CIRCADIAN AND HOMEOSTATIC REGULATION OF SLEEP IN CAST/EiJ AND C57BL/6J MICE

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Recommended Citation
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CIRCADIAN AND HOMEOSTATIC REGULATION OF SLEEP
IN CAST/EiJ AND C57BL/6J MICE

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
Requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences at the University of Kentucky

By

Peng Jiang

Lexington, Kentucky

Director: Dr. Bruce F. O’Hara, Associate Professor of Biology
Co-Director: Dr. Marilyn J. Duncan, Professor of Anatomy and Neurobiology

Lexington, KY

2011

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Sleep is essential for mammals and possibly for all animals. Advancing our knowledge of sleep regulation is crucial for the development of interventions in sleep-related health and social problems. With this aim, this study utilizes laboratory mice to explore sleep regulatory mechanisms at behavioral, molecular, and genetic levels.

Sleep is regulated by the interaction of circadian and homeostatic processes. The circadian clock facilitates sleep to occur at a favorable time of the day. Normal mice, such as the C57BL/6J (B6) strain, sleep mostly during the day and initiate activities at dark onset. Here, I show mice of the CAST/EiJ (CAST) strain initiate activity unusually early (hours before dark). The circadian gating of photic phase-shifting responses was phase-lagged in the CAST mice relative to their activity rhythms, implying an altered coupling between the clock and its output. Light failed to suppress activity in the CAST mice, allowing full expression of the early activity. A previously identified quantitative trait locus that contributes to the advanced circadian phase was also confirmed and refined to a smaller genomic region.

The circadian oscillation and light-induction of clock-genes Per1 and Per2 expression was not different between B6 and CAST mice in the suprachiasmatic nucleus (SCN) of the brain, where the mammalian master circadian clock is located. However, in the cerebral cortex and paraventricular hypothalamic nucleus of CAST mice, Per mRNA oscillations were phase-advanced coordinately with their advanced behavioral rhythms. These data thus provide direct evidence that the cause of the early runner phenotype is located downstream of the master circadian clock.

The rhythms of cortical Per expression may not be a result of direct SCN effector mechanisms, but rather driven by activity-rest and sleep-wake. I further show that prolonged waking induces cortical Per expression, and this induction persisted in SCN-lesioned animals. SCN Per expression in intact animals was not affected. Thus, a homeostatic drive, independent of the SCN clock, regulates cortical Per expression, although a possible circadian influence in the intact animals was also suggested by
detailed analyses. These data may suggest a molecular mechanism bridging the circadian and homeostatic processes for sleep regulation and functions.

KEYWORDS: Era1, phase response curve, Per expression, SCN, cerebral cortex
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Dedicated to my parents, Xueqin Huang and Liren Jiang, for their constant love and encouragement.
ACKNOWLEDGEMENTS

I would like to thank many people for the insights, directions, and support that they have provide throughout my graduate work.

First and foremost, I would like to express my sincere gratitude and appreciation to my advisor, Dr. Bruce O’Hara, who lead me into the fascinating world of quantitative genetics and sleep research. He always greatly supported and encouraged me. His wisdoms, broad knowledge, and timely guidance benefited me invaluably throughout my career path. I also would like to thank my co-advisor, Dr. Marilyn Duncan, for her guidance and support throughout the years, and for her instructive advice and comments that sharpened my thinking.

Next, I wish to thank my committee members, Drs. Elizabeth Debski and Randal Voss, for their advice and guidance. I also thank Dr. Melinda Wilson for taking her time to serve as the outside examiner.

As a significant portion of my graduate training was completed through collaborations, I would like to thank our collaborators Drs. Jonathan Wisor, Patrice Bourgin, Paul Franken, and people in their labs, for their contributions and insights. I also wish to thank Drs. Kazu Shimomura and Joe Takahashi for the sharing of data and for their advice.

I have to offer my sincere gratitude to the present and past members of the O’Hara and Duncan labs that I have worked with throughout my graduate study, Martin Striz, Ling Liu, Prashant Kaul, Fan Wu, Jason Passafiume, Ryan Thomason, Ting Zhang, and Verda Davis. Special thanks are due to Dr. Kathy Franklin, for her help and technical instructions, without which, a substantial part of my studies would not be completed.

I would like to thank the current and former Director of Graduate Studies, Drs. Brian Rymond and Pete Mirabito for their academic instructions and for their genuine enthusiasm and support to graduate students. Other faculty and course coordinators in the Biology Department include Drs. Vinnie Cassone, Becky Kellum, John Rawls, Doug Harrison, Robin Cooper, Brent Palmer, John Seabolt, Andy Bouwma-Gearhart. I appreciate their academic influence and their efforts that made the Biology Department such a wonderful place for graduate study as well as working as a teaching assistant. I also thank many graduate students and postdoctoral fellows, present or former, in the department, Drs Robert Page, Hui Li, Travis Sexton, Liqun Wang, Dianna Morris, Sonya Bierbower, Gang Wang, and Jiffin Paulose, Shanshan Pei, Hou-Fu Guo, Wenhui Wu, Daipayan Banerjee (Deep), Swagata Ghosh, Min Chen, Ye Li, Lingfeng Tang, and many others for the exchange of scientific ideas and for all the good times we had together in the Biology Department. Furthermore, I would like to thank Beverly Taulbee, Cheryl Edwards, and Michael Adams for their administrative work and help.

Finally, I wish to give my appreciation and love to my parents and my girlfriend Qian Chen for all their love and support.
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CHAPTER ONE

Background

Lack of movement, reduced consciousness, decreased responsiveness, and vivid dreams—all of these characteristics of sleep are familiar to us, as sleep occupies a third of our life time. Sleep is also a common and fundamental need of most if not all animals, lack of which causes serious physiological consequences, even death. A considerable body of research has provided important information about the health implications of sleep. However, we are still not able to fully answer fundamental questions about sleep: what is sleep, what are the functions of sleep, and how is it regulated? On the other hand, despite the general awareness that a good night of sleep is necessary for optimal performance the next day, sufficient sleep (that meets the average basal need of 7 to 8 hours per night) has unfortunately become a luxury of modern life in industrialized societies. It has been estimated that 18-26% of adults in the U.S. (Kapur et al., 2002; Strine & Chapman, 2005) and more than 23% in Japan (Liu et al., 2000) do not get sufficient sleep. Adolescents require 9 to 10 hours of sleep each night (Carskadon et al., 1993; Mercer et al., 1998; Carskadon et al., 2004), although the majority of them (>85%) reported sleeping less than 8.5 hours and 26% of them even sleep less than 6.5 hours each weeknight because of delayed bed time (Wolfson & Carskadon, 1998). Time that should be spent asleep is occupied by social interactions, academic work, recreational activities, and watching TV (Wolfson & Carskadon, 1998; Johnson et al., 2004). With the fast growth of the internet, video games and electronic devices, one can only expect this situation to get even worse. The negative impacts of not getting enough sleep are enormous. Acute (e.g. hours and days of) sleep
deprivation (SD) under controlled lab conditions causes sympathetic activation, hypertension, reduced glucose tolerance, hormonal changes, and increased inflammation, while chronic (e.g., years of) sleep loss (<7 hours of sleep a day) is associated with increased mortality, cardiovascular disease, obesity, diabetes, anxiety, and alcohol use (Alvarez & Ayas, 2004; Colten & Altevogt, 2006; Mullington et al., 2009). Moreover, insufficient sleep causes excessive day-time (or work-time) sleepiness, impaired judgments, and prolonged reaction time, which may lead to operational errors at the workplace. In fact, sleep loss is thought to have contributed to some of the most devastating accidents in human history, such as the nuclear reactor meltdown at Chernobyl and the Challenger space shuttle explosion (Mahowald & Schenck, 2005; Colten & Altevogt, 2006). More than 100,000 automobile crashes each year in the U.S. resulted from falling asleep behind the wheel (Mahowald & Schenck, 2005). The majority of drivers who fall asleep and crash are younger than 25 years old, and the fatality rate of such an accident is almost as high as drunk driving (~2%; (Pack et al., 1995)). Insufficient sleep is also associated with poor academic performance in adolescents (Wolfson & Carskadon, 1998). Furthermore, it is estimated that 50-70 million people in the U.S. suffer from a chronic sleep disorder, which, in addition to inadequate sleep, causes various health problems and immense economic burden (Colten & Altevogt, 2006). There are nearly 100 identified sleep disorders, which jeopardize health and well-being of the patients and cost billions of dollars of medical bills a year (Hillman et al., 2006). Solving these sleep and sleep disorder related health, social and economic issues requires a comprehensive understanding of sleep, its functions and regulations, which currently is still lacking. Therefore, research in both human and model organisms must continue to advance our knowledge about sleep. With this aim, this work utilizes laboratory mice (Mus musculus) as a model organism to explore the regulatory mechanisms of sleep at behavioral, genetic, and molecular levels.
The Two-Process Model

Borbély is largely responsible for advancing the two process model of sleep regulation, which has gained wide acceptance (Borbely, 1982; Daan et al., 1984; Borbely & Achermann, 1992; Borbely, 1998). In this model, sleep is regulated by a circadian process (Process C) that restrains the timing of sleep and wake in a 24-hour rhythmic pattern and a sleep homeostatic process (Process S) representing sleep need, which accumulates during wake and dissipates during sleep. These two processes develop independently from each other, but their interaction determines the timing, propensity and quality of sleep.

