 kHz and filtered at 10 kHz. In order to block synaptic transmission (as in Fig. 4-5): bicuculline (30µM) and CdCl2 (200µM) (Sigma-Aldritch, St. Louis, MO), D-AP5 (50µM) and CNQX (10µM)

(Abcam, Cambrige, MA), and TTX (1µM) (Tocris Biosciences, Minneanapolis, MN) were added to the bath solution. To isolate GIRK currents (Fig. 4-5) the concentration of KCl was increased from 3.5 mM to 30 mM in order to increase potassium conductance across the membrane (as in (Fu et al., 2004)). The GIRK channel antagonist Tertiapin-Q (0.2 µM) (Alamone Labs, Israel) was used for experiments in Fig. 3-5. For experiments shown in Fig. 3-4, cells were treated with either vehicle (water) or melatonin (2 µM; Sigma-Aldritch, St. Louis, MO). For experiments shown in Fig. 5 cells were treated with either vehicle (20 nM ethanol) or ramelteon (10 pM; Biotang Inc., Lexington, MA). All data were collected within 6 min of membrane rupture to minimize any potential washout effects from the whole-cell recording (Schaap et al., 1999). Average spike rate was calculated from at least 1 min of the 2-min trace. For all electrophysiological experiments, at least 3 biological replicates with at least 4 cells per animal were used. There was no specific regional bias when recording within the SCN.

Statistical Analysis

All statistical analysis was performed with SPSS 22. For comparisons of means, an independent samples t-test or ANOVA was used for comparisons between two means or two or more means, respectively. Two factor designs were analyzed with a two-way ANOVA with repeated measures (using a linear mixed model) when appropriate. In cases of a non-normal distribution, a nonparametric Kruskal-Wallis test was used, followed by median test for post hoc analyses. For all other tests, significance was ascribed at $P < 0.05$.

Results

GIRK2 is necessary for melatonin-induced phase advances of behavioral rhythms

It has been previously shown that three-day subcutaneous administration of melatonin in the late day (CT10) phase advances wheel-running behavioral rhythms of mice (Dubocovich et al., 2005). In order to test the hypothesis that these phase advances are mediated by GIRK channels, we performed a similar experiment using GIRK2 KO animals as a model of disrupted GIRK channel signaling, compared to WT littermate controls. Administration of melatonin to WT mice induced significantly larger phase advances in wheel running behavior compared to vehicle (linear mixed model ANOVA, main effect of treatment, $F(1, 29.3) = 4.88$, $P = 0.035$; WT: n = 12-14 per group). Moreover, KO animals responded similarly to melatonin and vehicle injections, and these shifts were overall reduced compared to WT animals (main effect of genotype, $F(1, 29.3) = 5.29$, $P = 0.029$; genotype x treatment interaction, $F(1,29.3) = 3.32$, $P = 0.079$; KO: n = 7-8 per group), indicating that GIRK2 is necessary for the phase-advancing effects of melatonin on behavior (Fig. 1,2). Similar to our previous findings (Hablitz et al., 2014), GIRK2 KO animals exhibited a longer free-running period compared to WT controls (WT: 23.74 ± 0.04h, KO: 23.87 ± 0.04h; independent samples t-test, $t(37) = 2.094$, $P = 0.043$). Finally, melatonin increased free-running period equally in both WT and KO animals (post vehicle mean: 23.77 ± 0.04h, post melatonin mean: 23.85 ± 0.05h; repeated measures ANOVA, main effect of treatment, $F(1, 34) = 4.324$, $P = 0.045$), indicating that GIRK2 is not necessary for melatonin-induced effects on period length.

Melatonin-induced suppression of SCN neuronal activity is mediated by GIRK currents

Previous studies have shown that melatonin suppresses action potential rates of SCN neurons both in rat (Jiang et al., 1995; Zhou et al., 2000; van den Top et al., 2001) and mouse (Scott et al., 2010). Given that GIRK channels are necessary for the phase-advancing effects of late-day melatonin on wheel-running behavior, we tested the hypothesis that GIRK channels are necessary for the inhibitory effects of melatonin in the SCN. First, we performed loose patch electrophysiology within the SCN. Melatonin application (2 μM) in the late day (ZT10-12) significantly decreased the spontaneous action potential frequencies of SCN neurons (mean ± SEM, Veh: 4.51 ± 0.47 Hz, Mel: 3.07 ± 0.36 Hz; Kruskal–Wallis test, $H(3) = 8.035$, $P = 0.045$; median post hoc test, $P = 0.048$ for Veh vs. Mel). This effect was lost in the presence of tertiapin-Q (TPQ), a GIRK channel antagonist (TPQ + Mel: 5.04 ± 0.54 Hz, median post hoc test, $P = 0.009$ for TPQ + Mel vs. Mel). TPQ alone had no influence on spike rate of SCN neurons (TPQ: 4.84 ± 0.53 Hz; Median post hoc test, $P = 0.755$, Veh vs. TPQ; n = >28 cells/group). Similar to TPQ, melatonin application to SCN slices from GIRK2 KO mice did not suppress firing rate when applied during the late day (KO Veh: 4.62 ± 0.45 Hz, KO Mel: 5.07 ± 0.48 Hz; independent samples t-test, $t(88) = -0.692$, $P = 0.49$, n= >44 cells per group), indicating that GIRK channels are necessary for the decreased firing rate in response to melatonin (Fig. 3).

Melatonin receptor activation has been shown to activate GIRK channels in a Xenopus oocyte expression system (Nelson et al., 1996), yet it remains unknown as to whether melatonin directly activates GIRK channels within SCN neurons. To measure a melatonin-sensitive GIRK current in SCN neurons, we used whole-cell, voltage-clamp

electrophysiology and pharmacological inhibition of synaptic transmission (1µM TTX, 30µM bicuculline, 50µM D-AP5, 10µM CNQX, and 200µM CdCl2) along with increased KCl (30mM; in order to increase potassium conductance as in (Hablitz et al., 2014)), in response to a slow ramp (2.5 s) from -140 mV to -20 mV. We found that melatonin increased peak inward current during this protocol (mean ± SEM, Veh: -56.64 ± 5.79 pA, Mel: -113.59 ± 10.21 pA; Kruskal–Wallis test, H(3) = 20.98, P = 0.0001; median post hoc test, P = 0.001 for Veh vs. Mel), and this effect was lost in the presence of TPQ (TPQ + Mel: -61.35 ± 8.59 pA, median post hoc test, P = 0.001 for TPQ + Mel vs. Mel; n= >17 cells per group), demonstrating that melatonin directly activates GIRK channels in SCN neurons (Fig. 4).

GIRK channels mediate the effects of the melatonin agonist Ramelteon within the SCN

Ramelteon is a MT1/2 receptor agonist used in treating sleep disorders, depression, and delirium (Borja and Daniel, 2006; Hatta et al., 2014). This therapeutic agent can cause similar phase-shifts to the circadian cycle (Rawashdeh et al., 2011), and has a higher affinity for melatonin receptors than melatonin (Kato et al., 2005; Miyamoto, 2009). Here, we tested whether ramelteon acts upon similar mechanisms as melatonin within the SCN, thereby inducing GIRK currents and suppressing action potential rates. The results indicated that 2-hr ramelteon application (10 pM, from ZT10-12) significantly decreased the spike rates of SCN neurons (mean ± SEM, Veh: 3.49 ± 0.4 Hz, Ram: 1.99 ± 0.3 Hz) and this effect was gone in the presence of TPQ (TPQ: 3.52 ± 0.5, TPQ + Ram: 3.29 ± 0.5; Kruskal–Wallis test, H(3) = 14.89, P = 0.002, Fig. 5). Moreover, this inhibition corresponded to an increased inward current that was sensitive to TPQ (two-

way ANOVA, ramelteon by TPQ interaction: $F(1,119) = 8.122$, $P = 0.005$; simple effects analysis: ramelteon vs. vehicle: $P = 0.0001$, Ramelteon vs. TPQ: $P = 0.842$; Fig. 5; n= >23 cells per group), similar to melatonin treatment (Fig. 5). Altogether, these results indicate that GIRK channels are a downstream target of melatonin receptor activation by ramelteon.

Discussion

The molecular mechanisms underlying decreased neuronal firing and phase shifts induced by late-day melatonin within the SCN are largely unknown. Here, we propose that GIRK channels mediate this suppression of firing rate, and are necessary for melatonin induced phase shifts. Indeed, we show that melatonin fails to phase advance circadian activity rhythms of mice in the absence of GIRK2 channels. Furthermore, melatonin did not suppress spontaneous action potential rates of SCN neurons when GIRK channels were blocked or genetically ablated. This melatonin-induced decrease in spike rate corresponded to activation of a TPQ¬-sensitive GIRK current. These results support the hypothesis that GIRK channel activation is necessary within the SCN to convey the phase shifting properties of melatonin on the circadian clock. Because melatonin is a key molecule to treat sleep disorders, depression, and several other diseases, we examined the effects of ramelteon, a clinical sleep aid and potent MT1/2 receptor agonist, on SCN neurophysiological response. We found that ramelteon required GIRK channel activation to suppress SCN firing rate. To our knowledge, our study is the first to investigate this acute inhibitory effect of ramelteon on neuronal excitability.

Altogether, these results indicate that GIRK channels are a potential therapeutic target in diseases where melatonin signaling has been disrupted.

Similar to melatonin, previous work from our lab shows that neuropeptide Y (NPY) signaling within the SCN is also mediated through GIRK channels (Hablitz et al., 2014). Importantly, activation of GIRK channels during the day is sufficient to phase advance organotypic SCN cultures (as reported by PER2::luciferase), similar to in vivo phase response curves for both NPY (Huhman and Albers, 1994; Besing et al., 2012) and melatonin (McArthur et al., 1991; Hastings et al., 1992; Benloucif and Dubocovich, 1996; Hunt et al., 2001; Dubocovich, 2007). This type of phase response curve with maximal phase advances during the day is characteristic of several neurotransmitters that signal for the presence of 'nonphotic' stimuli (i.e., not driven by light-induced activation of the retinohypothalamic tract), such as arousal and exercise (Challet, 2007). Interestingly, these neurotransmitters (melatonin, NPY, serotonin and GABA) are mediated through Gi/o heterotrimeric G protein signaling (Muraki et al., 2004; Dubocovich, 2007; Fowler et al., 2007; van den Pol, 2012), indicating that there may be shared common mechanisms for resetting circadian clock phase. Here, we show that GIRK channels are necessary for melatonin-induced SCN neuronal response and behavioral phase advances. Taken together with our prior study, we propose that GIRK channel activation is a putative conserved mechanism for nonphotic signals to influence the circadian clock.

Although the present study found that melatonin-induced changes in SCN neurophysiology were mediated through GIRK channels, little is known about how exogenous melatonin may regulate the molecular clock, a transcription-translation

feedback loop (Roenneberg and Merrow, 2005; Partch et al., 2014) that drives circadian rhythms on a molecular level throughout different tissue types. A single injection of melatonin in the late day does not influence molecular clock levels on the first day, but does significantly change expression of Per1, Per3, Bmal1 and AVP on the second day (Poirel et al., 2003). Nuclear orphan receptors such as Reverb-α, which have been implicated in melatonin signaling (Agez et al., 2007), may mediate this effect. Also, mice with a CLOCK mutation show normalization of period length in the presence of melatonin or ramelteon (Shimomura et al., 2010). Future studies should investigate whether GIRK channel activation ultimately influences components of the molecular clock such as regulation of Reverb-α (Agez et al., 2007; Agez et al., 2009) influencing redox state of the cell (Bonnefont-Rousselot and Collin, 2010; Luchetti et al., 2010; Garcia et al., 2014), or second messenger pathways like PKC activation (Luchetti et al., 2010), all of which have been implicated in melatonin signaling. It is important to emphasize that although GIRK channels are necessary for melatonin-induced phase advances, this does not preclude the involvement of co-activation of PKC in response to melatonin. Indeed, studies measuring SCN ensemble firing in rats and rhythmic PKC expression in cell culture have demonstrated that PKC activation via MT1/2 receptors plays a key role in the phase shifting effects of melatonin (McArthur et al., 1997; Rivera-Bermudez et al., 2003; Rivera-Bermudez et al., 2004).

In addition to resetting circadian phase, endogenous melatonin also provides a seasonal cue, signaling photoperiodic day length via changes in hormonal circulation patterns released from the pineal gland (Coomans et al., 2014). Classic studies have shown that changes in the length of the photoperiod cause long-lasting effects on period

length, such that longer or shorter photoperiods lengthen or shorten period, respectively (Pittendrigh and Daan, 1976). In the present study, we found that administration of melatonin for three consecutive days at CT10 was sufficient to significantly increase period length by approximately 5 minutes, indicating that acute pulses of melatonin may provide day-length information. Although this result may seem biologically insignificant, it may provide insight into circadian timing disruption in affective disorders such as seasonal affective disorder and depression, which have been successfully treated with melatonin receptor agonists (Srinivasan et al., 2012) or have shown evidence of disrupted melatonin receptor disruption within the SCN in postmortem brain tissue (Wu et al., 2013).

In conclusion, melatonin signaling has been shown to influence neuronal excitability, metabolic state, time-of-day, inflammation, and much more (Dubocovich et al., 2003; Dubocovich, 2007; Uberos et al., 2010; Paulis et al., 2012; Srinivasan et al., 2012). Ramelteon is clinically available and has been used to treat depression, insomnia, delirium, and Alzheimer's disease symptoms (Borja and Daniel, 2006; Furuya et al., 2012; Hatta et al., 2014). Here, we show that the effects of both melatonin and ramelteon on the neurophysiological function of SCN neurons are mediated by GIRK channels. Diseases such as addiction, Down's syndrome, and epilepsy, which have strong circadian components (Loddenkemper et al., 2011; Zarowski et al., 2011; Cho, 2012; Churchill et al., 2012; Lott, 2012; Stores and Stores, 2012; Ramgopal et al., 2013; Parekh et al., 2015; Webb et al., 2015), are characterized by aberrant GIRK channel function (Kobayashi and Ikeda, 2006; Luscher and Slesinger, 2010; Arora et al., 2011; Kaufmann et al., 2013). Future studies could investigate whether pharmacological regulators of GIRK channels,

such as TPQ or ML297 (a GIRK channel agonist (Days et al., 2010; Wydeven et al., 2014)), could be used as therapeutic agents in diseases presenting with circadian rhythm disruption. In support of this future research area, one study has shown that ML297 is effective in reducing signs of epilepsy in mice (Kaufmann et al., 2013).