The Process C is a daily rhythmic process regardless of prior sleep/wake distribution. The rhythm is governed by an endogenous circadian clock, which free-funs under constant conditions with a period of approximately 24 hours and can be entrained (i.e. synchronized) by external stimuli, such as light. The circadian clock consists of an input pathway, a self-sustained master pacemaker, and downstream pathways. In mammals, the master circadian pacemaker is located in the suprachiasmatic nucleus (SCN). Circadian rhythmicity is abolished by lesions of the SCN and can be restored by SCN transplants with a circadian period of the donor (Ralph et al., 1990), supporting that the SCN is both necessary and sufficient for the generation of circadian rhythms. The SCN is a paired hypothalamic nuclei; each consists of approximately 10,000 neurons. Each individual SCN neuron is a circadian oscillator, which fires in a ~24-hour rhythmic pattern driven by transcriptional-translational feedback loops (Reppert & Weaver, 2001; Lowrey & Takahashi, 2004). These SCN neurons retain rhythmicity when isolated, and are orchestrally coupled in the SCN in order to function as a clock [for a review, see (Welsh et al., 2010)]. The SCN is reset on a daily basis by direct inputs from the eye via the retinohypothalamic tract (Provencio et al., 1998). Canonic photo receptors (rods and
cones) as well as melanopsin containing retinal ganglion cells in the eye are involved in the resetting of the SCN clock by light (Panda et al., 2002; Ruby et al., 2002; Hattar et al., 2003). The SCN sends direct projections to many regions of the brain. However, the majority of SCN projections end at the neighboring subparaventricular zone and the signals are relayed to the dorsomedial hypothalamic nucleus, from which projections are then sent to other hypothalamic regions, including inhibitory GABAergic projections to the sleep-promoting ventrolateral preoptic area (VLPO) and excitatory glutamatergic projections to the wake-promoting orexin-containing neurons in the lateral hypothalamic area (Saper et al., 2005b). Diffusible factors have also been suggested as part of the circadian output mechanisms, as encapsulated SCN-transplants are capable of rescuing the loss of locomotor rhythmicity caused by SCN lesions (Silver et al., 1996). To date, three molecules have been proposed as SCN output signals, including Transforming growth factor alpha [TGFα; (Kramer et al., 2001)], Prokineticin 2 (Cheng et al., 2002) and Cardiotrophin-Like Cytokine [CLC; (Kraves & Weitz, 2006)]. Although detailed mechanisms are still lacking, outputs from the SCN impose ~24-hour rhythmicities on sleep and wake and other mammalian behavioral and physiological activities, including body temperature, energy metabolism, hormone release (such as cortisol/corticosterone and melatonin), and many others.

Process S, on the other hand, is sometimes referred to as an “hourglass”-like process as opposite to the clock-like Process C (Dijk et al., 1987). The propensity of initiating sleep increases as wake prolongs. The mechanisms, especially the molecular mechanisms of sleep homeostasis are not yet clear, although several hypotheses have been formed. In one such hypothesis, adenosine, which is directly linked to cellular metabolism, has been proposed as the key sleep homeostasis molecule. SD increases extracellular levels of adenosine in the basal forebrain, and increased adenosine levels
facilitate recovery sleep after SD [for a review, see (Porkka-Heiskanen & Kalinchuk, 2011)]. However, this hypothesis has difficulties in explaining cortical sleep homeostasis (see below), especially its local properties and detailed time course. Sleep/wake dependent gene expression studies have also suggested several plausible hypotheses about homeostatic regulation of sleep and wake, which will be discussed later in this chapter.

Electroencephalographic (EEG) slow-wave activity (SWA; spectra power density in the <4 Hz band) recorded from the cerebral cortex provides so far the best correlate of Process S. SWA is a hallmark of deep (stage 3, 4) non-rapid eye movement (NREM) sleep, and is a result of synchronized rhythmic burst firing of hyperpolarized thalamocortical neurons (Steriade et al., 1993; McCormick & Bal, 1997). In both humans and mice, SWA is enhanced as the duration of previous waking increases (Borbely et al., 1981; Dijk et al., 1987; Dijk et al., 1990a; Franken et al., 1999; Franken et al., 2001). Also, SWA decreases exponentially as sleep progresses through multiple NREM/REM cycles (Dijk et al., 1990b). As an example in humans, a daytime or early-evening nap decreases the total SWA during subsequent nighttime sleep by the amount that has already occurred during the previous nap (Feinberg et al., 1992; Werth et al., 1996b). These data suggest that SWA changes as a function of prior sleep and wake. Indeed, in mice and humans, the prior sleep/wake distribution alone is sufficient to mathematically predict the time course of SWA, and the calculated SWA dynamics matched well with experimental values under baseline, SD, and napping (in humans) conditions (Huber et al., 2000; Franken et al., 2001; Wisor et al., 2002; Achermann & Borbely, 2003).

It is worth noting that, although SWA faithfully reflects prior sleep/wake distribution (i.e. a marker of the Process S), it may not be responsible for determining sleep or wake (i.e. the Process S per se). In humans, selective SWA deprivation does
not result in increased sleep duration despite a SWA rebound (Dijk & Beersma, 1989). During the recovery period after SD in rats, sleep duration remains elevated after rebound SWA dissipated (Franken et al., 1991; Schwierin et al., 1999b). Also, rapid eye movement (REM) sleep is not addressed in the two-process model, although it is also regulated by both circadian and homeostatic components. The homeostatic regulation of REM sleep depends on the total REM loss during both waking and NREM sleep (Franken, 2002). Lastly, more recent studies have suggested the necessity to incorporate ultradian [e.g., the NREM/REM cycles; (Achermann & Borbely, 1990; Achermann et al., 1993)], regional-specific (Schwierin et al., 1999a; Zavada et al., 2009), and genetic components (Franken et al., 2001). Like any other models, refining the details of the two-process model to accommodate emerging data seems “inevitable” (Borbely, 2009).

Nonetheless, the two-process model provides a framework that reliably describes the dynamics of sleep and wake under various conditions. For example, when exposed to different photoperiods, rats exhibits dramatic change in the daily rhythmic pattern of sleep and wake, but no difference in either the total amount of baseline sleep or the homeostatic response to SD [such as increased SWA and sleep rebound; (Franken et al., 1995)]. Furthermore, in SCN lesioned animals, the circadian rhythms of sleep and wake are abolished, while the rebound of total sleep and SWA after SD remains unaffected (Mistlberger et al., 1983; Tobler et al., 1983; Trachsel et al., 1992). Lastly, the two-process model has been further validated in a vital study using a “forced desynchrony” protocol (Dijk & Czeisler, 1994; 1995). In this study, human subjects were assigned to a 28-hour sleep/wake cycle (9.33-hour sleep/18.66-hour wake), to which their endogenous clock failed to entrain. Thus, sleep initiated at various circadian phases, allowing detailed studies of the interaction between the two processes. Under
these conditions, the EEG SWA was mostly driven by sleep/wake history as it progressively decreased during the courses of all sleep episodes. Although a small circadian influence on SWA was also observed, it did not parallel with the circadian course of sleep propensity but more likely reflected the quality of previous sleep episodes. Moreover, the data also suggested that sleep propensity was indeed regulated by the interaction of Process C and S: sleep was most consolidated during the circadian phase of habitual bedtime and during the first few hours of each sleep episode. Collectively, these animal and human data confirm that the two independent processes interact to govern sleep and wake. Under entrained conditions, sleep is maintained by the increasing circadian-dependent sleep drive even though homeostatic pressure dissipates. On the other hand, during waking, the circadian-depended sleep drive is decreasing, thus canceling out the accumulating homeostatic sleep pressure (Daan et al., 1984; Borbely & Achermann, 1992; Borbely, 1998).

Circadian Entrainment

The circadian clock is thought to be adaptive by anticipating daily rhythmic changes in the environment. To achieve this function, the endogenous clock must be entrained to the external timing cues. The light/dark (LD) cycle is the most prominent timing cue, or Zeitgeber. The resetting effect of light on the clock can be demonstrated under laboratory conditions. When housed under 12-hour light/12-hour dark (LD12:12) conditions with access to a running wheel, a mouse is active predominantly during the dark phase, as is typical for nocturnal animals. When released to constant dark (DD), this wheel-running activity rhythm persists with an intrinsic circadian period. In normal mice, the endogenous circadian period under DD is slightly shorter than 24 hours. Light
resets the clock by producing phase-shifts with different directions and amplitudes according to the time of light exposure. This phase-shifting response can be illustrated by a phase response cure (PRC), in which the amplitudes of phase-shifts (positive values for phase-advances and negative values for phase-delays) are plotted against the time at which a light pulse is given. The overall shape of the photic PRC is highly conserved across species, including nocturnal animals such as mice and diurnal species such as humans (Pittendrigh & Daan, 1976a; Minors et al., 1991; Khalsa et al., 2003).

During the subjective day (i.e., the internal time of the day analogous to the daytime under entrained conditions), the PRC exhibits a “dead zone” when the light produces little or no phase-shifts. However, phase-delays result when the animal is exposed to light during early subjective night and phase-advances result when the animal is exposed to light during the late subjective night.

The PRC provides a mechanism for circadian entrainment, known as “non-parametric entrainment” (Pittendrigh, 1965; Pittendrigh & Daan, 1976b). First of all, the light-induced phase-shifts correct for the deviation of the endogenous circadian period from 24 hours. The clock is phase-delayed by the light at dusk and phase-advanced by the light at dawn. The amount of time that the circadian period is shorter or longer than 24 hours is counterbalanced respectively by the overall phase-delay or phase-advance produced every day. Further, by tracking the dusk and dawn, this mechanism ensures the clock functions properly during the short days of winter as well as long days of summer. Finally, the interaction of circadian period and PRC dictates the phase of the LD cycles at which the clock is entrained: the shorter the circadian period, the earlier the entrained circadian phase. In order to synchronize to the 24-hour LD cycles, a circadian clock with a shorter intrinsic period requires a larger daily phase-delay, which will typically occur with more exposure to light during a larger portion of the early subjective
night. Thus, in an animal with a shorter circadian period, the clock and the overt rhythms are synchronized at a more advanced phase relative to the LD cycles. Such a correlation between circadian period and entrained phase has been reported in population studies, an extreme case in a human circadian-sleep disorder, and in animals bearing genetic mutations that alter circadian period (Aschoff, 1981; Ralph & Menaker, 1988; Jones et al., 1999; Duffy et al., 2001).

In humans, the circadian clock facilitates a 24-hour rhythmic alternation of sleep and wake. Under entrained conditions, most people go to bed after sunset and wake up around sunrise, although a large range of variations in the timing of sleep and wake exists (Horne & Ostberg, 1977; Roenneberg et al., 2007). Some of these variations may represent a poor or even pathological coordination of the cycles of the endogenous clock and/or other overt rhythms. Similar situations happen in shift work and jet lag, where sleep/wake and other physiological processes are forced to occur at non-habitual or non-preferred circadian phases. Shift-workers, who represent 20% of the workforce in the U.S., together with hundreds of thousands of civilian and military aircrews, are frequently exposed to these conflicts, which lead to impaired physiological and cognitive performance as well as increased susceptibility to sleep problems, heart disease, gastrointestinal disorders and cancer (Arendt et al., 2000; Monk, 2000; Davis & Mirick, 2006). Moreover, in cases of human circadian-sleep disorders, such as advanced or delayed sleep phase syndrome (ASPS or DSPS) and non-24-h sleep syndrome (also known as free-running disorder), daily bed time can be substantially (e.g. 4-6 hours) earlier or later than normal, or even desynchronized from the 24-hour cycles. ASPS and DSPS cause difficulties with social demands. Sleep deprivation often results when patients try to catch up with the normal work and social schedule, thus hindering performance and health (Baker & Zee, 2000; Sack et al., 2007). In all of these above
mentioned conditions, the circadian clock and/or its overt rhythms fail to align with the external LD cycles at a favorable phase. Therefore, solving the problems related to circadian-sleep disorders, jet lag, and shift work require adjustments of the circadian clock and/or overt rhythms to synchronize at a more appropriate phase. This cannot be achieved without a better understanding of the physiological and molecular mechanisms of circadian phase determination. In Chapter Two of this work, I describe a novel mouse model of human ASPS, CAST/EiJ mice (CAST), the study of which provides insight into the mechanisms of circadian phase alignment of activity/rest as well as sleep/wake rhythms relative to the LD cycles.

Molecular Mechanisms of the Circadian Clock

In mammalian SCN neurons, the circadian oscillation is generated by transcriptional and translational feedback loops [for reviews, see (Reppert & Weaver, 2001; Lowrey & Takahashi, 2004)]. Two basic helix-loop-helix (bHLH)-PAS domain proteins, CLOCK and BMAL1 dimerize through their PAS domain and act on a consensus cis-regulatory sequence called E-box, activating the transcription of downstream clock-controlled genes as well as the negative regulators Period genes (Per1, 2, 3) and Cryptochrome genes (Cry1, 2). After translation, PERs interact with CRYs, translocate back into the nucleus, and suppress the activity of CLOCK-BMAL1 heterodimers thus shutting down their own transcription as well as the transcription of clock-controlled genes. Following the degradation of PERs and CRYs, the repression of CLOCK-BMAL1 activity is released, and the cycle starts again. This entire process thereby oscillates with a period of roughly 24 hours. The circadian transcriptional activation is associated with the acetyltransferase activity of CLOCK, which acetylates
histone H3 and BMAL1 (Doi et al., 2006; Hirayama et al., 2007). CRY1 and CRY2 are the main suppressor of CLOCK-BMAL1 perhaps through inhibiting the acetylation of histone H3 (Etchegaray et al., 2003), while their interaction with PER proteins may facilitate nuclear entry (Miyazaki et al., 2001) and regulate the interaction between PER-CRY and CLOCK-BMAL (Langmesser et al., 2008). This main feedback loop is stabilized by an additional feedback loop which constitutes two transcriptional regulators of Bmal1, RORA and REV-ERBα. These are two orphan nuclear receptors and the expression of both is under the control of CLOCK-BMAL1, yet they exert opposing effects on Bmal1 transcription: RORA activates and REV-ERBα represses Bmal1 expression (Preitner et al., 2002; Sato et al., 2004). In addition to transcriptional and translational regulations, post-translational mechanisms are also involved [for a review, see (Gallego & Virshup, 2007)]. Particularly, phosphorylation mediated by Casein kinase 1 δ and ε (CSNK1D and CSNK1E) and perhaps also other kinases regulates the transcriptional activity, nuclear entry and retention, as well as stability of clock-proteins, all of which are crucial for the proper function of the clock (Vielhaber et al., 2000; Lee et al., 2001; Eide et al., 2002; Vanselow et al., 2006; Xu et al., 2007). These clock-genes are also expressed in other regions of the brain as well as peripheral tissues, providing a molecular mechanism for oscillators downstream of the SCN.

The transcriptional and translational feedback loops in SCN neurons generate a ~24-hour oscillation of transcript levels of clock-genes and clock-controlled genes (Reppert & Weaver, 2001). Per and Cry mRNAs peak during the mid subjective day, anti-phase to the peak of Bmal1 transcripts, while Clock is constitutively expressed in the SCN. There is a lag of ~6 hours between the protein peaks and mRNA peaks of Per and Cry. Although the mechanisms are still unclear, this lag is thought to be important for an oscillation with a ~24-hour period. The molecular oscillations of clock-genes in
SCN neurons are self-sustained without external stimuli, but can be reset by light (Reppert & Weaver, 2001; Meijer & Schwartz, 2003; Morin & Allen, 2006). Upon stimulus by light, glutamate and a modulating molecule PACAP (pituitary adenylate cyclase-activating polypeptide) are released at the synapses of retina-to-SCN projections, resulting in a post-synaptic depolarization and increased cytoplasmic Ca\(^{2+}\) in SCN neurons. Increased intracellular Ca\(^{2+}\) triggers a series of phosphorylation events, leading to the activation of Ca\(^{2+}/\)cAMP response element binding protein (CREB). Activated CREB binds to the Ca\(^{2+}/\)cAMP response element (CRE) located in the promoters of Per1 and Per2, and enhances their transcription. Thereby, light induces Per1 and Per2 expression in the SCN and synchronizes the oscillations of Per1 and Per2 mRNAs peaking at the mid-day (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). In my mouse model of ASPS (i.e., the CAST mice), behavioral rhythms are aligned at an earlier phase of the LD cycles. It is important to know whether this advanced phase alignment is produced by changes in the synchronization of Per1 and Per2 mRNA oscillations in the SCN relative to the LD cycles. To address this question, I describe in Chapter Three the dynamics of mRNA oscillations and light-induced expression of Per1 and Per2 in the SCN of CAST mice. I also examine the oscillations of Per1 and Per2 mRNA levels in other brain regions, such as the cerebral cortex and the paraventricular hypothalamic nucleus, where the peaks of these oscillations normally occur when animals are active.

Mutations in clock-genes disrupt circadian rhythms. Knockout (KO) of Bmal1, double KO of Per1 and Per2, or double KO of Cry1 and Cry2 completely abolishes circadian rhythms under DD, while single KO of Per1, Per2, Cry1 or Cry2 affects the endogenous circadian period [for a review, see (Lowrey & Takahashi, 2004)]. Also, Clock was in fact identified in a random mutagenesis screening, and the homozygous
mutant mice has a circadian period ~4 hours longer than normal (Vitaterna et al., 1994; Antoch et al., 1997; King et al., 1997). Although a KO of Clock does not substantially disturb circadian rhythmicity, the rhythms are abolished when Clock and its paralog Npas2 are both deleted, suggesting a compensatory role of Npas2 in Clock KO mice (DeBruyne et al., 2006; DeBruyne et al., 2007). In addition, the hamster tau mutant producing an extremely short circadian period has been mapped to the Csnk1e gene (Lowrey et al., 2000). This mutation leads to a deficient phosphorylation activity of CSNK1E protein and thus decreased phosphorylation of PER proteins.