Acknowledgments

This work was supported by National Institutes of Health Grants R01NS082413 to K.L.G and F31NS084683 to L.M.H. The authors would like to thank Russell Johnson Jr. for help with animal maintenance. Also, thank you to Drs. Rita Cowell, Catherine Fuller, Martin Young, and Linda Overstreet-Wadiche for discussing organization of the paper.

References

Agez L, Laurent V, Pevet P, Masson-Pevet M, Gauer F (2007) Melatonin affects nuclear orphan receptors mRNA in the rat suprachiasmatic nuclei. Neuroscience 144:522-530.

Agez L, Laurent V, Guerrero HY, Pevet P, Masson-Pevet M, Gauer F (2009) Endogenous melatonin provides an effective circadian message to both the suprachiasmatic nuclei and the pars tuberalis of the rat. J Pineal Res 46:95-105.

Arora D, Hearing M, Haluk DM, Mirkovic K, Fajardo-Serrano A, Wessendorf MW, Watanabe M, Lujan R, Wickman K (2011) Acute cocaine exposure weakens GABA(B) receptor-dependent G-protein-gated inwardly rectifying K+ signaling in dopamine neurons of the ventral tegmental area. J Neurosci 31:12251-12257.

Banach M, Gurdziel E, Jedrych M, Borowicz KK (2011) Melatonin in experimental seizures and epilepsy. Pharmacol Rep 63:1-11.

Benloucif S, Dubocovich ML (1996) Melatonin and light induce phase shifts of circadian activity rhythms in the C3H/HeN mouse. J Biol Rhythms 11:113-125.

Besing RC, Hablitz LM, Paul JR, Johnson RL, Prosser RA, Gamble KL (2012) Neuropeptide Y-induced phase shifts of PER2::LUC rhythms are mediated by long-term

suppression of neuronal excitability in a phase-specific manner. Chronobiol Int 29:91-102.

Biello SM (2009) Circadian clock resetting in the mouse changes with age. Age (Dordr) 31:293-303.

Bonnefont-Rousselot D, Collin F (2010) Melatonin: action as antioxidant and potential applications in human disease and aging. In: Toxicology, pp 55-67. Ireland: 2010 Elsevier Ireland Ltd.

Borja NL, Daniel KL (2006) Ramelteon for the treatment of insomnia. Clin Ther 28:1540-1555.

Campos Costa I, Nogueira Carvalho H, Fernandes L (2013) Aging, circadian rhythms and depressive disorders: a review. Am J Neurodegener Dis 2:228-246.

Challet E (2007) Minireview: Entrainment of the suprachiasmatic clockwork in diurnal and nocturnal mammals. Endocrinology 148:5648-5655.

Cho CH (2012) Molecular mechanism of circadian rhythmicity of seizures in temporal lobe epilepsy. Front Cell Neurosci 6:55.

Churchill SS, Kieckhefer GM, Landis CA, Ward TM (2012) Sleep measurement and monitoring in children with Down syndrome: a review of the literature, 1960-2010. Sleep Med Rev 16:477-488.

Comai S, Gobbi G (2014) Unveiling the role of melatonin MT2 receptors in sleep, anxiety and other neuropsychiatric diseases: a novel target in psychopharmacology. In: J Psychiatry Neurosci, pp 6-21. Canada.

Coomans CP, Ramkisoensing A, Meijer JH (2014) The suprachiasmatic nuclei as a seasonal clock. Front Neuroendocrinol.

Days E, Kaufmann K, Romaine I, Niswender C, Lewis M, Utley T, Du Y, Sliwoski G, Morrison R, Dawson ES, Engers JL, Denton J, Daniels JS, Sulikowski GA, Lindsley CW, Weaver CD (2010) Discovery and Characterization of a Selective Activator of the G-Protein Activated Inward-Rectifying Potassium (GIRK) Channel. In: Probe Reports from the NIH Molecular Libraries Program. Bethesda (MD): National Center for Biotechnology Information (US).

Dubocovich ML (2007) Melatonin receptors: role on sleep and circadian rhythm regulation. Sleep Med 8 Suppl 3:34-42.

Dubocovich ML, Markowska M (2005) Functional MT1 and MT2 melatonin receptors in mammals. In: Endocrine, pp 101-110. United States.

Dubocovich ML, Rivera-Bermudez MA, Gerdin MJ, Masana MI (2003) Molecular pharmacology, regulation and function of mammalian melatonin receptors. Front Biosci 8:d1093-1108.

Dubocovich ML, Hudson RL, Sumaya IC, Masana MI, Manna E (2005) Effect of MT1 melatonin receptor deletion on melatonin-mediated phase shift of circadian rhythms in the C57BL/6 mouse. J Pineal Res 39:113-120.

Fowler CE, Aryal P, Suen KF, Slesinger PA (2007) Evidence for association of GABA(B) receptors with Kir3 channels and regulators. J Physiol 580:51-65.

Fu LY, Acuna-Goycolea C, van den Pol AN (2004) Neuropeptide Y inhibits hypocretin/orexin neurons by multiple presynaptic and postsynaptic mechanisms: tonic depression of the hypothalamic arousal system. J Neurosci 24:8741-8751.

Furuya M, Miyaoka T, Yasuda H, Yamashita S, Tanaka I, Otsuka S, Wake R, Horiguchi J (2012) Marked improvement in delirium with ramelteon: five case reports. Psychogeriatrics 12:259-262.

Garcia JJ, Lopez-Pingarron L, Almeida-Souza P, Tres A, Escudero P, Garcia-Gil FA, Tan DX, Reiter RJ, Ramirez JM, Bernal-Perez M (2014) Protective effects of melatonin in reducing oxidative stress and in preserving the fluidity of biological membranes: a review. J Pineal Res 56:225-237.

Hablitz LM, Molzof HE, Paul JR, Johnson RL, Gamble KL (2014) Suprachiasmatic nucleus function and circadian entrainment are modulated by G protein-coupled inwardly rectifying (GIRK) channels. J Physiol 592:5079-5092.

Hastings MH, Mead SM, Vindlacheruvu RR, Ebling FJ, Maywood ES, Grosse J (1992) Non-photic phase shifting of the circadian activity rhythm of Syrian hamsters: the relative potency of arousal and melatonin. In: Brain Res, pp 20-26. Netherlands.

Hatta K, Kishi Y, Wada K, Takeuchi T, Odawara T, Usui C, Nakamura H (2014) Preventive effects of ramelteon on delirium: a randomized placebo-controlled trial. JAMA Psychiatry 71:397-403.

Huhman KL, Albers HE (1994) Neuropeptide Y microinjected into the suprachiasmatic region phase shifts circadian rhythms in constant darkness. Peptides 15:1475-1478.

Hunt AE, Al-Ghoul WM, Gillette MU, Dubocovich ML (2001) Activation of MT(2) melatonin receptors in rat suprachiasmatic nucleus phase advances the circadian clock. Am J Physiol Cell Physiol 280:C110-118.

Jain S, Besag FM (2013) Does melatonin affect epileptic seizures? Drug Saf 36:207-215.

Jiang ZG, Nelson CS, Allen CN (1995) Melatonin activates an outward current and inhibits Ih in rat suprachiasmatic nucleus neurons. Brain Res 687:125-132.

Kato K, Hirai K, Nishiyama K, Uchikawa O, Fukatsu K, Ohkawa S, Kawamata Y, Hinuma S, Miyamoto M (2005) Neurochemical properties of ramelteon (TAK-375), a selective MT1/MT2 receptor agonist. Neuropharmacology 48:301-310.

Kaufmann K, Romaine I, Days E, Pascual C, Malik A, Yang L, Zou B, Du Y, Sliwoski G, Morrison RD, Denton J, Niswender CM, Daniels JS, Sulikowski GA, Xie XS, Lindsley CW, Weaver CD (2013) ML297 (VU0456810), the First Potent and Selective Activator of the GIRK Potassium Channel, Displays Antiepileptic Properties in Mice. ACS Chem Neurosci 4:1278-1286.

Kobayashi T, Ikeda K (2006) G protein-activated inwardly rectifying potassium channels as potential therapeutic targets. Curr Pharm Des 12:4513-4523.

Kuhlman SJ, McMahon DG (2004) Rhythmic regulation of membrane potential and potassium current persists in SCN neurons in the absence of environmental input. Eur J Neurosci 20:1113-1117.

Laudon M, Frydman-Marom A (2014) Therapeutic effects of melatonin receptor agonists on sleep and comorbid disorders. In: Int J Mol Sci, pp 15924-15950. Switzerland.

Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK, Reppert SM (1997) Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. In: Neuron, pp 91-102. United States.

Loddenkemper T, Vendrame M, Zarowski M, Gregas M, Alexopoulos AV, Wyllie E, Kothare SV (2011) Circadian patterns of pediatric seizures. Neurology 76:145-153.

Lott IT (2012) Neurological phenotypes for Down syndrome across the life span. Prog Brain Res 197:101-121.

Luchetti F, Canonico B, Betti M, Arcangeletti M, Pilolli F, Piroddi M, Canesi L, Papa S, Galli F (2010) Melatonin signaling and cell protection function. In: FASEB J, pp 3603-3624. United States.

Luscher C, Slesinger PA (2010) Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. Nat Rev Neurosci 11:301-315.

McArthur AJ, Gillette MU, Prosser RA (1991) Melatonin directly resets the rat suprachiasmatic circadian clock in vitro. Brain Res 565:158-161.

McArthur AJ, Hunt AE, Gillette MU (1997) Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. Endocrinology 138:627-634.

Miyamoto M (2009) Pharmacology of ramelteon, a selective MT1/MT2 receptor agonist: a novel therapeutic drug for sleep disorders. CNS Neurosci Ther 15:32-51.

Mundey K, Benloucif S, Harsanyi K, Dubocovich ML, Zee PC (2005) Phase-dependent treatment of delayed sleep phase syndrome with melatonin. Sleep 28:1271-1278.

Muraki Y, Yamanaka A, Tsujino N, Kilduff TS, Goto K, Sakurai T (2004) Serotonergic regulation of the orexin/hypocretin neurons through the 5-HT1A. J Neurosci 24:7159-7166.

Nelson CS, Marino JL, Allen CN (1996) Melatonin receptors activate heteromeric G-protein coupled Kir3 channels. Neuroreport 7:717-720.

Parekh PK, Ozburn AR, McClung CA (2015) Circadian clock genes: Effects on dopamine, reward and addiction. Alcohol.

Partch CL, Green CB, Takahashi JS (2014) Molecular architecture of the mammalian circadian clock. Trends Cell Biol 24:90-99.

Paulis L, Simko F, Laudon M (2012) Cardiovascular effects of melatonin receptor agonists. Expert Opin Investig Drugs 21:1661-1678.

Pittendrigh CS, Daan S (1976) A functional analysis of circadian pacemakers in nocturnal rodents. In, pp 223-252. Journal of Comparative Physiology: Springer-Verlag.

Poirel VJ, Boggio V, Dardente H, Pevet P, Masson-Pevet M, Gauer F (2003) Contrary to other non-photic cues, acute melatonin injection does not induce immediate changes of clock gene mRNA expression in the rat suprachiasmatic nuclei. Neuroscience 120:745-755.

Racagni G, Riva MA, Popoli M (2007) The interaction between the internal clock and antidepressant efficacy. In: Int Clin Psychopharmacol, pp S9-S14. England.

Ramgopal S, Thome-Souza S, Loddenkemper T (2013) Chronopharmacology of anti-convulsive therapy. Curr Neurol Neurosci Rep 13:339.

Rawashdeh O, Hudson RL, Stepien I, Dubocovich ML (2011) Circadian periods of sensitivity for ramelteon on the onset of running-wheel activity and the peak of suprachiasmatic nucleus neuronal firing rhythms in C3H/HeN mice. Chronobiol Int 28:31-38.

Rivera-Bermudez MA, Gerdin MJ, Earnest DJ, Dubocovich ML (2003) Regulation of basal rhythmicity in protein kinase C activity by melatonin in immortalized rat suprachiasmatic nucleus cells. In: Neurosci Lett, pp 37-40. Ireland.

Rivera-Bermudez MA, Masana MI, Brown GM, Earnest DJ, Dubocovich ML (2004) Immortalized cells from the rat suprachiasmatic nucleus express functional melatonin receptors. In: Brain Res, pp 21-27. Netherlands.

Roenneberg T, Merrow M (2005) Circadian clocks - the fall and rise of physiology. In: Nat Rev Mol Cell Biol, pp 965-971. England.

Ruiz de Elvira MC, Persaud R, Coen CW (1992) Use of running wheels regulates the effects of the ovaries on circadian rhythms. Physiol Behav 52:277-284.

Schaap J, Bos NP, de Jeu MT, Geurtsen AM, Meijer JH, Pennartz CM (1999) Neurons of the rat suprachiasmatic nucleus show a circadian rhythm in membrane properties that is lost during prolonged whole-cell recording. Brain Res 815:154-166.

Scott FF, Belle MD, Delagrange P, Piggins HD (2010) Electrophysiological effects of melatonin on mouse Per1 and non-Per1 suprachiasmatic nuclei neurones in vitro. J Neuroendocrinol 22:1148-1156.

Shimomura K, Lowrey PL, Vitaterna MH, Buhr ED, Kumar V, Hanna P, Omura C, Izumo M, Low SS, Barrett RK, LaRue SI, Green CB, Takahashi JS (2010) Genetic suppression of the circadian Clock mutation by the melatonin biosynthesis pathway. Proc Natl Acad Sci U S A 107:8399-8403.