Furthermore, in many cases, the human circadian-sleep disorders DSPS and ASPS are also influenced or determined by genetic variations (Sack et al., 2007; Takahashi et al., 2008). DSPS is characterized by a 3 to 6-hour delayed timing of daily sleep and wake. DSPS and/or more generally, eveningness have been associated with the variable-number tandem-repeat (VNTR) polymorphism in the human Per3 gene (Ebisawa et al., 2001; Archer et al., 2003) and a SNP (i.e., single nucleotide polymorphism) in the 3'-UTR (i.e., untranslated region) of the human Clock gene although with some debate (Katzenberg et al., 1998; Iwase et al., 2002; Robilliard et al., 2002; Mishima et al., 2005). In addition, an amino acid substitution in the human CSNK1E protein has been reported to inversely associate with DSPS indicating a perhaps protective role, and a SNP in the 5'-UTR of the human Per2 gene distinguishes extreme evening preference from extreme morning type (Carpen et al., 2005). On the other hand, ASPS features a daily sleep and wake time earlier than normal by 3-6 hours. In some cases, ASPS exhibits a Medelian inheritance pattern within pedigrees, known as familial ASPS [FASPS; (Jones et al., 1999)]. In one FASPS family, the extreme advanced sleep/wake timing is linked to a mutation in the human Per2 gene at a CSNK1 dependent phosphorylation site (Toh et al., 2001). In another FASPS family, ASPS
results from a missense mutation in the human Csnk1d gene hindering phosphorylation activity of its encoded protein (Xu et al., 2005). Both of these two mutations cause an advanced timing of sleep/wake cycles by shortening the circadian period, similar to the hamster tau mutant (Lowrey et al., 2000). However, altering circadian period may not be the only means to vary the preference of chronotype in humans (Brown et al., 2008), and perhaps does not even account for most cases. Further, in some populations, ASPS and DSPS failed to associate with any polymorphisms of the above mentioned clock-genes, suggesting genetic heterogeneity of ASPS and DSPS (Robilliard et al., 2002; Satoh et al., 2003). Therefore, further studies of the genetic mechanisms of ASPS and DSPS are needed. In Chapter Two of this work I also take a forward genetic approach and fine-map a mouse quantitative trait locus (QTL) that underlies an advanced phase alignment of mouse behavioral rhythms relative to the LD cycles, similar to human ASPS.

**Homeostatic Changes in Gene Expression during Sleep and Wake in Rodents**

Accumulating data, especially those obtained from genome-wide microarray studies, suggest the mRNA level of a large number of genes is sleep/wake dependent. These state-dependent changes in gene expression provide important clues to sleep function and regulatory mechanisms. For example, the first genome-wide screen for sleep/wake dependent transcripts in rodents found about 5% of the detectable transcripts in the cerebral cortex changed between waking and sleeping animals (Cirelli et al., 2004). Among these genes whose cortical expression is sleep/wake dependent, genes involved in energy metabolism, transcriptional up-regulation (including immediate early genes, or IEGs), and cellular stress/unfolded protein responses exhibit higher expression during waking, while genes involved in cholesterol biosynthesis, protein
translation, and membrane trafficking exhibit higher expression during sleep. Moreover, genes whose functions are associated with synaptic plasticity and cell signaling are differentially regulated by sleep and wake: wake up-regulates (or, sleep down-regulates) synaptic plasticity genes associated with information acquisition and synaptic potentiation, genes associated with glutamatergic neurotransmission, and genes sensitive to depolarization; sleep up-regulates (or, wake down-regulates) synaptic plasticity genes associated with memory consolidation and synaptic depression, genes involved in GABAergic neurotransmission, and genes promoting hyperpolarization.

These findings are generally consistent with other studies in rodents. In fact, IEG (such as c-fos, NGFI-A) expression was found to be sleep/wake dependent even before large-scale gene expression studies were possible (O'Hara et al., 1993; Grassi-Zucconi et al., 1994; Pompeiano et al., 1994). The mRNA and protein levels of most IEGs are elevated by increased neuronal activity. Thus, the expression of IEGs has been used to map brain regions with sleep/wake dependent activities, leading to the identification of sleep-active regions such as the VLPO (Sherin et al., 1996) as well as the nNOS (neuronal nitric oxide synthase) expressing GABAergic neurons in the cortex (Gerashchenko et al., 2008). The VLPO is a part of the hypothalamic “sleep/wake switch” and shuts down the wake-active areas by GABAergic projections (Saper et al., 2005b), while the cortical nNOS-GABAergic neurons might have an important role in the synchronization of neuronal firing that underlies EEG SWA. On the other hand, most of the IEGs are transcription factors, and thus the increased expression of IEGs might function to trigger complex molecular pathways that underlies the cellular function and homeostatic regulation of sleep and wake (Morgan & Curran, 1991). Similar to IEGs, transcript levels of genes involved in cellular stress response are consistently elevated during waking (Terao et al., 2003; Cirelli et al., 2004; Terao et al., 2006; Mackiewicz et
al., 2007; Maret et al., 2007). These data suggest accumulating cellular stress during prolonged waking, which may eventually facilitate the shutdown of wakefulness (Mackiewicz et al., 2008a; Naidoo, 2009).

Sleep/wake dependent cortical mRNA levels of synaptic plasticity related genes (such as BDNF, Arc, Camk4) highlight a possible role of sleep in synaptic plasticity and memory consolidation (Stickgold, 2005). These findings are later incorporated into the “synaptic homeostasis” hypothesis of sleep/wake control (Tononi & Cirelli, 2003; 2006). In this hypothesis, wakefulness is associated with plastic processes that result in a net increase of synaptic strength at a high cost of energy in many brain regions. Sleep, on the other hand, homeostatically down-scales synaptic strength thus optimizing signal-noise ratios and enhancing efficiency of neural circuits. In support of this hypothesis, for example, the increase of cortical BDNF (i.e., brain derived neurotrophic factor) transcript level is nicely correlated with the amount of exploratory behavior during wakefulness and the homeostatic response of SWA during subsequent sleep (Huber et al., 2007). Direct interactions between sleep and neuronal plasticity have also been addressed by investigations of changes in gene expression during a sleep bout following learning and memory tasks. For instance, the expression of an IEG, Zif-268 (also known as Krox-24, NGFI-A, Ngf1, Egr1), is induced in multiple brain regions during REM sleep by rich sensorimotor experience and hippocampal long-term potentiation (LTP) during previous waking (Ribeiro et al., 1999; Ribeiro et al., 2002). Further in a screening for secondary activation of genes in hippocampus following Zif-268 induction, hippocampal transcript levels of plasticity-related genes (such as CaMKI) and other genes involved in signal transduction, neurotransmission, and cell adhesion were modulated only in the LTP treated hemisphere and only when sleep is allowed (Romcy-Pereira et al., 2009).
However, the molecular mechanisms might be complicated for a role of plasticity-related genes during sleep and wake. One study has addressed the issue of genetic background in sleep/wake dependent gene expression, using three different inbred strains of mice (Maret et al., 2007). Many of the changes in gene expression, including BDNF and other plasticity genes, are strain specific. However, Homer1a together with a small number of other transcripts show consistent changes across strains. Homer1a is a truncated transcript isoform of the Homer1 gene and encodes for a negative regulator of the products of the long isoforms (e.g., Homer1b and Homer1c), which are involved in regulatory protein complexes coupling metabotropic glutamate receptors and NMDA receptors (Xiao et al., 2000). Thus, it is possible that elevated Homer1a transcript levels after prolonged waking may facilitate subsequent sleep by modulating glutamate signaling. Also, HOMER1 proteins regulate cellular calcium homeostasis (Tu et al., 1998; Worley et al., 2007), which might be important for neuronal recovery or optimization following a period of waking. Additional evidence for a role of Homer1 in sleep homeostasis come from the finding that it is the most plausible candidate gene underlying a QTL that influences recovery from SD (Franken et al., 2001; Maret et al., 2007; Mackiewicz et al., 2008b). Lastly, the Drosophila homer gene has only one transcript isoform, similar to the long isoforms of mammalian Homer1 (e.g., Homer1b, c) but not the short negative form (e.g., Homer1a). The mRNA level of Drosophila homer is elevated during rest (a sleep-like behavioral state) and decreased during activity, suggesting a possible conserved role of homer across phyla (Zimmerman et al., 2006).

Other interesting changes across sleep and prolonged wake include an increase of mRNAs that are involved in macromolecule synthesis and transport during sleep (Mackiewicz et al., 2007). This is consistent with the idea that sleep may have a
restorative function for certain molecular components, which are perhaps depleted during wake, thus imposing a limit on the duration of waking (Mackiewicz et al., 2008a).

Several concerns arise over these sleep gene expression studies. For example, most investigations of sleep/wake dependent gene expression in rodents were performed under the condition of total sleep deprivation. Also, most studies utilized coarse-dissected brain regions (or whole brain tissue). However, sleep has two distinct stages NREM and REM, which may have different functions and are likely to be separately regulated. At least one microarray study focused on selective deprivation of REM sleep and identified an interesting set of paradoxical sleep (mostly REM sleep) related genes (Guindalini et al., 2009). Further, the brain is a highly heterogeneous organ. Different regions of the brain have different functional roles and thus are likely to express a different set of genes during sleep and wake. Indeed, a recent study utilizing microarrays to examine mRNA from laser micro-dissected tissues and a high-throughput in situ hybridization technique has provided much greater detail in brain-region and cell-type specific changes in gene expression during SD, sleep, and wake (Thompson et al., 2010).

In addition, a common confound of these sleep/wake dependent gene expression studies comes from the fact that SD induces an increase in plasma “stress hormones” such as corticosterone in rodents (Gip et al., 2004; Tartar et al., 2009), which in turn can affect gene expression. A recent study revealed that adrenalectomy (ADX) attenuated the amplitude of changes in as many as 76% of the transcripts that were previously affected by SD (Mongrain et al., 2010). Many of these genes are involved in processes that have been indicated as homeostatically regulated during sleep and wake, including biomolecule (lipid, cholesterol, protein) synthesis, energy metabolism, and stress responses. The SD-induced changes in gene expression were even completely
eliminated in some cases, such as the energy metabolism gene *Pdk4* and the stress response gene *Sgk1*. Also, this study identified a set of SD-induced gene expression changes in ADX mice that was not observed in intact animals, indicating that corticosterone may have masking, in addition to amplifying, effects on the homeostatic regulation of gene expression during sleep and wake. On the other hand, ADX did not affect SWA (baseline and homeostatic response to SD) or SD-induced expression changes in synaptic plasticity related genes (such as *Homer1a, Arc, BDNF*), IEGs (such as *c-fos, Egr1, 3*) and heatshock protein genes (*Hspa1a, Hspa5*). A core of 78 such transcripts provided a more reliable molecular signature of sleep homeostatic process.

Collectively, sleep/wake dependent changes in gene expression suggest that synaptic plasticity related processes, IEG dependent transcriptional activation, heatshock protein mediated cellular stress responses, and perhaps large molecule synthesis may be involved in the cellular functions and/or homeostatic regulations of sleep. However, it is important to note that these gene expression changes may or may not underlie the sleep homeostatic process itself. First of all, the state-dependent gene expression observed in all these studies discussed above are likely to be merely responses to sleep/wake behavioral state, and there is so far no direct evidence for a causal role of alterations in gene expression in variations of sleep characteristics and homeostasis (such as duration, EEG features, etc.). Further, there is a time course issue, particularly in rodents (O'Hara *et al.*, 2007). Sleep and wake episodes typically last minutes or even seconds in rats and mice. Also, sleep pressure can build up as fast as within one hour of waking (Franken *et al.*, 2001; Franken, 2002). Gene expression mediated functional alterations in the brain, however, are likely to take more time, particularly for those IEG-induced secondary expression changes. At least for REM sleep, both short-term and long-term processes have been suggested in its homeostatic
control (Franken, 2002). It is thus possible that both short-term (e.g., fast) and long-term homeostatic (e.g., slow) mechanisms may generally exist to regulate sleep and wake states. The alternations between short sleep and wake periods as well as the immediate accumulation of sleep pressure during the first hour of waking may be associated with fast processes such as protein phosphorylation. Indeed, rapid changes in cortical protein levels and phosphorylations have been observed in rats during short (e.g. 10 min) spontaneous sleep and wake bouts, independent of transcript levels (Vazquez et al., 2008). Nonetheless, changes in mRNA levels may underlie the slower mechanisms. Presumably, changes in these mRNAs leads to a change in protein populations presented in the neurons, and may thus alter the basic property of the neuron (such as excitability) facilitating or preventing the transition between sleep and wake over longer periods.

**Clock-genes and Sleep Homeostasis**

Interestingly, another class of genes with sleep/wake dependent cortical expression is the clock-genes. While faithfully oscillating with a ~24-hour period as a clock in the SCN, *Per1* and *Per2* expression in the cerebral cortex of rat and mouse exhibits an hourglass-like pattern determined by the prior sleep/wake distribution (Cirelli et al., 2004; Franken et al., 2007; Wisor et al., 2008; Thompson et al., 2010). Generally, *Per1* and *Per2* mRNA levels are elevated after prolonged waking and fall back to baseline after recovery sleep. As glucocorticoid response elements (GRE) are found in both *Per1* and *Per2* promoters (Yamamoto et al., 2005; So et al., 2009), it is not surprising that their increased expression after SD is reduced in ADX mice, although SD still leads to a significant elevation in *Per2* and an increasing trend in *Per1* mRNA levels.
after ADX (Mongrain et al., 2010). Thus, SD-induced corticosterone release may serve to amplify the change in *Per2* (and perhaps *Per1*) expression, which is sleep/wake dependent *per se*. In addition to the changes in *Per1* and *Per2* mRNAs, SD also induces a decrease in the mRNA level of a CLOCK-BMAL1 controlled transcription factor *Dbp* (Cirelli et al., 2004; Franken et al., 2007). Increased mRNA levels of *Cry2, Bmal1, Clock, Npas2*, and *Csnk1e* after 6 hours of SD have also been reported, although these changes are likely to be specific to AKR/J mice but not C57BL/6J or DBA/2 (Wisor et al., 2008). These data raise the hypothesis that the molecular clock machinery, particularly PER2 and perhaps also PER1 may be involved in the homeostatic process of sleep in the cerebral cortex (and perhaps also other regions of the brain), in addition to their clock function in the SCN.

Stronger support for this hypothesis has been obtained in studies of clock-gene mutants and KOs, which produced interesting alterations in sleep homeostasis, in addition to their circadian phenotypes (Franken & Dijk, 2009). For example, *Cry1,2* double KO mice exhibit longer NREM sleep bouts and increased SWA under baseline conditions, indicating elevated homeostatic sleep pressure (Wisor et al., 2002). Two independent studies on *Per1, Per2* or *Per3* null alleles and *Per1,2* double KO mice concluded no disruption of sleep homeostasis (Kopp Caroline, 2002; Shiromani et al., 2004), although the data in those studies have suggested otherwise, such as a prolonged time course of SWA rebound after SD (O’Hara et al., 2007). The above-mentioned human *Per3* VNTR polymorphism also affects sleep and waking performance (Viola et al., 2007), in addition to its association with DSPS. Subjects with *Per3*<sup>4/4</sup> genotype, the genotype associated with evening preference and DSPS (Archer et al., 2003), exhibit lower SWA during the first 2 hours of both baseline and recovery sleep compared to *Per3*<sup>5/5</sup> subjects, indicating a possibly slower build-up of sleep pressure in
Per3^{4/4} subjects. Furthermore, mutations or deletions of the positive regulators of the circadian feedback loop also lead to changes in sleep homeostasis. Deletion of Npas2 gene reduces the late-night napping that is typical in mice, and decreases the SD-induced homeostatic rebound of SWA and NREM with interesting genotype-sex interactions (Franken et al., 2006). Also, the Clock mutant mice exhibit less NREM sleep under baseline conditions (Naylor et al., 2000). In contrast, increased SWA, NREM sleep, and REM sleep have been found in Bmal1 KO, together with a reduced rebound sleep (both NREM and REM) after SD (Laposky, 2005). Lastly, KO of the clock-controlled gene Dbp causes a shorter circadian period as well as more fragmented NREM sleep and attenuated accumulation of SWA during baseline nights (Franken et al., 2000). Interestingly, there might be an association between Per1 and Per2 expression levels in these clock-gene mutants/KOs and their sleep phenotypes. The low amounts of NREM coincide with low baseline brain expression of Per2 in the Clock mutant (Oishi et al., 2000) while elevated baseline cortical expression of Per1 and Per2 in Cry1,2 double KO mice (Wisor et al., 2002) is accompanied by higher sleep drive. Similarly, the reduced NREM and SWA rebound after SD in Npas2 KO mice may also be correlated with a decrease in the SD-induced elevation of Per2 transcript levels (Franken et al., 2006). These data may suggest a central role of Per1 and Per2 in sleep homeostatic mechanisms that involves clock machinery.

If the cortical molecular clock machinery is involved in sleep homeostasis, and if this involvement is centered on the sleep/wake dependent expression of Per1 and Per2, one would predict that the dynamics of Per1 and Per2 expression mimics that of Process S, similar to SWA. However, under entrained baseline conditions, cortical Per1 and Per2 in nocturnal rodents are also expressed with a 24-hour rhythmic pattern peaking at the middle of the night, anti-phase to their expression in the SCN. This expression
pattern may be driven by a direct circadian input from the SCN master clock and/or a state dependent input as mice and rats are predominantly active during the night and sleep mostly during the day. Therefore, it is difficult to quantitatively describe the dynamics of homeostatic drive of cortical Per1 and Per2 expression even when data are collected at different SD durations. One solution to this problem is to isolate the circadian and state-dependent drive of Per1 and Per2 expression. This has been done in Cry1,2 double KO mice (Wisor et al., 2002), in which the direct circadian impact is eliminated. The amplitude of Per1 and Per2 induction by 6 hours of SD in Cry1,2 double KO mice is similar to those that have been reported in intact mice (Franken et al., 2006; Franken et al., 2007). However, as described above, the baseline Per1 and Per2 mRNA levels as well as homeostatic sleep pressure in Cry1,2 double KO mice are already high, and thus may interfere with further homeostatic responses of gene expression and rebound sleep induced by SD. Indeed, the amount of rebound SWA and total NREM after SD in Cry1,2 double KO mice is not as high as in wildtype animals (Wisor et al., 2002). In Chapter Four of this work, I take a different approach by using SCN lesioned (SCNx) mice, in which direct circadian control from the SCN is abolished without affecting the cellular machinery in other regions of the brain. By comparing SCNx and intact animals, I show that the cortical expression of Per1 and Per2 are mostly driven by prior sleep and wake, although an impact from the SCN clock may also exist. Therefore, while process S and process C may be generally independent, the clock genes may underlie both, and thereby provide a fundamental link between these two basic processes.