Signorini S, Liao YJ, Duncan SA, Jan LY, Stoffel M (1997) Normal cerebellar development but susceptibility to seizures in mice lacking G protein-coupled, inwardly rectifying K+ channel GIRK2. Proc Natl Acad Sci U S A 94:923-927.

Siuciak JA, Fang JM, Dubocovich ML (1990) Autoradiographic localization of 2-[125I]iodomelatonin binding sites in the brains of C3H/HeN and C57BL/6J strains of mice. Eur J Pharmacol 180:387-390.

Srinivasan V, De Berardis D, Shillcutt SD, Brzezinski A (2012) Role of melatonin in mood disorders and the antidepressant effects of agomelatine. Expert Opin Investig Drugs 21:1503-1522.

Stores G, Stores R (2012) Sleep disorders and their clinical significance in children with Down syndrome. Dev Med Child Neurol.

Turek FW, Penev P, Zhang Y, van Reeth O, Zee P (1995) Effects of age on the circadian system. Neurosci Biobehav Rev 19:53-58.

Uberos J, Romero J, Molina-Carballo A, Munoz-Hoyos A (2010) Melatonin and elimination of kynurenines in children with Down's syndrome. J Pediatr Endocrinol Metab 23:277-282.

van den Pol AN (2012) Neuropeptide transmission in brain circuits. Neuron 76:98-115.

van den Top M, Buijs RM, Ruijter JM, Delagrange P, Spanswick D, Hermes ML (2001) Melatonin generates an outward potassium current in rat suprachiasmatic nucleus neurones in vitro independent of their circadian rhythm. Neuroscience 107:99-108.

Vyazovskiy VV, Kopp C, Wigger E, Jones ME, Simpson ER, Tobler I (2006) Sleep and rest regulation in young and old oestrogen-deficient female mice. J Neuroendocrinol 18:567-576.

Webb IC, Lehman MN, Coolen LM (2015) Diurnal and circadian regulation of reward-related neurophysiology and behavior. Physiol Behav 143C:58-69.

Wu YH, Ursinus J, Zhou JN, Scheer FA, Ai-Min B, Jockers R, van Heerikhuize J, Swaab DF (2013) Alterations of melatonin receptors MT1 and MT2 in the hypothalamic suprachiasmatic nucleus during depression. J Affect Disord 148:357-367.

Wydeven N, Marron Fernandez de Velasco E, Du Y, Benneyworth MA, Hearing MC, Fischer RA, Thomas MJ, Weaver CD, Wickman K (2014) Mechanisms underlying the activation of G-protein-gated inwardly rectifying K+ (GIRK) channels by the novel anxiolytic drug, ML297. Proc Natl Acad Sci U S A 111:10755-10760.

Zarowski M, Loddenkemper T, Vendrame M, Alexopoulos AV, Wyllie E, Kothare SV (2011) Circadian distribution and sleep/wake patterns of generalized seizures in. Epilepsia 52:1076-1083.

Zhou XJ, Jiang XH, Yu GD, Yin QZ (2000) Modulation of circadian rhythm of discharges of suprachiasmatic nucleus neurons in rat hypothalamic slices by melatonin. Sheng Li Xue Bao 52:215-219.

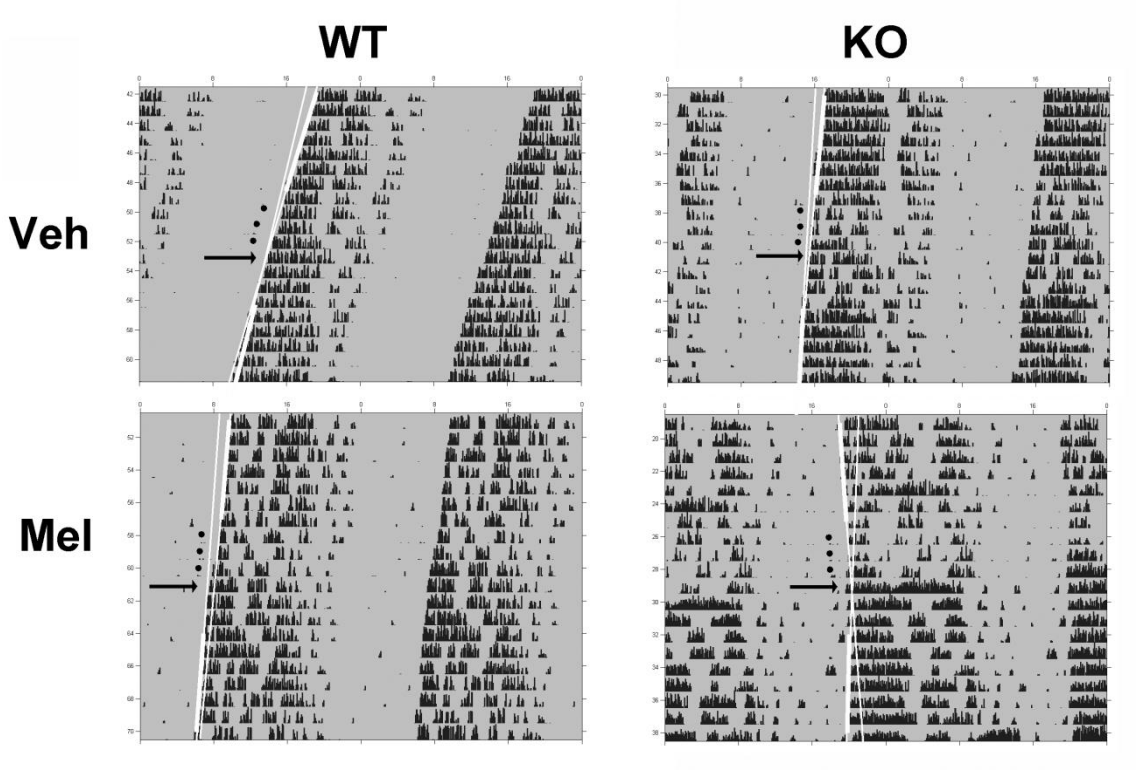

Figure 1: Melatonin-induced phase shifts in circadian activity of WT and GIRK2 KO mice. Representative, double-plotted actograms of individual WT (A,C) or KO (B,D) mice treated with vehicle (A,B) or melatonin (C,D). WT and KO mice were housed in constant darkness (indicated by gray shading) and treated 3 consecutive days at CT10 with either injections of vehicle (3% ethanol/saline, s.c.) or melatonin (90μg/mouse, s.c.). Treatment is indicated by white circles; best fit lines to activity onsets are shown in gray with the extended fit lines in black. White arrows indicate the day after injections (used in the phase shift predictions; see Methods). Black tick marks indicate activity counts.

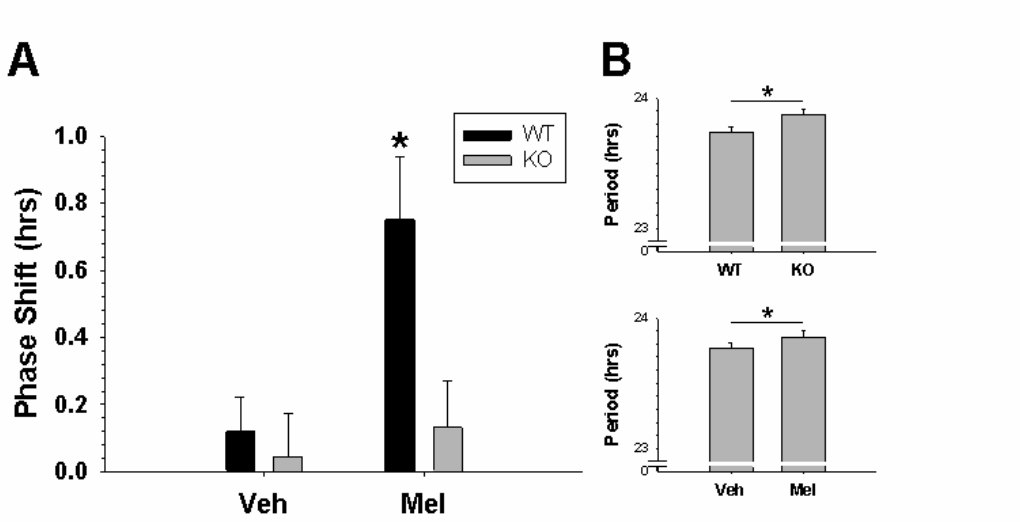

Figure 2: GIRK2 is necessary for the phase-advancing effects of melatonin on wheel running activity. A) Average phase shifts (mean ± SEM) of wheel-running activity rhythms in response to 3 days of subcutaneous injections at CT10 with either vehicle (3% ethanol/saline) or melatonin (90µg/mouse) in WT and KO mice. B) Free-running period (mean ± SEM) for KO and WT mice before treatments. C) Cumulative free-running period (mean ± SEM) post treatment with either vehicle or melatonin. *P < 0.05.

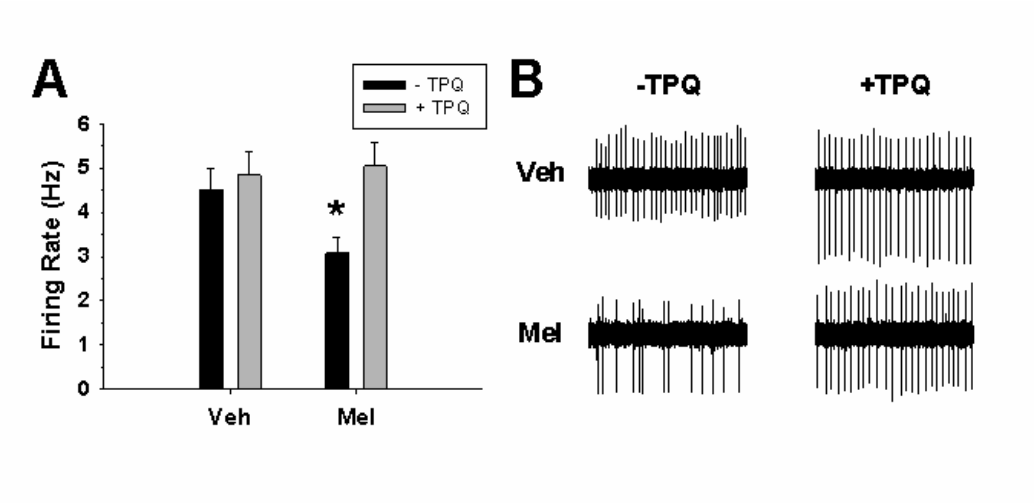

Figure 3: GIRK channels are necessary for melatonin-induced suppression of SCN action potential firing. A) Action potential rates (mean ± SEM) for vehicle and melatonin (2 µM) treated cells during ZT10-12 in the presence and absence of TPQ (0.2 µM). *P < 0.05. B) Representative loose patch traces (5 sec) of neurons in (A).

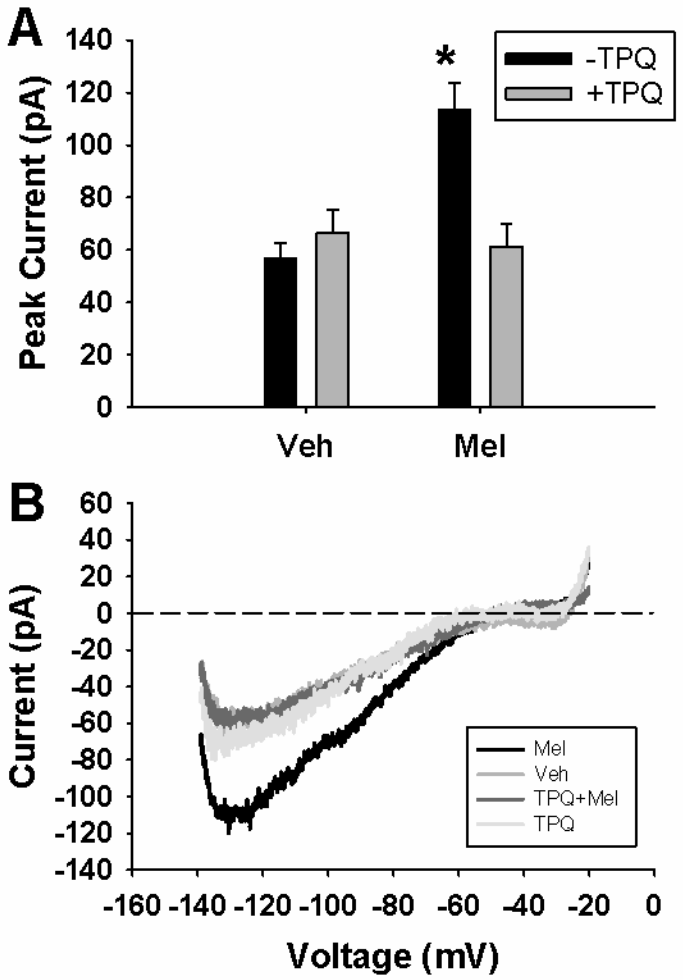

Figure 4: Melatonin induced an inward GIRK current in SCN neurons. A) Peak inward current (mean ± SEM) for vehicle and melatonin (2 µM) treated cells during ZT10-12 in the presence and absence of TPQ (0.2 µM). *P < 0.05. B) Representative voltage-clamp ramp traces. Recordings were done in the presence of TTX (1µM), bicuculline (30µM), D-AP5 (50µM), CNQX (10µM), and CdCl2 (200µM) in order to block synaptic transmission and 30 mM KCl to increase potassium conductance.