In summary, accumulating data have provided intriguing clues to the function of sleep as well as the circadian and homeostatic mechanisms of sleep regulation.
Nonetheless, as emerging evidence continues to reveal the health, social and economical consequences related to sleep loss and sleep disorders, the goal of gaining a comprehensive understanding of sleep regulation has become increasingly demanding. In the following chapters of this dissertation, I describe in detail my studies using laboratory mice as a model system to explore the circadian as well as homeostatic mechanisms of sleep regulation. I first establish a novel animal model, CAST inbred mice, for human ASPS. I show that the change in the phase alignment of sleep/wake cycles relative to the LD cycles in CAST mice result from altered coupling between the clock and its outputs. I also refine a genetic locus that contributes to phase alignment of sleep/wake (and activity/rest) cycles. Later, I demonstrate different dynamics of clock-genes \textit{Per1} and \textit{Per2} expression in the SCN and other brain regions in CAST mice. Finally, I illustrate a more general role of \textit{Per1} and \textit{Per2} in sleep homeostasis. The contributions of circadian and homeostatic input driving cortical \textit{Per1} and \textit{Per2} expression are quantitatively estimated. The ultimate goal of my research is to provide a blueprint of molecular networks that underlies the circadian and homeostatic processes as well as their interactions, and thereby facilitate the development of interventions and therapeutics for sleep-related problems and disorders.
Introduction

Sleep is regulated by a homeostatic process and a circadian process. The latter is thought to be adaptive by restricting sleep to a favorable time of day (i.e., phase). However, in humans, the timing of sleep and activity exhibit considerable variation. In extreme cases, such as advanced or delayed sleep phase syndrome (ASPS or DSPS), sleep phases are several hours early or late, generating conflicts with social demands as well as negative consequences to physical health (Baker & Zee, 2000; Zisapel, 2001; Ando et al., 2002; Sack et al., 2007). These disorders are thought to reflect misalignment between endogenous circadian rhythms and external timing cues.

In mammals, circadian rhythms are generated by an endogenous clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which free-runs under constant conditions with a period of ~24 h and can be synchronized, or entrained, to the external light/dark (LD) cycle. Light entrains the clock by inducing phase delays during early subjective night and phase advances during late subjective day, as can be described in the phase response curve (PRC). These light-induced responses ensure that the clock adjusts to seasonal changes in the timing of dawn and dusk, but also correct for the deviation of the internal circadian period from 24 h (Johnson et al., 2003). If the circadian period is longer or shorter than 24 h, the clock must be advanced or delayed, respectively, on a daily basis to maintain entrainment.
The PRC also dictates the phase angle (i.e., phase relation) between the clock and the LD cycles to which the animal is entrained (Pittendrigh & Daan, 1976b). With shorter circadian periods, entrainment occurs at an earlier phase angle, as synchronization will only be achieved with greater magnitude phase delays. Such phase delays are only achieved when the clock is exposed to light during a larger portion of the early subjective night. This model of circadian phase determination has been supported by studies in both animal models and humans (Aschoff, 1981; Ralph & Menaker, 1988; Duffy et al., 2001). The extreme morningness in one patient with familial ASPS (FASPS), whose endogenous period was assessed, was also found to be associated with a shorter circadian period (Jones et al., 1999). Despite these successes, alterations in circadian period may not be sufficient to explain all (or even most) circadian phase variations. A recent study in a more general population suggested that human “morning larks” and “evening owls” may be attributed to other properties of the clock (Brown et al., 2008).

Forward genetic approaches have been taken to elucidate molecular mechanisms of circadian phase determination. The hamster tau mutation, which confers an extremely short period and thus a pronounced early phase, was mapped to the Casein kinase 1-epsilon (Csnk1e) gene, the product of which phosphorylates the core-clock PERIOD proteins (Lowrey et al., 2000). In two kindreds of FASPS, extreme morningness was linked in different pedigrees to mutations in human Period 2 (Per2) or Casein kinase 1-delta (Csnk1d) genes, both causing shortened periods (Toh et al., 2001; Xu et al., 2005). These and other circadian genetic studies focused mostly on the molecular determinants of circadian period, but did not provide insights into the phase alignment of overt rhythms relative to cycles of entraining signals such as light.
A previous study in our lab has taken an alternative forward genetic approach, quantitative trait loci (QTL) analysis, which exploits natural genetic variation that accumulated over many years of evolution, to search for genetic determinants of circadian phase in a backcross population originated from two genetically divergent mouse strains, CAST/EiJ (CAST) and C57BL/6J (B6) (Wisor et al., 2007). Mouse "early runner" behavior similar to human ASPS and a QTL on chromosome 18, named Era1, which contributes to this phenotype, were identified. In this chapter, I show that several circadian endophenotypes, circadian clock properties that may contribute to the early runner phenotype, are found in the parental CAST strain and the B6.CAST.18 congenic mice (Iakoubova et al., 2001; Davis et al., 2007), in which a portion of chromosome 18 has been transferred from the CAST strain onto the B6 background. My results suggest that other factors, aside from endogenous period, influence circadian phase determination in CAST mice. CAST is a unique animal model for the study of ASPS, circadian entrainment, phase relationships, and other poorly understood aspects of the circadian system.

**Materials and Methods**

**Animals**

Six-week old male and female C57BL/6J (B6) and CAST/EiJ (CAST) mice were purchased from the Jackson Laboratory, Bar Harbor, ME. CAST/EiJ is an inbred strain derived from the subspecies *Mus musculus castaneus*. 54 (B6 x CAST) x CAST backcross mice were bred by crossing female B6 mice to male CAST mice and subsequently backcrossing the female F1 progeny to male CAST mice. Two strains of B6.CAST congenic mice [also known as Genome-Tagged Mice, GTM (Davis et al.,
were obtained from the University of California, Los Angeles. They carry CAST segments of proximal and middle portions of chromosome 18 introgressed on an otherwise 99% B6 background (B6.CAST.18P and B6.CAST.18M, respectively). These two congenic strains were chosen because the CAST segments in these strains overlap with the mapped Era1 QTL region (Figure 2.7A). Female and male B6.CAST congenic mice were mated in the animal facility at the University of Kentucky. All animal procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

**Wheel-Running Activity Recording**

Mouse wheel-running activity was monitored in light-tight chambers (Phenome Technologies, Lincolnshire, IL). Male mice ≥8 weeks old were individually housed at 23±1°C with unrestricted access to a running wheel, the rotations of which were detected by a mechanical switch connected to a desktop computer. Food and water were provided *ad libitum*. Data were recorded and analyzed using Clocklab software (Coulbourn Instruments, Whitehall, PA). Green LED light (526 nm max, 50% bandwidth ± 20 nm, 11.8 μW/cm², approximately 83 lux as measured inside of mouse cages, 100 lux as measured at the bottom of the light chamber) and white fluorescent light (22.3 μW/cm², approximately 160 lux as measured inside the mouse cage) were provided 24.5 cm above the cage floor. Cages were changed every 7 days. Data from days on which cage changes occurred were excluded from analysis.

**Activity Onset, Phase Angle of Entrainment, and Circadian Period**

Mice, including CAST (n = 10), B6 (n = 8), (B6 x CAST) x CAST (n = 54), B6.CAST.18P (n = 9), and B6.CAST.18M (n = 8), were first housed in a 12-h light/12-h dark (LD12:12) cycle of green light ≥21 days prior to all experimental manipulations of
lighting conditions. Daily wheel running activity onset was first defined by Clocklab software as the time of day that best approximates a pattern of 6 h of inactivity (i.e., lower than the 20th percentile activity level) followed by a 6-h period of high activity. The computer-defined activity onsets were then adjusted by eye for any obvious mis-scored values, and averaged over the last 10 LD cycles.

Mice of the CAST, B6, B6.CAST.18P and B6.CAST.18M strains were then subjected to ≥14 days of constant dark (DD) housing for measurement of the phase angle of entrainment and circadian period. Phase angle of entrainment was defined by extrapolating, to the last day of LD12:12 housing, a line fitted through activity onsets during the first 14 days of DD housing. Free running period in DD was determined by chi-square periodogram analysis of data from days 1-14 in DD, using Clocklab software.

**Photic Phase Response Curve and Tau Response Curve**

Subsequent to the measurement of phase angle of entrainment and circadian period in DD, CAST mice and B6 mice were exposed to a single 30-min green light pulse once every 2 weeks. All mice were subjected to the light pulse simultaneously, and as a result the circadian time of the pulse was random for each animal. Individual mice were subjected to anywhere from 3 to 9 light pulses and thus were in constant darkness punctuated by 30-min green light pulses for up to 193 days. Because the number and circadian times of light pulses administered to each animal were random, it was not possible to analyze the data in a repeated measures design. The phase shifts induced by these light pulses were treated as independent measures. To determine the magnitude of phase shifts in response to light pulses, 2 fitted lines were drawn through activity onsets (defined as described above) during the 10 circadian cycles preceding light exposure, and cycles 4-13 subsequent to light exposure, respectively. The phase
shifts were calculated similarly as described by Spoelstra et al (Spoelstra et al., 2004).

Briefly, $\Delta \phi = \phi_2 - \phi_1$, where $\phi_1 = (((t_{LP} - t_{Ons1}) \mod \tau_1) \times 24/\tau_1 + 12) \mod 24$, and $\phi_2 = (((t_{LP} - t_{Ons2}) \mod \tau_2) \times 24/\tau_2 + 12) \mod 24$, both expressed in circadian hours. $t_{LP}$ is the time when the light pulse is given, while $t_{Ons}$ and $\tau$ are the time of activity onset and free running period before ($t_{Ons1}, \tau_1$) and after ($t_{Ons2}, \tau_2$) light exposure, determined by the 2 fitted lines described above. A 2-harmonic Fourier function (Comas et al., 2006) with a fixed period of 24 circadian hours was fitted to the phase response curves for CAST and B6 strains, using the curve fitting tool of the Matlab software package (MathWorks, Inc. Natick, MA.). The magnitude of changes in circadian period in response to light pulses was determined simply by $\Delta \tau = \tau_2 - \tau_1$, where the values of $\tau_1$ and $\tau_2$ were determined as described above.

**Masking and Activity Density Before Dark**

Negative masking effects of white and green light were assessed by exposing CAST (n = 5), B6.CAST.18P (n = 5), B6.CAST.18M (n = 5), and B6 (n = 4) mice to light during a 60-min interval beginning 30 min after dark onset in an LD12:12 cycle of green light. Each mouse was exposed to one 60-min white light exposure and one 60-min green light exposure, both of which were preceded by 6 uninterrupted LD cycles. The magnitude of negative masking by light exposure was quantified as the “percentage of activity remaining” during the 60-min light exposure at ZT12.5-13.5. This variable was measured by dividing the number of wheel revolutions during light exposure by the number of wheel revolutions during the analogous time interval averaged over the previous 2 nights and multiplying the resulting number by 100%.

The same 5 CAST mice were also used to study the effect of lighting intensity and/or spectra on activity density before dark. Mice were first housed in LD12:12 cycles
with white light at ~160 lux for 3 weeks, and then in the same LD cycles with green light at ~83 lux for another 3 weeks. Activity density before dark under white and green light housing was measured by averaging total wheel revolutions occurring between the wheel running activity onset and dark onset over the last 10 cycles in LD12:12, and then dividing this number by the total minutes during which the animal was active before dark onset.

**Recording of Sleep and Wake Behavior**

A subset of (B6 x CAST) x CAST backcross mice (n = 14) was also tested on an automated piezoelectric system, which has been shown to have >90% sensitivity relative to electroencephalographically defined sleep (Flores et al., 2007; Donohue et al., 2008). Sleep and wake behavior were continuously recorded over more than 10 LD12:12 cycles. Time of awakening for the major wake bout each day was defined as the time between ZT4 and ZT15 when the percent of wake first reached 75% of the night time average for a sustained period.

**Sequencing the cDNA of candidate genes and bioinformatic analysis**

Total RNA were extracted from brains (a rostral piece of the forebrain) of two B6 and two (B6 x CAST) x CAST backcross mice that have been genotyped as homozygous CAST, using TRIzol® Reagent (Invitrogen, Carlsbad, CA). Total brain RNA was then purified with Turbo DNA-free™ (Ambion, Foster City, CA) to remove of possible genomic DNA contamination, and 3 μg of purified total RNA was subject to cDNA synthesis, using Oligo(dT)$_{12-18}$, and Superscript II™ (Invitrogen, Carlsbad, CA). Two genes of interest (e.g., *Csnk1a1* and *Grp*) was amplified using PCR with gene-specific primers (Table 2.1) and a high-fidelity DNA polymerase (GeneAmp®, Applied Biosystems, Foster City, CA). After gel-purification, PRC fragments of *Csnk1a1* from the
4 animals described above were sent for sequencing at Genetic Variation and Gene Discovery Core Facility of the Cincinnati Children's Hospital Research Foundation (Cincinnati, OH), and Grp fragments from one B6 and one backcross mouse described above were sent to ACGT, Inc (Wheeling, IL) for sequencing. Sequencing was performed in both strands. Csnk1a1 sequence was obtained using 4 overlapping pieces at each strand, while Grp was sequenced using a pair of internal primers (Table 2.2).

CAST and B6 sequences were compared to each other and to the reference sequences in the GenBank database (Csnk1a1, NM_146087; Grp, NM_175012.2). Translated GRP precursor peptide sequences of B6 and CAST genotypes were also aligned with references GRP precursor sequences of rat (NP_598254.2) and human isoforms (NP_002082.2, NP_001012530.1, NP_001012531.1), using ClustalW2 with default settings at the European Bioinformatics Institute (EBI) website.

**Statistical analysis**

In comparisons of the parental CAST and B6 strains (wheel running activity onset, phase angle of entrainment, maximum phase delay and phase advance magnitudes, tau response magnitude), Student's $t$ for independent measures was applied. For within-strain comparisons of green and white light effects (wheel running activity onset, activity density, magnitude of negative masking), Student's $t$ for paired measures was applied. In comparisons of both parental strains and the two chromosome 18 congenic lines, (free running period in DD, phase angle of entrainment), one-way analysis of variance (ANOVA) for independent measures was applied, and was followed by Student's $t$ for independent measures when significant. Statistical analyses were performed using SAS software, version 9.2.
Results

Early Runner Phenotype in CAST Mice

When CAST and B6 mice were housed in LD12:12, using monochromatic green light, the timing of wheel running relative to the LD cycles differed strikingly between the two strains (Figure 2.1C). Wheel running onset was 2.70±0.35 h (n = 10) before dark onset in CAST mice, and nearly coincided with dark onset in B6 mice (0.086±0.030 h after dark onset, n = 8; P < 0.001 vs. CAST). This finding was also observed independently by Drs. Kazu Shimomura and Joe Takahashi at Northwestern University and UT Southwestern using similar lighting conditions (Kazu Shimomura, personal communications). As light exposure can also acutely affect activity in mice (i.e., masking), the true phase angle of entrainment for wheel-running activity rhythm needs to be assessed by extrapolation of a line drawn through the onsets of activity in constant dark (DD) subsequent to LD12:12. Upon release into DD, B6 and CAST mice also differed significantly in the phase angle of entrainment (Figure 2.1D; P < 0.001). Only one B6 male exhibited a negative phase angle of entrainment (0.5 h before dark in this animal); this value was >3 SD from the mean of B6. When the data from this mouse were excluded from analysis, the phase angle of entrainment for the remaining B6 population (n = 7) was even more delayed (0.360±0.073 h after dark onset). Meanwhile, phase angle of entrainment in CAST mice was 1.93±0.30 h before lights-off, and was strongly correlated with the timing of wheel running onset in LD12:12 across animals within the CAST strain (Pearson r = 0.8732, P = 0.0021), indicating that the early activity onset in CAST mice under LD12:12 conditions was a valid measure of entrained phase. These data demonstrate that the early runner phenotype, characterized by an advanced activity onset in LD and an advanced phase angle of entrainment, can be detected in inbred CAST mice under green light.
I also tested the wheel running activity of 54 (B6 x CAST) x CAST backcross mice under LD 12:12 cycles using these green lighting conditions. Compared to a previous study from our lab using more intense white lighting conditions, a higher percentage of (B6 x CAST) x CAST backcross mice exhibited the early runner phenotype under green light (data not shown), indicating that green light may have increased the penetrance of alleles that contribute to the early runner phenotype. Studies have shown that phase preference in mammals may be altered by the availability of a running wheel (Kas & Edgar, 1999; Mistlberger & Holmes, 2000). To test this possibility, a subset of (B6 x CAST) x CAST backcross mice (n = 14) were also phenotyped using a piezoelectric system which scores the percentage of time spent asleep (Flores et al., 2007; Donohue et al., 2008). Without access to a running wheel, the early activity onset under entrained conditions persisted as an earlier daily awakening time (Figure 2.2). These data clearly demonstrate that the early runner phenotype represents entrainment at an advanced phase for activity/rest, as well as sleep/wake, regardless of the availability of a running wheel.

**Lack of Correlation Between Period and Phase Within the CAST Strain**

Circadian period can influence the phase angle of entrainment. To test whether the early activity onset in CAST mice can be attributed to a shorter circadian period, I transferred mice to constant dark to assess their endogenous period (Figure 2.3A). The circadian period in CAST mice (23.17±0.10 h) was indeed shorter than that of B6 (23.66±0.04 h, P = 0.001). However, CAST mice with similar circadian period exhibited wide variations in activity onset under LD as well as in phase angle of entrainment. There was no significant correlation between circadian period and either phase of activity onset under LD12:12 (Pearson r = -0.3078, P = 0.42, Figure 2.3B) or phase angle of entrainment (Pearson r = -0.06375, P = 0.87, Figure 2.3C) within the CAST
population. In addition, CAST mice whose period length fell in the range of B6 mice initiated their daily activity 2-3 h earlier than B6 mice. Thus, factors other than endogenous period must contribute to the early activity onset in CAST mice under entrained conditions.