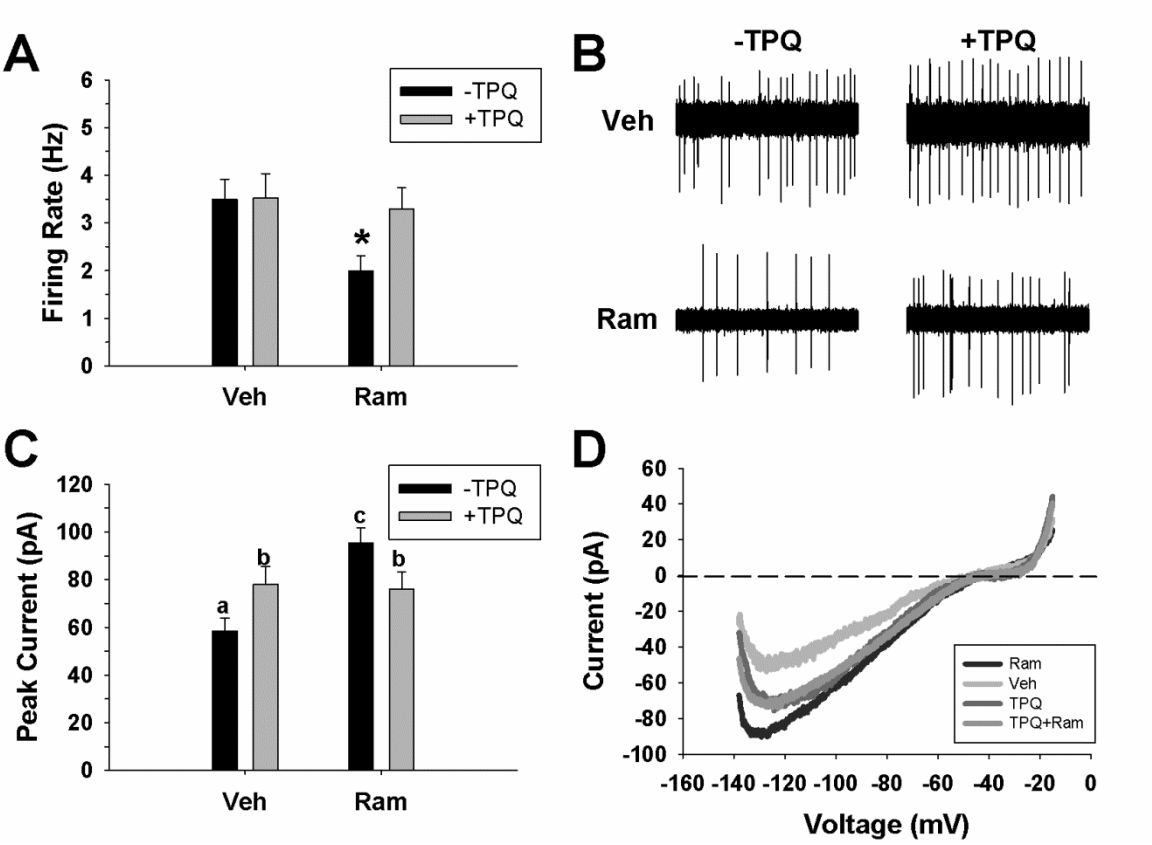

Figure 5: GIRK channels mediated the inhibitory effects of ramelteon within the SCN. A) Action potential rates (mean ± SEM) for vehicle and ramelteon (10 pM) treated cells during ZT10-12 in the presence and absence of TPQ (0.2 µM). *P < 0.05. B) Representative loose patch traces (5 sec) of neurons in (A). C) Peak inward current (mean ± SEM) for vehicle and ramelteon (10 pM) treated cells during ZT10-12 in the presence and absence of TPQ (0.2 µM). *P < 0.05. B) Representative voltage-clamp ramp traces. Recordings for C-D were done in the presence of TTX (1µM), bicuculline (30µM), D-AP5 (50µM), CNQX (10µM), and CdCl2 (200µM) in order to block synaptic transmission. Recordings in C-D were done in 30 mM KCl to increase potassium conductance.

DISCUSSION

GIRK channels and nonphotic entrainment: a recap

In general, both photic and nonphotic information signals are transmitted to the SCN, altering cellular excitability and the timing of the molecular clock. Ultimately, this signaling changes the entrainment of the animal. This dissertation explores the role of GIRK channels in mediating these cues within the SCN (Figure 1). The main hypothesis of this dissertation is that **nonphotic stimuli activate GIRK channels which decrease neuronal excitability, modulating the timing of circadian rhythms.**

Chapter one of this dissertation focused on defining the role of GIRK channels in regulating the timing of circadian rhythms and regulation of SCN excitability. GIRK1 and GIRK2 protein was found in the SCN. GIRK2 protein levels were higher during the day, even under constant conditions, indicating direct regulation by the circadian clock. This high level of GIRK2 during the day corresponded with an increased inward current, as measured by whole-cell voltage clamp electrophysiology. Loss of GIRK2, utilizing a GIRK2 knockout mouse, eliminated this current and resulted in more depolarized daytime SCN neurons. GIRK2 knockout animals failed to shorten free-running period in response to running wheel access and shifted more rapidly to a 6-hour phase advance of the light cycle, suggesting a deficit of the nonphotic entrainment pathway. Indeed, the inhibitory effects of the nonphotic neurotransmitter, NPY, on SCN neuronal firing rate were diminished in GIRK2 KO brain slices. Finally, broad activation of GIRK channels via application of ML297 (a GIRK channel agonist) induced a nonphotic phase shift, indicating that activation of GIRK channels is sufficient to mimic a nonphotic cue.

Because GIRK channels mediate the effects of NPY within the SCN, and activation is sufficient to cause a nonphotic phase advance, Chapter 2 focuses on whether

GIRK channels mediate additional nonphotic cues; in this case, the circadian effects of the common sleep aid melatonin. Subcutaneous injections of melatonin in the subjective late day caused a phase advance of wheel running activity in mice. This effect was lost when GIRK2 was genetically ablated. In addition to the phase shifting effects of melatonin, melatonin application to SCN slices suppressed neuronal firing and elicited an inward current, both of which were dependent on GIRK channel function. Finally, we demonstrated that GIRK channels were necessary for the effects of ramelteon, a potent melatonin receptor agonist, on the electrical activity of SCN neurons. This study was the first to characterize the single-cell neurophysiological effects of ramelteon on any neuronal population.

Does GIRK regulation of excitability and phase extend to other brain areas?

Multiple areas of the brain exhibit rhythmicity in clock gene expression and/or excitability (46, 131), such as the hippocampus(132), and multiple regions of cortex (133). The medial habenula even has similar oscillatory waveforms and resting membrane potential regulation as the SCN (134). Yet, the regulation of these rhythms and underlying ion channels governing excitability has yet to be studied. GIRK channels are found throughout many of these brain regions (122), but it is unclear if and how they are regulated by the clock.

In addition to whether these neuronal oscillators regulate their excitability in a 24-hour pattern in a manner similar to the SCN, another fundamental question is how the oscillators maintain their phase relationship to the SCN. Future studies should include: 1) clearly defining the phase relationships of circadian rhythmicity between multiple

oscillators in the brain, 2) determining the sensitivity of these regions to external phase-shifting stimuli (*e.g.*, developing phase response curves), and 3) defining the mechanisms of entrainment for different brain regions. Perhaps GIRK channels regulate entrainment of these regions in a similar manner as the in SCN, such that integration of multiple $G_{i/o}$ coupled cues determines the phase relationship to the SCN. Understanding the phase relationships throughout the brain and the mechanisms underlying them could have huge ramifications for diseases such as schizophrenia, depression, and bipolar disorder, all of which exhibit signs of circadian disruption and involve integration of signals from multiple brain regions (135, 136).

Epilepsy & heart arrhythmias

GIRK channels have been implicated in epilepsy (118, 137-139), a disease of hyperexcitability and seizure generation within the brain. Temporal lobe epilepsy has time-of-day dependent seizure generation, with most seizures occurring in the late evening (5, 6, 140). In addition to epilepsy, GIRK channels, expressed in both the atria and ventricles, have been implicated in both long QT syndrome (141) and chronic atrial fibrillation (142, 143) in the heart. These arrhythmias, caused by dysregulation of excitability in the heart, have time-of-day dependent onset (8, 9, 144).

GIRK channels regulate intrinsic membrane properties of excitable cells throughout multiple peripheral oscillators in the brain and the body. In addition, a GIRK channel agonist, ML297, was able to increase time to seizure onset, increase survival, and decrease then number of convulsions in mice subjected to either a maximal electrical shock model of epilepsy, or a pentylenetetrazol (a $GABA_A$ antagonist) model of epilepsy

(139). Melatonin has been used both in rodent models and in humans to treat epilepsy, but with mixed results (145). Finally, NPY has anticonvulsant effects in animal models of both temporal lobe epilepsy and hippocampal kindling models (146), and application of NPY to hippocampal slices resected from epileptic patients results in decreased evoked action potentials from dentate granule cells (147). Future studies will determine if diurnal/circadian regulation of these channels extends to other oscillators in the brain and heart, and whether this could drive time-of-day dependencies of seizure and arrhythmia generation. Regulating time-of-day administration may increase the efficacy of antiepileptic and antiarrhythmic drugs.

Aging and development

GIRK channels are upregulated within the brain with age (120), and can be expressed differentially in cultured neurons in the presence of different neurotransmitters (113). In the cerebellar cortex, GIRK channel expression reaches maximum potential at P5 (148), and in cultured hippocampal neurons, GIRK channels mediate $GABA_B$-induced inward currents by day 14 in vitro (149). This tight control over GIRK channel expression during different developmental timelines could have interesting implications for circadian rhythms. For example, it is well known that sdolescents are typically phase-delayed compared to the general population (150). In addition, aging tends to dampen circadian rhythmicity and decreases the response to daily entrainment cues (151, 152). If GIRK channels are differentially expressed within the SCN across development, it could help explain these phenomena.

In addition to basic questions of whether GIRK channels are necessary in the development and maintenance of rhythms, they may also play a role in neurodevelopmental disorders such as Down syndrome (DS), which occurs when chromosome 21 is triplicated, or Rett syndrome (RTT), which is associated with loss-of-function mutations in the gene encoding the methyl CpG binding protein 2 (MeCP2). Both DS and RTT patients and mouse models (specifically the Ts65Dn model of DS where the mouse homolog of the "critical region" of human chromosome 21 is triplicated (153), and MeCP2$^{-/y}$ mice as a model of RTT (154)) exhibit sleep/wake and circadian rhythm disruption (155-162). In the case of DS, the GIRK2 gene is located on chromosome 21 and is thus triplicated, leading to overall decreases in neuronal excitability throughout the brain (163, 164). In RTT, MeCP2$^{-/y}$ mice show overall decreased excitability within the SCN (160). Also, some symptoms of RTT are alleviated with serotonin receptor agonists, which also activate GIRK channels (165, 166). This evidence suggests that GIRK channel regulation within the SCN may be disrupted in these developmental disorders and that further research is necessary to determine if recovery of proper GIRK channel regulation could help alleviate the sleep/wake and entrainment problems in these patients.

Implications for human entrainment

The results of this dissertation project have shown that NPY and melatonin are sufficient to modulate the timing of circadian rhythms via GIRK channel activation. Mice with a loss of GIRK2 fail to adjust their free running period in response to wheel running. We also demonstrate that loss of GIRK2 enhances entrainment to a 6-hour phase advance

of the light-dark cycle. These results highlight the importance of nonphotic entrainment and GIRK channels in multiple aspects of circadian timing.

The efficacy of nonphotic entrainment in humans has been debated, mainly due to the smaller magnitude of shifts and the prevalence of highly irregular lighting cycles in day-to-day life that may decrease the efficacy of nonphotic stimuli (167, 168). However, it is possible to entrain blind individuals to scheduled wake up times and brief 10 minute bicycle exercise (169). Additionally, musically enhanced bird song can shift human sleep/wake patterns when given in the early morning (170, 171). Interestingly, scheduled daytime exercise decreases the time to re-entrainment altered light cycles, either in a controlled circadian experiment or due to flights to a different time zone (172, 173). Exercise in older individuals also alleviates sleep disruption phenotypes (174-177). These experiments suggest that nonphotic stimuli, including music and exercise, are effective at entraining the circadian clock in humans, and could help improve sleep and mood in the older population or individuals with circadian disruption.

Bright light therapy (LT) is a noninvasive, fast, and efficacious way to reset the circadian clock. In patients with major depressive disorder, LT has been shown to alleviate some symptoms within one week, compared to antidepressant drugs that have a minimal time of two to three weeks before the effects are noticeable (178). LT has also been successfully used to treat seasonal affective disorder, insomnia, bipolar disorder, and epilepsy (179-183). However, LT is most effective in the morning, which is a problem for those patients with delayed phase angles, and is most often prescribed for a half hour every day (184-186). This strict regimen hampers adherence. Understanding how nonphotic cues, their downstream targets, and photic signaling interact could have

major implication for LT, potentially decreasing the time investment needed to be successful. Specifically, future studies should focus on the phase advancing effects of nonphotic cues, and timing these stimuli in such a way that does not block the effects of light.

Conclusions

This dissertation demonstrates that not only does NPY and melatonin-signaling require GIRK channel activation within the SCN, but that GIRK channel activation alone is sufficient to mimic a nonphotic phase advance, suggesting that GIRK channel activation may be a conserved response to $G_{i/o}$ coupled cues within the SCN (see Figure 1 for overall model of entrainment). GIRK channels have been implicated in a wide variety of diseases that show evidence of circadian disruption, and with proper timing and dosage, GIRK channel activation could be a powerful chronotherapeutic target. Future studies should investigate how GIRK channels regulate excitability in peripheral oscillators throughout the brain and body, how GIRK channels determine phase of the circadian clocks in these regions, if GIRK channels mediate development of circadian rhythms within the SCN, and finally, how GIRK channels modulate the nonphotic interactions with the photic entrainment system.

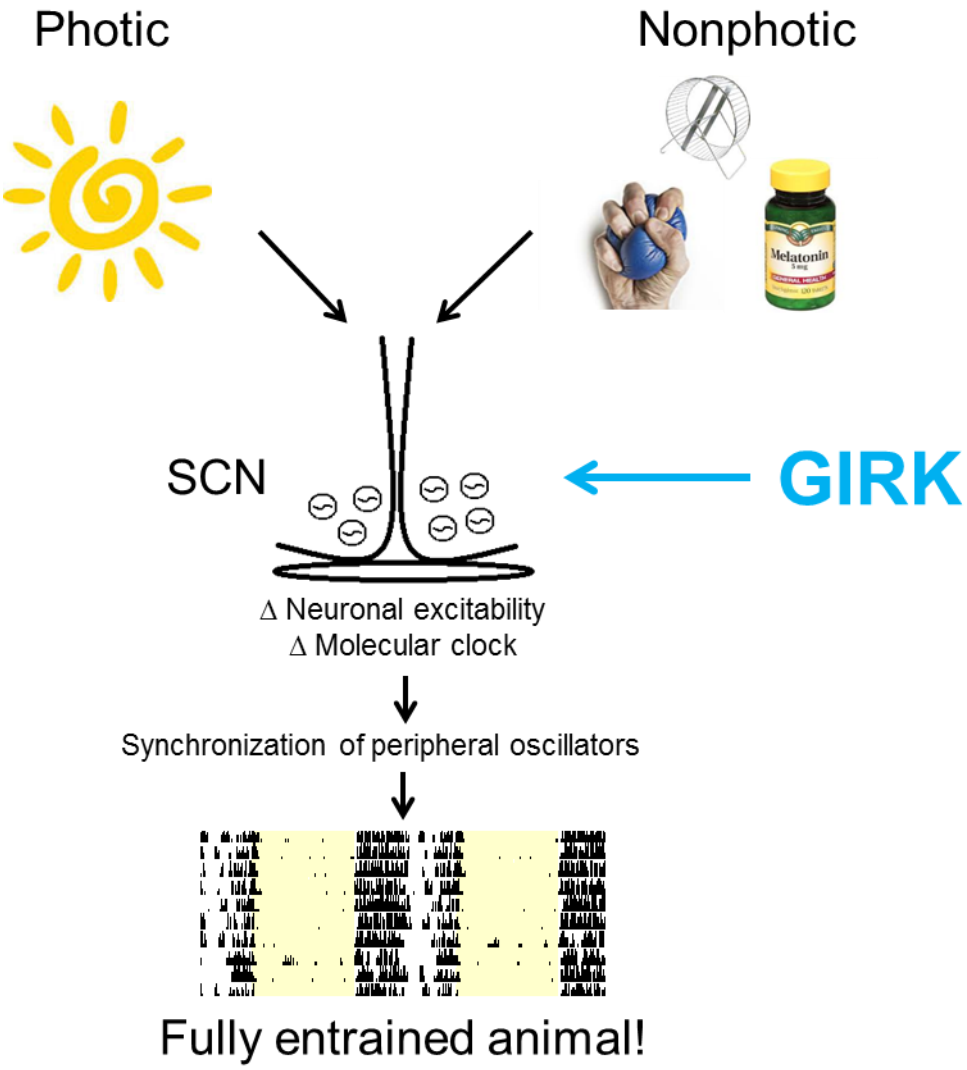

Figure 1: Model of entrainment. Photic and nonphotic stimuli interact and converge upon the SCN, a cluster of highly synchronized individual neuronal oscillators. These signals are coded by altering neuronal excitability and changing the timing of the molecular clock. The SCN signals through downstream cues to synchronize peripheral oscillators, eventually leading to a fully entrained animal. We hypothesize that GIRK channels modulate the change in activity of SCN neurons in response to nonphotic cues.

GENERAL LIST OF REFERENCES

1. Albrecht U. Timing to perfection: the biology of central and peripheral circadian clocks. Neuron. 2012;74(2):246-60. Epub 2012/05/01. doi: 10.1016/j.neuron.2012.04.006. PubMed PMID: 22542179.

2. Gamble KL, Young ME. Metabolism as an integral cog in the mammalian circadian clockwork. Crit Rev Biochem Mol Biol. 2013. Epub 2013/04/19. doi: 10.3109/10409238.2013.786672. PubMed PMID: 23594144.

3. Campos Costa I, Nogueira Carvalho H, Fernandes L. Aging, circadian rhythms and depressive disorders: a review. Am J Neurodegener Dis. 2013;2(4):228-46. Epub 2013/12/10. PubMed PMID: 24319642; PMCID: Pmc3852564.

4. Lee HJ, Son GH, Geum D. Circadian rhythm hypotheses of mixed features, antidepressant treatment resistance, and manic switching in bipolar disorder. Psychiatry Investig. 2013;10(3):225-32. Epub 2013/12/05. doi: 10.4306/pi.2013.10.3.225. PubMed PMID: 24302944; PMCID: Pmc3843013.

5. Loddenkemper T, Vendrame M, Zarowski M, Gregas M, Alexopoulos AV, Wyllie E, Kothare SV. Circadian patterns of pediatric seizures. Neurology. 2011;76(2):145-53. Epub 2011/01/12. doi: 10.1212/WNL.0b013e318206ca46. PubMed PMID: 21220719.

6. Zarowski M, Loddenkemper T, Vendrame M, Alexopoulos AV, Wyllie E, Kothare SV. Circadian distribution and sleep/wake patterns of generalized seizures in. Epilepsia. 2011;52(6):1076-83. Epub 2011/03/24. doi: 10.1111/j.1528-1167.2011.03023.x. PubMed PMID: 21426332.

7. Cho CH. Molecular mechanism of circadian rhythmicity of seizures in temporal lobe epilepsy. Front Cell Neurosci. 2012;6:55. doi: 10.3389/fncel.2012.00055. PubMed PMID: 23189039; PMCID: PMC3504933.

8. Capucci A, Calcagnini G, Mattei E, Triventi M, Bartolini P, Biancalana G, Gargaro A, Puglisi A, Censi F. Daily distribution of atrial arrhythmic episodes in sick sinus syndrome patients: implications for atrial arrhythmia monitoring. Europace. 2012;14(8):1117-24. Epub 2012/03/13. doi: 10.1093/europace/eus038. PubMed PMID: 22406397.

9. Shusterman V, Warman E, London B, Schwartzman D. Nocturnal peak in atrial tachyarrhythmia occurrence as a function of arrhythmia burden. J Cardiovasc Electrophysiol. 2012;23(6):604-11. Epub 2012/03/21. doi: 10.1111/j.1540-8167.2011.02263.x. PubMed PMID: 22429736.

10. Takigawa M, Kawamura M, Noda T, Yamada Y, Miyamoto K, Okamura H, Satomi K, Aiba T, Kamakura S, Sakaguchi T, Mizusawa Y, Itoh H, Horie M, Shimizu W. Seasonal and circadian distributions of cardiac events in genotyped patients with

congenital long QT syndrome. Circ J. 2012;76(9):2112-8. Epub 2012/07/13. PubMed PMID: 22785222.

11. Schroder EA, Lefta M, Zhang X, Bartos DC, Feng HZ, Zhao Y, Patwardhan A, Jin JP, Esser KA, Delisle BP. The cardiomyocyte molecular clock, regulation of Scn5a, and arrhythmia susceptibility. Am J Physiol Cell Physiol. 2013;304(10):C954-65. Epub 2013/02/01. doi: 10.1152/ajpcell.00383.2012. PubMed PMID: 23364267; PMCID: Pmc3651636.

12. Beckwith EJ, Yanovsky MJ. Circadian regulation of gene expression: at the crossroads of transcriptional and post-transcriptional regulatory networks. Curr Opin Genet Dev. 2014;27:35-42. Epub 2014/05/23. doi: 10.1016/j.gde.2014.03.007. PubMed PMID: 24846841.

13. Partch CL, Green CB, Takahashi JS. Molecular architecture of the mammalian circadian clock. Trends Cell Biol. 2014;24(2):90-9. Epub 2013/08/07. doi: 10.1016/j.tcb.2013.07.002. PubMed PMID: 23916625; PMCID: PMC3946763.

14. Hardin PE, Panda S. Circadian timekeeping and output mechanisms in animals. Curr Opin Neurobiol. 2013;23(5):724-31. Epub 2013/06/05. doi: 10.1016/j.conb.2013.02.018. PubMed PMID: 23731779; PMCID: PMC3973145.

15. Rusak B, Zucker I. Neural regulation of circadian rhythms. Physiol Rev. 1979;59(3):449-526. Epub 1979/07/01. PubMed PMID: 379886.

16. Schwartz WJ, Busis NA, Hedley-Whyte ET. A discrete lesion of ventral hypothalamus and optic chiasm that disturbed the daily temperature rhythm. J Neurol. 1986;233(1):1-4. Epub 1986/02/01. PubMed PMID: 3950658.

17. Cohen RA, Albers HE. Disruption of human circadian and cognitive regulation following a discrete hypothalamic lesion: a case study. Neurology. 1991;41(5):726-9. Epub 1991/05/01. PubMed PMID: 2027490.

18. Lehman MN, Silver R, Gladstone WR, Kahn RM, Gibson M, Bittman EL. Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. J Neurosci. 1987;7(6):1626-38. Epub 1987/06/01. PubMed PMID: 3598638.

19. Ralph MR, Foster RG, Davis FC, Menaker M. Transplanted suprachiasmatic nucleus determines circadian period. Science. 1990;247(4945):975-8. Epub 1990/02/23. PubMed PMID: 2305266.

20. Welsh DK, Logothetis DE, Meister M, Reppert SM. Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron. United States1995. p. 697-706.

21. Brown TM, Piggins HD. Electrophysiology of the suprachiasmatic circadian clock. Prog Neurobiol. 2007;82(5):229-55. Epub 2007/07/25. doi: 10.1016/j.pneurobio.2007.05.002. PubMed PMID: 17646042.

22. Kuhlman SJ, McMahon DG. Encoding the ins and outs of circadian pacemaking. J Biol Rhythms. 2006;21(6):470-81. Epub 2006/11/17. doi: 10.1177/0748730406294316. PubMed PMID: 17107937.

23. Pennartz CM, Bierlaagh MA, Geurtsen AM. Cellular mechanisms underlying spontaneous firing in rat suprachiasmatic nucleus: involvement of a slowly inactivating component of sodium current. J Neurophysiol. 1997;78(4):1811-25. Epub 1997/10/27. PubMed PMID: 9325350.

24. Jackson AC, Yao GL, Bean BP. Mechanism of spontaneous firing in dorsomedial suprachiasmatic nucleus neurons. J Neurosci. United States2004. p. 7985-98.

25. Belle MD, Diekman CO, Forger DB, Piggins HD. Daily electrical silencing in the mammalian circadian clock. Science. United States2009. p. 281-4.

26. Colwell CS. Circadian modulation of calcium levels in cells in the suprachiasmatic nucleus. Eur J Neurosci. 2000;12(2):571-6. Epub 2000/03/11. PubMed PMID: 10712636.

27. Pennartz CM, de Jeu MT, Bos NP, Schaap J, Geurtsen AM. Diurnal modulation of pacemaker potentials and calcium current in the mammalian circadian clock. Nature. England2002. p. 286-90.

28. Lundkvist GB, Kwak Y, Davis EK, Tei H, Block GD. A calcium flux is required for circadian rhythm generation in mammalian pacemaker neurons. J Neurosci. United States2005. p. 7682-6.

29. Cloues RK, Sather WA. Afterhyperpolarization regulates firing rate in neurons of the suprachiasmatic nucleus. J Neurosci. United States2003. p. 1593-604.

30. Teshima K, Kim SH, Allen CN. Characterization of an apamin-sensitive potassium current in suprachiasmatic nucleus neurons. Neuroscience. 2003;120(1):65-73. Epub 2003/07/10. PubMed PMID: 12849741.

31. Kuhlman SJ, McMahon DG. Rhythmic regulation of membrane potential and potassium current persists in SCN neurons in the absence of environmental input. Eur J Neurosci. 2004;20(4):1113-7. Epub 2004/08/13. doi: 10.1111/j.1460-9568.2004.03555.x. PubMed PMID: 15305881.

32. Itri JN, Michel S, Vansteensel MJ, Meijer JH, Colwell CS. Fast delayed rectifier potassium current is required for circadian neural activity. Nat Neurosci. United States2005. p. 650-6.

33. Meredith AL, Wiler SW, Miller BH, Takahashi JS, Fodor AA, Ruby NF, Aldrich RW. BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. Nat Neurosci. United States2006. p. 1041-9.

34. Pitts GR, Ohta H, McMahon DG. Daily rhythmicity of large-conductance Ca2+ -activated K+ currents in suprachiasmatic nucleus neurons. Brain Res. Netherlands2006. p. 54-62.

35. Itri JN, Vosko AM, Schroeder A, Dragich JM, Michel S, Colwell CS. Circadian regulation of a-type potassium currents in the suprachiasmatic nucleus. J Neurophysiol. 2010;103(2):632-40. Epub 2009/11/27. doi: 10.1152/jn.00670.2009. PubMed PMID: 19939959; PMCID: Pmc2822681.

36. Granados-Fuentes D, Norris AJ, Carrasquillo Y, Nerbonne JM, Herzog ED. I(A) channels encoded by Kv1.4 and Kv4.2 regulate neuronal firing in the suprachiasmatic nucleus and circadian rhythms in locomotor activity. J Neurosci. 2012;32(29):10045-52. Epub 2012/07/21. doi: 10.1523/jneurosci.0174-12.2012. PubMed PMID: 22815518; PMCID: Pmc3752070.

37. Colwell CS. Linking neural activity and molecular oscillations in the SCN. Nat Rev Neurosci. 2011;12(10):553-69. Epub 2011/09/03. doi: 10.1038/nrn3086. PubMed PMID: 21886186.

38. Marinc C, Derst C, Pruss H, Veh RW. Immunocytochemical localization of TASK-3 protein (K2P9.1) in the rat brain. Cell Mol Neurobiol. 2014;34(1):61-70. Epub 2013/10/01. doi: 10.1007/s10571-013-9987-7. PubMed PMID: 24077856.

39. Golombek DA, Rosenstein RE. Physiology of circadian entrainment. Physiol Rev. United States2010. p. 1063-102.

40. Morin LP, Shivers KY, Blanchard JH, Muscat L. Complex organization of mouse and rat suprachiasmatic nucleus. Neuroscience. United States2006. p. 1285-97.

41. Welsh DK, Takahashi JS, Kay SA. Suprachiasmatic nucleus: cell autonomy and network properties. Annu Rev Physiol. 2010;72:551-77. Epub 2010/02/13. doi: 10.1146/annurev-physiol-021909-135919. PubMed PMID: 20148688; PMCID: PMC3758475.

42. Webb AB, Angelo N, Huettner JE, Herzog ED. Intrinsic, nondeterministic circadian rhythm generation in identified mammalian neurons. Proc Natl Acad Sci U S A. United States2009. p. 16493-8.

43. Mohawk JA, Takahashi JS. Cell autonomy and synchrony of suprachiasmatic nucleus circadian oscillators. Trends Neurosci. England: 2011 Elsevier Ltd; 2011. p. 349-58.

44. Colwell CS, Michel S, Itri J, Rodriguez W, Tam J, Lelievre V, Hu Z, Liu X, Waschek JA. Disrupted circadian rhythms in VIP- and PHI-deficient mice. Am J Physiol Regul Integr Comp Physiol. United States2003. p. R939-49.

45. Aton SJ, Colwell CS, Harmar AJ, Waschek J, Herzog ED. Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. Nat Neurosci. United States2005. p. 476-83.

46. Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. Annu Rev Physiol. 2010;72:517-49. Epub 2010/02/13. doi: 10.1146/annurev-physiol-021909-135821. PubMed PMID: 20148687.

47. Challet E. Minireview: Entrainment of the suprachiasmatic clockwork in diurnal and nocturnal mammals. Endocrinology. 2007;148(12):5648-55. Epub 2007/09/29. doi: 10.1210/en.2007-0804. PubMed PMID: 17901231.

48. Smale L, Castleberry C, Nunez AA. Fos rhythms in the hypothalamus of Rattus and Arvicanthis that exhibit nocturnal and diurnal patterns of rhythmicity. Brain Res. Netherlands2001. p. 101-5.

49. Schwartz MD, Nunez AA, Smale L. Differences in the suprachiasmatic nucleus and lower subparaventricular zone of diurnal and nocturnal rodents. Neuroscience. United States2004. p. 13-23.

50. Schwartz MD, Nunez AA, Smale L. Rhythmic cFos expression in the ventral subparaventricular zone influences general activity rhythms in the Nile grass rat, Arvicanthis niloticus. Chronobiol Int. England2009. p. 1290-306.

51. Hastings MH, Duffield GE, Smith EJ, Maywood ES, Ebling FJ. Entrainment of the circadian system of mammals by nonphotic cues. Chronobiol Int. 1998;15(5):425-45. Epub 1998/10/27. PubMed PMID: 9787934.

52. Miller JD. On the nature of the circadian clock in mammals. Am J Physiol. 1993;264(5 Pt 2):R821-32. Epub 1993/05/01. PubMed PMID: 8388661.

53. Reebs SG, Mrosovsky N. Effects of induced wheel running on the circadian activity rhythms of Syrian hamsters: entrainment and phase response curve. J Biol Rhythms. 1989;4(1):39-48. Epub 1989/01/01. PubMed PMID: 2519579.

54. Hastings MH, Mead SM, Vindlacheruvu RR, Ebling FJ, Maywood ES, Grosse J. Non-photic phase shifting of the circadian activity rhythm of Syrian hamsters: the relative potency of arousal and melatonin. Brain Res. Netherlands1992. p. 20-6.

55. Mead S, Ebling FJ, Maywood ES, Humby T, Herbert J, Hastings MH. A nonphotic stimulus causes instantaneous phase advances of the light-entrainable circadian oscillator of the Syrian hamster but does not induce the expression of c-fos in the

suprachiasmatic nuclei. J Neurosci. 1992;12(7):2516-22. Epub 1992/07/01. PubMed PMID: 1613544.

56. Harrington ME, Rusak B. Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms. J Biol Rhythms. 1986;1(4):309-25. Epub 1986/01/01. PubMed PMID: 2979593.

57. Pickard GE, Ralph MR, Menaker M. The intergeniculate leaflet partially mediates effects of light on circadian rhythms. J Biol Rhythms. 1987;2(1):35-56. Epub 1987/01/01. PubMed PMID: 2979650.

58. Johnson RF, Moore RY, Morin LP. Lateral geniculate lesions alter circadian activity rhythms in the hamster. Brain Res Bull. 1989;22(2):411-22. Epub 1989/02/01. PubMed PMID: 2650808.

59. Pickard GE. Entrainment of the circadian rhythm of wheel-running activity is phase shifted by ablation of the intergeniculate leaflet. Brain Res. 1989;494(1):151-4. Epub 1989/08/07. PubMed PMID: 2765914.

60. Pickard GE. Intergeniculate leaflet ablation alters circadian rhythms in the mouse. Neuroreport. 1994;5(16):2186-8. Epub 1994/10/27. PubMed PMID: 7865773.

61. Maywood ES, Smith E, Hall SJ, Hastings MH. A thalamic contribution to arousal-induced, non-photic entrainment of the circadian clock of the Syrian hamster. Eur J Neurosci. 1997;9(8):1739-47. Epub 1997/08/01. PubMed PMID: 9283828.

62. Yannielli PC, Harrington ME. Neuropeptide Y applied in vitro can block the phase shifts induced by light in vivo. Neuroreport. 2000;11(7):1587-91. Epub 2000/06/07. PubMed PMID: 10841381.

63. Lall GS, Biello SM. Attenuation of circadian light induced phase advances and delays by neuropeptide Y and a neuropeptide Y Y1/Y5 receptor agonist. Neuroscience. 2003;119(2):611-8. Epub 2003/05/29. PubMed PMID: 12770573.

64. Soscia SJ, Harrington ME. Neuropeptide Y attenuates NMDA-induced phase shifts in the SCN of NPY Y1 receptor knockout mice in vitro. Brain Res. 2004;1023(1):148-53. Epub 2004/09/15. doi: 10.1016/j.brainres.2004.07.037. PubMed PMID: 15364030.

65. Yannielli PC, Brewer JM, Harrington ME. Blockade of the NPY Y5 receptor potentiates circadian responses to light: complementary in vivo and in vitro studies. Eur J Neurosci. 2004;19(4):891-7. Epub 2004/03/11. PubMed PMID: 15009136.

66. Janik D, Mikkelsen JD, Mrosovsky N. Cellular colocalization of Fos and neuropeptide Y in the intergeniculate leaflet after nonphotic phase-shifting events. Brain Res. Netherlands1995. p. 137-45.

67. Huhman KL, Albers HE. Neuropeptide Y microinjected into the suprachiasmatic region phase shifts circadian rhythms in constant darkness. Peptides. 1994;15(8):1475-8. Epub 1994/01/01. PubMed PMID: 7700850.

68. Yannielli P, Harrington ME. Let there be "more" light: enhancement of light actions on the circadian system. Prog Neurobiol. 2004;74(1):59-76. Epub 2004/09/24. doi: 10.1016/j.pneurobio.2004.06.001. PubMed PMID: 15381317.

69. Biello SM, Janik D, Mrosovsky N. Neuropeptide Y and behaviorally induced phase shifts. Neuroscience. 1994;62(1):273-9. Epub 1994/09/01. PubMed PMID: 7816205.

70. Huhman KL, Gillespie CF, Marvel CL, Albers HE. Neuropeptide Y phase shifts circadian rhythms in vivo via a Y2 receptor. Neuroreport. 1996;7(7):1249-52. Epub 1996/05/17. PubMed PMID: 8817542.

71. Harrington M, Molyneux P, Soscia S, Prabakar C, McKinley-Brewer J, Lall G. Behavioral and neurochemical sources of variability of circadian period and phase: studies of circadian rhythms of npy-/- mice. Am J Physiol Regul Integr Comp Physiol. 2007;292(3):R1306-14. Epub 2006/11/04. doi: 10.1152/ajpregu.00383.2006. PubMed PMID: 17082354.

72. van de Kar LD, Lorens SA. Differential serotonergic innervation of individual hypothalamic nuclei and other forebrain regions by the dorsal and median midbrain raphe nuclei. Brain Res. Netherlands1979. p. 45-54.

73. Levine JD, Rosenwasser AM, Yanovski JA, Adler NT. Circadian activity rhythms in rats with midbrain raphe lesions. Brain Res. Netherlands1986. p. 240-9.

74. Smale L, Michels KM, Moore RY, Morin LP. Destruction of the hamster serotonergic system by 5,7-DHT: effects on circadian rhythm phase, entrainment and response to triazolam. Brain Res. Netherlands1990. p. 9-19.

75. Morin LP, Blanchard J. Depletion of brain serotonin by 5,7-DHT modifies hamster circadian rhythm response to light. Brain Res. Netherlands1991. p. 173-85.

76. Edgar DM, Reid MS, Dement WC. Serotonergic afferents mediate activity-dependent entrainment of the mouse circadian clock. Am J Physiol. 1997;273(1 Pt 2):R265-9. Epub 1997/07/01. PubMed PMID: 9249559.

77. Antle MC, Marchant EG, Niel L, Mistlberger RE. Serotonin antagonists do not attenuate activity-induced phase shifts of circadian rhythms in the Syrian hamster. Brain Res. Netherlands: 1998 Published by Elsevier Science B.V.; 1998. p. 139-49.

78. Ugrumov MV, Popov AP, Vladimirov SV, Kasmambetova S, Novodjilova AP, Tramu G. Development of the suprachiasmatic nucleus in rats during ontogenesis: serotonin-immunopositive fibers. Neuroscience. England1994. p. 161-5.

79. Takatsuji K, Oyamada H, Tohyama M. Postnatal development of the substance P-, neuropeptide Y- and serotonin-containing fibers in the rat suprachiasmatic nucleus in relation to development of the retino-hypothalamic projection. Brain Res Dev Brain Res. 1995;84(2):261-70. Epub 1995/02/16. PubMed PMID: 7538055.

80. Takeuchi K, Mohammad S, Ozaki T, Morioka E, Kawaguchi K, Kim J, Jeong B, Hong JH, Lee KJ, Ikeda M. Serotonin-2C receptor involved serotonin-induced Ca(2)(+) mobilisations in neuronal progenitors and neurons in rat suprachiasmatic nucleus. Sci Rep. England2014. p. 4106.

81. Racagni G, Riva MA, Popoli M. The interaction between the internal clock and antidepressant efficacy. Int Clin Psychopharmacol. England2007. p. S9-S14.

82. Ciarleglio CM, Resuehr HE, McMahon DG. Interactions of the serotonin and circadian systems: nature and nurture in rhythms and blues. Neuroscience. United States: A 2011. Published by Elsevier Ltd.; 2011. p. 8-16.

83. Colecchia EF, Penev PD, Zee PC, Turek FW. Phase-shifting effects of a serotonin agonist in tau mutant hamsters. Brain Res. Netherlands1996. p. 227-31.

84. Medanic M, Gillette MU. Serotonin regulates the phase of the rat suprachiasmatic circadian pacemaker in vitro only during the subjective day. J Physiol. 1992;450:629-42. Epub 1992/05/01. PubMed PMID: 1432721; PMCID: PMC1176142.

85. Prosser RA, Dean RR, Edgar DM, Heller HC, Miller JD. Serotonin and the mammalian circadian system: I. In vitro phase shifts by serotonergic agonists and antagonists. J Biol Rhythms. 1993;8(1):1-16. Epub 1993/01/01. PubMed PMID: 8490207.

86. Coomans CP, Ramkisoensing A, Meijer JH. The suprachiasmatic nuclei as a seasonal clock. Front Neuroendocrinol. 2014. Epub 2014/12/03. doi: 10.1016/j.yfrne.2014.11.002. PubMed PMID: 25451984.

87. Pevet P, Jacob N, Vuillez P. Suprachiasmatic nuclei, intergeniculate leaflet, and photoperiod. Adv Exp Med Biol. 1999;460:233-45. Epub 2000/05/16. PubMed PMID: 10810519.

88. Dubocovich ML, Rivera-Bermudez MA, Gerdin MJ, Masana MI. Molecular pharmacology, regulation and function of mammalian melatonin receptors. Front Biosci. 2003;8:d1093-108. Epub 2003/09/06. PubMed PMID: 12957828.

89. Dubocovich ML. Melatonin receptors: role on sleep and circadian rhythm regulation. Sleep Med. Netherlands2007. p. 34-42.

90. Hardeland R, Poeggeler B. Melatonin and synthetic melatonergic agonists: actions and metabolism in the central nervous system. Cent Nerv Syst Agents Med Chem. Netherlands2012. p. 189-216.

91. Saper CB, Scammell TE, Lu J. Hypothalamic regulation of sleep and circadian rhythms. Nature. England2005. p. 1257-63.

92. Benloucif S, Dubocovich ML. Melatonin and light induce phase shifts of circadian activity rhythms in the C3H/HeN mouse. J Biol Rhythms. 1996;11(2):113-25. Epub 1996/06/01. PubMed PMID: 8744239.

93. Hunt AE, Al-Ghoul WM, Gillette MU, Dubocovich ML. Activation of MT(2) melatonin receptors in rat suprachiasmatic nucleus phase advances the circadian clock. Am J Physiol Cell Physiol. 2001;280(1):C110-8. Epub 2000/12/21. PubMed PMID: 11121382.

94. Dubocovich ML, Hudson RL, Sumaya IC, Masana MI, Manna E. Effect of MT1 melatonin receptor deletion on melatonin-mediated phase shift of circadian rhythms in the C57BL/6 mouse. J Pineal Res. 2005;39(2):113-20. Epub 2005/08/16. doi: 10.1111/j.1600-079X.2005.00230.x. PubMed PMID: 16098087.

95. McArthur AJ, Gillette MU, Prosser RA. Melatonin directly resets the rat suprachiasmatic circadian clock in vitro. Brain Res. 1991;565(1):158-61. Epub 1991/11/22. PubMed PMID: 1773352.

96. Lewy AJ, Ahmed S, Jackson JM, Sack RL. Melatonin shifts human circadian rhythms according to a phase-response curve. Chronobiol Int. 1992;9(5):380-92. Epub 1992/10/01. PubMed PMID: 1394610.

97. Lewy AJ, Bauer VK, Ahmed S, Thomas KH, Cutler NL, Singer CM, Moffit MT, Sack RL. The human phase response curve (PRC) to melatonin is about 12 hours out of phase with the PRC to light. Chronobiol Int. 1998;15(1):71-83. Epub 1998/03/11. PubMed PMID: 9493716.

98. Burgess HJ, Revell VL, Molina TA, Eastman CI. Human phase response curves to three days of daily melatonin: 0.5 mg versus 3.0 mg. J Clin Endocrinol Metab. United States2010. p. 3325-31.

99. Weber ET, Rea MA. Neuropeptide Y blocks light-induced phase advances but not delays of the circadian activity rhythm in hamsters. Neurosci Lett. 1997;231(3):159-62. Epub 1997/08/15. PubMed PMID: 9300646.

100. Brewer JM, Yannielli PC, Harrington ME. Neuropeptide Y differentially suppresses per1 and per2 mRNA induced by light in the suprachiasmatic nuclei of the golden hamster. J Biol Rhythms. 2002;17(1):28-39. Epub 2002/02/12. PubMed PMID: 11837945.

101. Mistlberger RE, Antle MC. Behavioral inhibition of light-induced circadian phase resetting is phase and serotonin dependent. Brain Res. Netherlands: 1998 Elsevier Science B.V.; 1998. p. 31-8.

102. Pickard GE, Weber ET, Scott PA, Riberdy AF, Rea MA. 5HT1B receptor agonists inhibit light-induced phase shifts of behavioral circadian rhythms and expression of the immediate-early gene c-fos in the suprachiasmatic nucleus. J Neurosci. 1996;16(24):8208-20. Epub 1996/12/15. PubMed PMID: 8987845.

103. Jiang ZG, Nelson CS, Allen CN. Melatonin activates an outward current and inhibits Ih in rat suprachiasmatic nucleus neurons. Brain Res. 1995;687(1-2):125-32. Epub 1995/07/31. PubMed PMID: 7583297.

104. Scott FF, Belle MD, Delagrange P, Piggins HD. Electrophysiological effects of melatonin on mouse Per1 and non-Per1 suprachiasmatic nuclei neurones in vitro. J Neuroendocrinol. 2010;22(11):1148-56. Epub 2010/09/08. doi: 10.1111/j.1365-2826.2010.02063.x. PubMed PMID: 20819119.

105. van den Pol AN, Obrietan K, Chen G, Belousov AB. Neuropeptide Y-mediated long-term depression of excitatory activity in suprachiasmatic nucleus neurons. J Neurosci. 1996;16(18):5883-95. Epub 1996/09/15. PubMed PMID: 8795640.

106. Belle MD, Hughes AT, Bechtold DA, Cunningham P, Pierucci M, Burdakov D, Piggins HD. Acute suppressive and long-term phase modulation actions of orexin on the mammalian circadian clock. J Neurosci. 2014;34(10):3607-21. Epub 2014/03/07. doi: 10.1523/jneurosci.3388-13.2014. PubMed PMID: 24599460; PMCID: Pmc3942578.

107. Smith VM, Sterniczuk R, Phillips CI, Antle MC. Altered photic and non-photic phase shifts in 5-HT(1A) receptor knockout mice. Neuroscience. United States2008. p. 513-23.

108. Shelton J, Yun S, Losee Olson S, Turek F, Bonaventure P, Dvorak C, Lovenberg T, Dugovic C. Selective pharmacological blockade of the 5-HT7 receptor attenuates light and 8-OH-DPAT induced phase shifts of mouse circadian wheel running activity. Front Behav Neurosci. 2014;8:453. Epub 2015/02/03. doi: 10.3389/fnbeh.2014.00453. PubMed PMID: 25642174; PMCID: PMC4295543.

109. Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK, Reppert SM. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. Neuron. United States1997. p. 91-102.

110. van den Top M, Buijs RM, Ruijter JM, Delagrange P, Spanswick D, Hermes ML. Melatonin generates an outward potassium current in rat suprachiasmatic nucleus neurones in vitro independent of their circadian rhythm. Neuroscience. 2001;107(1):99-108. Epub 2001/12/18. PubMed PMID: 11744250.

111. Paredes MF, Greenwood J, Baraban SC. Neuropeptide Y modulates a G protein-coupled inwardly rectifying potassium current in the mouse hippocampus. Neurosci Lett. 2003;340(1):9-12. Epub 2003/03/22. PubMed PMID: 12648746.

112. Fu LY, Acuna-Goycolea C, van den Pol AN. Neuropeptide Y inhibits hypocretin/orexin neurons by multiple presynaptic and postsynaptic mechanisms: tonic

depression of the hypothalamic arousal system. J Neurosci. 2004;24(40):8741-51. Epub 2004/10/08. doi: 10.1523/jneurosci.2268-04.2004. PubMed PMID: 15470140.

113. Sickmann T, Alzheimer C. Agonist-specific maturation of GIRK current responses in acutely isolated pyramidal neurons of rat neocortex. Brain Res. Netherlands2002. p. 166-74.

114. Levita L, Hammack SE, Mania I, Li XY, Davis M, Rainnie DG. 5-hydroxytryptamine1A-like receptor activation in the bed nucleus of the stria terminalis: electrophysiological and behavioral studies. Neuroscience. United States2004. p. 583-96.

115. Obrietan K, van den Pol AN. Neuropeptide Y depresses GABA-mediated calcium transients in developing suprachiasmatic nucleus neurons: a novel form of calcium long-term depression. J Neurosci. 1996;16(10):3521-33. Epub 1996/05/15. PubMed PMID: 8627385.

116. Bobrzynska KJ, Godfrey MH, Mrosovsky N. Serotonergic stimulation and nonphotic phase-shifting in hamsters. Physiol Behav. United States1996. p. 221-30.

117. Horikawa K, Yokota S, Fuji K, Akiyama M, Moriya T, Okamura H, Shibata S. Nonphotic entrainment by 5-HT1A/7 receptor agonists accompanied by reduced Per1 and Per2 mRNA levels in the suprachiasmatic nuclei. J Neurosci. United States2000. p. 5867-73.

118. Luscher C, Slesinger PA. Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. Nat Rev Neurosci. 2010;11(5):301-15. Epub 2010/04/15. doi: 10.1038/nrn2834. PubMed PMID: 20389305; PMCID: 3052907.

119. Koyrakh L, Lujan R, Colon J, Karschin C, Kurachi Y, Karschin A, Wickman K. Molecular and cellular diversity of neuronal G-protein-gated potassium channels. J Neurosci. 2005;25(49):11468-78. Epub 2005/12/13. doi: 10.1523/jneurosci.3484-05.2005. PubMed PMID: 16339040.

120. Fernandez-Alacid L, Watanabe M, Molnar E, Wickman K, Lujan R. Developmental regulation of G protein-gated inwardly-rectifying K+ (GIRK/Kir3) channel subunits in the brain. Eur J Neurosci. 2011;34(11):1724-36. Epub 2011/11/22. doi: 10.1111/j.1460-9568.2011.07886.x. PubMed PMID: 22098295; PMCID: PMC3936682.

121. Doupnik CA. GPCR-Kir channel signaling complexes: defining rules of engagement. J Recept Signal Transduct Res. 2008;28(1-2):83-91. Epub 2008/04/26. doi: 10.1080/10799890801941970. PubMed PMID: 18437632.

122. Saenz del Burgo L, Cortes R, Mengod G, Zarate J, Echevarria E, Salles J. Distribution and neurochemical characterization of neurons expressing GIRK channels in the rat brain. J Comp Neurol. 2008;510(6):581-606. Epub 2008/08/14. doi: 10.1002/cne.21810. PubMed PMID: 18698588.

123. Wickman K, Karschin C, Karschin A, Picciotto MR, Clapham DE. Brain localization and behavioral impact of the G-protein-gated K+ channel subunit GIRK4. J Neurosci. 2000;20(15):5608-15. Epub 2000/07/26. PubMed PMID: 10908597.

124. Kloukina V, Herzer S, Karlsson N, Perez M, Daraio T, Meister B. G-protein-gated inwardly rectifying K+ channel 4 (GIRK4) immunoreactivity in chemically defined neurons of the hypothalamic arcuate nucleus that control body weight. J Chem Neuroanat. 2012;44(1):14-23. Epub 2012/04/03. doi: 10.1016/j.jchemneu.2012.03.003. PubMed PMID: 22465809.

125. Hibino H, Inanobe A, Furutani K, Murakami S, Findlay I, Kurachi Y. Inwardly rectifying potassium channels: their structure, function, and physiological roles. Physiol Rev. 2010;90(1):291-366. Epub 2010/01/21. doi: 10.1152/physrev.00021.2009. PubMed PMID: 20086079.

126. Kim CS, Johnston D. A1 adenosine receptor-mediated GIRK channels contributes to the resting conductance of CA1 neurons in the dorsal hippocampus. J Neurophysiol: Journal of Neurophysiology.; 2015. p. jn 00951 2014.

127. Yamamoto R, Hatano N, Sugai T, Kato N. Serotonin induces depolarization in lateral amygdala neurons by activation of TRPC-like current and inhibition of GIRK current depending on 5-HT(2C) receptor. Neuropharmacology. 2014;82:49-58. Epub 2014/03/26. doi: 10.1016/j.neuropharm.2014.03.007. PubMed PMID: 24662600.

128. Zhang X, van den Pol AN. Direct inhibition of arcuate proopiomelanocortin neurons: a potential mechanism for the orexigenic actions of dynorphin. J Physiol. England2013. p. 1731-47.

129. Lalive AL, Munoz MB, Bellone C, Slesinger PA, Luscher C, Tan KR. Firing modes of dopamine neurons drive bidirectional GIRK channel plasticity. J Neurosci. United States2014. p. 5107-14.

130. Duffield GE, Hastings MH, Ebling FJ. Investigation into the regulation of the circadian system by dopamine and melatonin in the adult Siberian hamster (Phodopus sungorus). J Neuroendocrinol. 1998;10(11):871-84. Epub 1998/11/27. PubMed PMID: 9831263.

131. Iyer R, Wang TA, Gillette MU. Circadian gating of neuronal functionality: a basis for iterative metaplasticity. Front Syst Neurosci. 2014;8:164. Epub 2014/10/07. doi: 10.3389/fnsys.2014.00164. PubMed PMID: 25285070; PMCID: PMC4168688.

132. Wang LM, Dragich JM, Kudo T, Odom IH, Welsh DK, O'Dell TJ, Colwell CS. Expression of the circadian clock gene Period2 in the hippocampus: possible implications for synaptic plasticity and learned behaviour. ASN Neuro. 2009;1(3). Epub 2009/07/03. doi: 10.1042/an20090020. PubMed PMID: 19570032; PMCID: PMC2695588.

133. Rath MF, Rovsing L, Moller M. Circadian oscillators in the mouse brain: molecular clock components in the neocortex and cerebellar cortex. Cell Tissue Res.

2014;357(3):743-55. Epub 2014/05/21. doi: 10.1007/s00441-014-1878-9. PubMed PMID: 24842045.

134. Sakhi K, Belle MD, Gossan N, Delagrange P, Piggins HD. Daily variation in the electrophysiological activity of mouse medial habenula neurones. J Physiol. England2014. p. 587-603.

135. Baron KG, Reid KJ. Circadian misalignment and health. Int Rev Psychiatry. 2014;26(2):139-54. Epub 2014/06/04. doi: 10.3109/09540261.2014.911149. PubMed PMID: 24892891.

136. Karatsoreos IN. Links between Circadian Rhythms and Psychiatric Disease. Front Behav Neurosci. 2014;8:162. Epub 2014/05/17. doi: 10.3389/fnbeh.2014.00162. PubMed PMID: 24834040; PMCID: PMC4018537.

137. Kobayashi T, Ikeda K. G protein-activated inwardly rectifying potassium channels as potential therapeutic targets. Curr Pharm Des. 2006;12(34):4513-23. Epub 2006/12/16. PubMed PMID: 17168757.

138. D'Adamo MC, Catacuzzeno L, Di Giovanni G, Franciolini F, Pessia M. K(+) channelepsy: progress in the neurobiology of potassium channels and epilepsy. Front Cell Neurosci. 2013;7:134. Epub 2013/09/26. doi: 10.3389/fncel.2013.00134. PubMed PMID: 24062639; PMCID: PMC3772396.

139. Kaufmann K, Romaine I, Days E, Pascual C, Malik A, Yang L, Zou B, Du Y, Sliwoski G, Morrison RD, Denton J, Niswender CM, Daniels JS, Sulikowski GA, Xie XS, Lindsley CW, Weaver CD. ML297 (VU0456810), the First Potent and Selective Activator of the GIRK Potassium Channel, Displays Antiepileptic Properties in Mice. ACS Chem Neurosci. 2013;4(9):1278-86. Epub 2013/06/05. doi: 10.1021/cn400062a. PubMed PMID: 23730969; PMCID: Pmc3778424.

140. Stanley DA, Talathi SS, Parekh MB, Cordiner DJ, Zhou J, Mareci TH, Ditto WL, Carney PR. Phase shift in the 24-hour rhythm of hippocampal EEG spiking activity in a rat model of temporal lobe epilepsy. J Neurophysiol. 2013. Epub 2013/05/17. doi: 10.1152/jn.00911.2012. PubMed PMID: 23678009.

141. Yang Y, Liang B, Liu J, Li J, Grunnet M, Olesen SP, Rasmussen HB, Ellinor PT, Gao L, Lin X, Li L, Wang L, Xiao J, Liu Y, Zhang S, Liang D, Peng L, Jespersen T, Chen YH. Identification of a Kir3.4 mutation in congenital long QT syndrome. Am J Hum Genet. 2010;86(6):872-80. Epub 2010/06/22. PubMed PMID: 20560207; PMCID: Pmc3032079.

142. Dobrev D, Friedrich A, Voigt N, Jost N, Wettwer E, Christ T, Knaut M, Ravens U. The G protein-gated potassium current I(K,ACh) is constitutively active in patients with chronic atrial fibrillation. Circulation. 2005;112(24):3697-706. Epub 2005/12/07. doi: 10.1161/circulationaha.105.575332. PubMed PMID: 16330682.

143. Jabbari J, Olesen MS, Holst AG, Nielsen JB, Haunso S, Svendsen JH. Common polymorphisms in KCNJ5 [corrected] are associated with early-onset lone atrial fibrillation in Caucasians. Cardiology. 2011;118(2):116-20. Epub 2011/05/11. doi: 10.1159/000323840. PubMed PMID: 21555883.

144. Portaluppi F, Tiseo R, Smolensky MH, Hermida RC, Ayala DE, Fabbian F. Circadian rhythms and cardiovascular health. Sleep Med Rev. 2012;16(2):151-66. Epub 2011/06/07. doi: 10.1016/j.smrv.2011.04.003. PubMed PMID: 21641838.

145. Banach M, Gurdziel E, Jedrych M, Borowicz KK. Melatonin in experimental seizures and epilepsy. Pharmacol Rep. 2011;63(1):1-11. Epub 2011/03/29. PubMed PMID: 21441606.

146. Kovac S, Walker MC. Neuropeptides in epilepsy. Neuropeptides. 2013;47(6):467-75. Epub 2013/11/12. doi: 10.1016/j.npep.2013.10.015. PubMed PMID: 24210141.

147. Patrylo PR, van den Pol AN, Spencer DD, Williamson A. NPY inhibits glutamatergic excitation in the epileptic human dentate gyrus. J Neurophysiol. 1999;82(1):478-83. Epub 1999/07/13. PubMed PMID: 10400974.

148. Aguado C, Fernandez-Alacid L, Cabanero MJ, Yanagawa Y, Schilling K, Watanabe M, Fritschy JM, Lujan R. Differential maturation of GIRK2-expressing neurons in the mouse cerebellum. J Chem Neuroanat. 2013;47:79-89. Epub 2012/12/25. doi: 10.1016/j.jchemneu.2012.11.001. PubMed PMID: 23261870.

149. Correa SA, Munton R, Nishimune A, Fitzjohn S, Henley JM. Development of GABAB subunits and functional GABAB receptors in rat cultured hippocampal neurons. Neuropharmacology. England2004. p. 475-84.

150. Roenneberg T, Merrow M. Entrainment of the human circadian clock. Cold Spring Harb Symp Quant Biol. 2007;72:293-9. Epub 2008/04/19. doi: 10.1101/sqb.2007.72.043. PubMed PMID: 18419286.

151. Turek FW, Penev P, Zhang Y, van Reeth O, Zee P. Effects of age on the circadian system. Neurosci Biobehav Rev. 1995;19(1):53-8. Epub 1995/01/01. PubMed PMID: 7770197.

152. Biello SM. Circadian clock resetting in the mouse changes with age. Age (Dordr). 2009;31(4):293-303. Epub 2009/06/27. doi: 10.1007/s11357-009-9102-7. PubMed PMID: 19557547; PMCID: 2813053.

153. Reeves RH, Irving NG, Moran TH, Wohn A, Kitt C, Sisodia SS, Schmidt C, Bronson RT, Davisson MT. A mouse model for Down syndrome exhibits learning and behaviour deficits. Nat Genet. 1995;11(2):177-84. Epub 1995/10/01. doi: 10.1038/ng1095-177. PubMed PMID: 7550346.

154. Guy J, Hendrich B, Holmes M, Martin JE, Bird A. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat Genet. 2001;27(3):322-6. Epub 2001/03/10. doi: 10.1038/85899. PubMed PMID: 11242117.

155. Breslin JH, Edgin JO, Bootzin RR, Goodwin JL, Nadel L. Parental report of sleep problems in Down syndrome. J Intellect Disabil Res. 2011;55(11):1086-91. Epub 2011/07/06. doi: 10.1111/j.1365-2788.2011.01435.x. PubMed PMID: 21726315.

156. Churchill SS, Kieckhefer GM, Landis CA, Ward TM. Sleep measurement and monitoring in children with Down syndrome: a review of the literature, 1960-2010. Sleep Med Rev. 2012;16(5):477-88. Epub 2012/03/14. doi: 10.1016/j.smrv.2011.10.003. PubMed PMID: 22410159; PMCID: 3408773.

157. Stores G, Stores R. Sleep disorders and their clinical significance in children with Down syndrome. Dev Med Child Neurol. 2012. Epub 2012/09/04. doi: 10.1111/j.1469-8749.2012.04422.x. PubMed PMID: 22937986.

158. Anderson A, Wong K, Jacoby P, Downs J, Leonard H. Twenty years of surveillance in Rett syndrome: what does this tell us? Orphanet J Rare Dis. England2014. p. 87.

159. Wong K, Leonard H, Jacoby P, Ellaway C, Downs J. The trajectories of sleep disturbances in Rett syndrome. J Sleep Res. 2015;24(2):223-33. Epub 2014/09/16. doi: 10.1111/jsr.12240. PubMed PMID: 25219940; PMCID: PMC4351186.

160. Li Q, Loh DH, Kudo T, Truong D, Derakhshesh M, Kaswan ZM, Ghiani CA, Tsoa R, Cheng Y, Sun YE, Colwell CS. Circadian rhythm disruption in a mouse model of Rett syndrome circadian disruption in RTT. Neurobiol Dis. 2015;77:155-64. Epub 2015/03/18. doi: 10.1016/j.nbd.2015.03.009. PubMed PMID: 25779967.

161. Wither RG, Colic S, Wu C, Bardakjian BL, Zhang L, Eubanks JH. Daily rhythmic behaviors and thermoregulatory patterns are disrupted in adult female MeCP2-deficient mice. PLoS One. United States2012. p. e35396.

162. Stewart LS, Persinger MA, Cortez MA, Snead OC, 3rd. Chronobiometry of behavioral activity in the Ts65Dn model of Down syndrome. Behav Genet. 2007;37(2):388-98. Epub 2006/12/06. doi: 10.1007/s10519-006-9119-y. PubMed PMID: 17146725.

163. Harashima C, Jacobowitz DM, Witta J, Borke RC, Best TK, Siarey RJ, Galdzicki Z. Abnormal expression of the G-protein-activated inwardly rectifying potassium channel 2 (GIRK2) in hippocampus, frontal cortex, and substantia nigra of Ts65Dn mouse: a model of Down syndrome. J Comp Neurol. 2006;494(5):815-33. Epub 2005/12/24. doi: 10.1002/cne.20844. PubMed PMID: 16374808; PMCID: PMC2929960.

164. Best TK, Cramer NP, Chakrabarti L, Haydar TF, Galdzicki Z. Dysfunctional hippocampal inhibition in the Ts65Dn mouse model of Down syndrome. Exp Neurol. United States: Published by Elsevier Inc.; 2012. p. 749-57.

165. Levitt ES, Hunnicutt BJ, Knopp SJ, Williams JT, Bissonnette JM. A selective 5-HT1a receptor agonist improves respiration in a mouse model of Rett syndrome. J Appl Physiol (1985). United States2013. p. 1626-33.

166. De Filippis B, Nativio P, Fabbri A, Ricceri L, Adriani W, Lacivita E, Leopoldo M, Passarelli F, Fuso A, Laviola G. Pharmacological stimulation of the brain serotonin receptor 7 as a novel therapeutic approach for Rett syndrome. Neuropsychopharmacology. England2014. p. 2506-18.

167. Mistlberger RE, Skene DJ. Nonphotic entrainment in humans? J Biol Rhythms. 2005;20(4):339-52. Epub 2005/08/04. doi: 10.1177/0748730405277982. PubMed PMID: 16077153.

168. Pauley SM. Lighting for the human circadian clock: recent research indicates that lighting has become a public health issue. Med Hypotheses. Scotland: 2004 Elsevier Ltd.; 2004. p. 588-96.

169. Klerman EB, Rimmer DW, Dijk DJ, Kronauer RE, Rizzo JF, 3rd, Czeisler CA. Nonphotic entrainment of the human circadian pacemaker. Am J Physiol. 1998;274(4 Pt 2):R991-6. Epub 1998/05/12. PubMed PMID: 9575961.

170. Goel N. An arousing, musically enhanced bird song stimulus mediates circadian rhythm phase advances in dim light. Am J Physiol Regul Integr Comp Physiol. United States2006. p. R822-7.

171. Goel N. Late-night presentation of an auditory stimulus phase delays human circadian rhythms. Am J Physiol Regul Integr Comp Physiol. United States2005. p. R209-16.

172. Yamanaka Y, Hashimoto S, Masubuchi S, Natsubori A, Nishide SY, Honma S, Honma K. Differential regulation of circadian melatonin rhythm and sleep-wake cycle by bright lights and nonphotic time cues in humans. Am J Physiol Regul Integr Comp Physiol. United States: 2014 the American Physiological Society.; 2014. p. R546-57.

173. Shiota M, Sudou M, Ohshima M. Using outdoor exercise to decrease jet lag in airline crewmembers. Aviat Space Environ Med. 1996;67(12):1155-60. Epub 1996/12/01. PubMed PMID: 8968481.

174. Li F, Fisher KJ, Harmer P, Irbe D, Tearse RG, Weimer C. Tai chi and self-rated quality of sleep and daytime sleepiness in older adults: a randomized controlled trial. J Am Geriatr Soc. United States2004. p. 892-900.

175. Melancon MO, Lorrain D, Dionne IJ. Sleep depth and continuity before and after chronic exercise in older men: electrophysiological evidence. Physiol Behav. 2015;140:203-8. Epub 2014/12/30. doi: 10.1016/j.physbeh.2014.12.031. PubMed PMID: 25540930.

176. Yang PY, Ho KH, Chen HC, Chien MY. Exercise training improves sleep quality in middle-aged and older adults with sleep problems: a systematic review. J Physiother. Australia: 2012 Australian Physiotherapy Association. Published by . 2012. p. 157-63.

177. Pa J, Goodson W, Bloch A, King AC, Yaffe K, Barnes DE. Effect of exercise and cognitive activity on self-reported sleep quality in community-dwelling older adults with cognitive complaints: a randomized controlled trial. J Am Geriatr Soc. 2014;62(12):2319-26. Epub 2014/12/18. doi: 10.1111/jgs.13158. PubMed PMID: 25516028; PMCID: PMC4356237.

178. Oldham MA, Ciraulo DA. Bright light therapy for depression: a review of its effects on chronobiology and the autonomic nervous system. Chronobiol Int. 2014;31(3):305-19. Epub 2014/01/09. doi: 10.3109/07420528.2013.833935. PubMed PMID: 24397276.

179. Baxendale S, O'Sullivan J, Heaney D. Bright light therapy as an add on treatment for medically intractable epilepsy. Epilepsy Behav. United States: 2012 Elsevier Inc; 2012. p. 359-64.

180. Baxendale S, O'Sullivan J, Heaney D. Bright light therapy for symptoms of anxiety and depression in focal epilepsy: randomised controlled trial. Br J Psychiatry. England2013. p. 352-6.

181. Pail G, Huf W, Pjrek E, Winkler D, Willeit M, Praschak-Rieder N, Kasper S. Bright-light therapy in the treatment of mood disorders. Neuropsychobiology. Switzerland: Basel.; 2011. p. 152-62.

182. Naus T, Burger A, Malkoc A, Molendijk M, Haffmans J. Is there a difference in clinical efficacy of bright light therapy for different types of depression? A pilot study. J Affect Disord. 2013;151(3):1135-7. Epub 2013/08/27. doi: 10.1016/j.jad.2013.07.017. PubMed PMID: 23972661.

183. Sahlem GL, Kalivas B, Fox JB, Lamb K, Roper A, Williams EN, Williams NR, Korte JE, Zuschlag ZD, El Sabbagh S, Guille C, Barth KS, Uhde TW, George MS, Short EB. Adjunctive triple chronotherapy (combined total sleep deprivation, sleep phase advance, and bright light therapy) rapidly improves mood and suicidality in suicidal depressed inpatients: an open label pilot study. J Psychiatr Res. 2014;59:101-7. Epub 2014/09/19. doi: 10.1016/j.jpsychires.2014.08.015. PubMed PMID: 25231629; PMCID: PMC4252537.

184. Wirz-Justice A, Benedetti F, Berger M, Lam RW, Martiny K, Terman M, Wu JC. Chronotherapeutics (light and wake therapy) in affective disorders. Psychol Med. 2005;35(7):939-44. Epub 2005/07/28. PubMed PMID: 16045060.

185. Wirz-Justice A, Benedetti F, Terman M. Chronotherapeutics for Affective Disorders: A Clinician's Manual for Light and Wake Therapy. Switzerland: S. Karger AG; 2009. 124 p.

186. Wirz-Justice A, Terman M. Chronotherapeutics (light and wake therapy) as a class of interventions for affective disorders. Handb Clin Neurol. Netherlands2012. p. 697-713.

APPENDIX I

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 29-Jan-2015

TO: Gamble, Karen Lynnette

FROM: Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 29-Jan-2015.

Protocol PI: Gamble, Karen Lynnette

Title: GIRK Channel Modulation of SCN Excitability and Circadian Rhythms (Lauren Hablitz)

Sponsor: National Institute of Neurological Disorders and Stroke/NIH/DHHS

Animal Project Number (APN): IACUC-00453

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188 |