A Phase-Lagged PRC in CAST Mice

Because the circadian period and the PRC together generally determine the phase at which animals will be entrained to the LD cycles, I hypothesize that CAST mice differ from B6 in their PRC, which then contributes to the early activity onset in CAST mice. To test this hypothesis, CAST and B6 mice were housed under DD conditions, and were randomly given a 30-min green light pulse every 2 weeks. Both CAST and B6 mice exhibited photic phase shifts in response to 30-min green light pulses (Figure 2.4A, B, C). However, there were significant strain differences in the timing of circadian responses to light. Unlike B6 mice, light exposure close to activity onset in CAST mice did not phase delay the activity pattern, while light that fell several hours after activity onset induced significant phase delays. I fitted the phase response data from each strain using 2-harmonic Fourier curves (Figure 2.4C). The circadian time (CT, with CT12 defined as activity onset in DD for nocturnal animals) of maximum phase delay was CT16.41 in B6 mice and 3.05 h later at CT19.46 in CAST mice. The circadian time of maximum phase advance was CT23.92 in B6 mice and 3.46 h later at CT3.38 in CAST mice. Although the timing of maximal photic phase responses was shifted in CAST relative to B6 mice, the magnitude of maximal phase shifts (i.e., those occurring in response to light pulses delivered within ±1 h of the maximum delay and advance points on the PRC) did not differ between the strains. Maximum phase delays were -2.45±0.50 h in CAST (n = 7) and -1.31±0.31 h in B6 mice (n = 5; P = 0.083 vs. CAST). Maximum
phase advances were 0.98±0.43 h in CAST (n = 4) and 0.17±0.16 h in B6 mice (n = 6; P = 0.153 vs. CAST).

Noting that the difference between the strains in the timing of peak delays and advances in the PRC was very similar in magnitude to the difference between the strains in the timing of wheel running onset in an LD12:12 cycle, I reasoned that the photic PRC might have a distinct phase relationship to wheel running in the two strains and be otherwise identical. To account for this possibility, I defined circadian time according to the phase of wheel running relative to the daily onset of darkness under LD12:12, (e.g., activity onset of an animal in DD is defined as CT10 if this animal initiates activity at ZT10 in LD12:12), and plotted the PRC accordingly (Figure 2.4D). When the data were transformed in this manner, the PRCs were nearly identical in the two strains. The circadian time of maximum phase delay was at CT17.12 in CAST mice, less than one hour later than the maximum phase delay in B6 mice. The circadian time of maximum phase advance was CT0.79 in CAST mice, less than one hour later than the maximum phase advance in B6 mice. The magnitude of maximal phase delays and advances did not differ between the strains. These data demonstrate that the photic PRC differs between CAST and B6 mice primarily in its phase relationship to wheel running, and that the traditional definition of activity onset as CT12 in nocturnal rodents is probably inappropriate in CAST mice.

In addition to causing an acute phase shift, light exposure can also alter circadian period in the longer term. To document this effect of photic stimulation, I measured a photic tau response curve, in which the change in circadian period after a light pulse is plotted against the circadian time at which the light pulse is delivered (Figure 2.4E). Circadian period was selectively increased by light exposure only in CAST (P = 0.01) and only when light was administered during the first few hours after the onset of wheel
running. The mean increase in circadian period was 0.25±0.08 h (n = 12) in CAST mice exposed to light during CT12-15 (the circadian phase at which light exposure overlaps with wheel running in an LD12:12 cycle, using the traditional definition of CT12 = activity onset in DD for CAST mice). There was no consistent effect of light pulses on circadian period in B6 mice whether they were exposed to light at CT12-15 (0.056±0.058 h, n = 5) or at any other phase of the circadian cycle (Figure 2.4E).

**Blunted Light-Induced Masking Responses in CAST Mice**

Normally, light exposure acutely suppresses wheel-running activity in mice. The fact that CAST mice exhibit wheel running during the light phase of the LD12:12 cycle indicates that masking is at least attenuated to some degree in these mice. To quantify the suppression of wheel running by light, mice of both strains were exposed to 1-h light pulses beginning at 30 min into the dark portion of the LD12:12 cycle (Figure 2.5). B6 mice exhibited a near total (>95%) suppression of wheel running during light exposure whether the light was green at ~83 lux (P < 0.001, light exposure vs. baseline) or white at ~160 lux (P < 0.001, light exposure vs. baseline). By contrast, CAST mice exhibited no significant suppression of wheel running by green light but a modest 30% suppression of wheel running by white light (P < 0.05, light exposure vs. baseline). These data demonstrate that the negative masking effect of light is strongly attenuated in CAST mice.

As the sensitivity of light-induced masking responses in CAST mice is affected by the spectra and/or intensity of lighting conditions, I reasoned that lighting spectra and/or intensity may also modulate the stability of wheel-running activity before dark in CAST mice. To evaluate this effect, I housed CAST mice under LD conditions first using white light at ~160 lux and then green light at ~83 lux. Activity onset was delayed by
approximately three-quarters of an hour when CAST mice were housed in white light relative to green light (Figure 2.6B; P < 0.05). The intensity of wheel running at the daily activity onset was also influenced by the cage lighting: activity density (the number of wheel revolutions occurring per unit time between daily wheel running onset and dark onset) was greater in CAST mice under green light than under white light (Figure 2.6C; P < 0.05). Thus, the early runner phenotype in CAST mice is modulated by the intensity and/or wavelength of environmental lighting.

**Wheel-Running Phenotypes in Chromosome 18 Congenic Strains**

Using a (B6 x CAST) x CAST backcross population, previous work from our lab identified a QTL, named *Era1*, which contributes to 10% of the phenotypic variation in activity onset under LD conditions (Wisor *et al.*, 2007). To validate this QTL, wheel running phenotypes were measured in two congenic mouse strains in which proximal (B6.CAST.18P) and middle (B6.CAST.18M) segments of chromosome 18 have been transferred from the CAST to the B6 background by selective breeding (Figure 2.7A). Mice of both congenic strains exhibited entrainment to a LD12:12 cycle with the bulk of wheel running activity restricted to the dark portion of the cycle (Figure 2.7B, C). The phase angle of entrainment, as assessed by extrapolation of a line drawn through the daily onset of wheel running activity in constant dark subsequent to entrainment, was intermediate between the parental strains for both congenic lines (Figure 2.7D). The phase angle of entrainment was advanced by an average of 0.53 h in B6.CAST.18P relative to B6 mice (P < 0.001) and by an average of 0.70 h in B6.CAST.18M relative to B6 mice (P < 0.001). The phase angle of entrainment was significantly delayed in both congenic strains relative to CAST mice (P < 0.001). These data demonstrate that a modest but significant portion of the advanced phase angle of entrainment in CAST mice can be attributed to a locus (or loci) on the mid-portion of chromosome 18. Moreover,
the Era1 QTL is now mapped to a genomic region where the CAST segments in B6.CAST.18P and B6.CAST.18M overlap.

Negative masking of wheel running activity was also measured in B6.CAST.18P and B6.CAST.18M congenic lines during the dark portion of the LD cycles, as it was in the parental strains. These congenic lines appeared to have intact negative masking, as they exhibited >95% suppression of wheel-running activity when exposed to either a ~83 lux green light pulse or a ~160 lux white light at ZT12.5-13.5, identical to B6 mice.

When animals of the B6.CAST.18P and B6.CAST.18M strains were housed in constant dark, robust circadian rhythmicity was maintained (Figure 2.7B, C). Circadian period was unexpectedly longer in the congenic strains than in both parental strains (Figure 2.7E). Circadian period was longer by 0.26 h in B6.CAST.18P relative to B6 mice (P < 0.001) and by 0.15 h in B6.CAST.18M relative to B6 mice (P < 0.01). Circadian period was also significantly longer in both strains than in the CAST parental strain (P < 0.001). These data demonstrate that the shorter circadian period in CAST relative to B6 mice is not conferred by genetic material on proximal or mid-chromosome 18, and that the earlier phase angle of entrainment in the two congenic strains is not caused by a shorter circadian period.

**Sequence variations between B6 and CAST in the candidate genes of the Era1 QTL**

The genomic region (~10 Mb) where CAST segments of the two congenic strains overlap contains 73 annotated and several predicted genes. Among the candidate loci are *Casein kinase 1-alpha 1* (*Csnk1a1*) and *Gastrin-releasing peptide* (*Grp*). The former is functionally similar to two other casein kinases, CSNK1D and CSNK1E, known to influence circadian rhythms (Lowrey *et al.*, 2000; Xu *et al.*, 2005), while the later has been proposed as the signal communicating across functionally heterogeneous SCN
sub-regions (Antle et al., 2005). I sequenced the cDNA of the two genes in both CAST and B6 genotypes and found several single nucleotide polymorphisms (SNPs) between the two strains (Table 2.3). The SNPs found in Csnk1a1 did not appear to cause differences in the protein sequences between the two strains. SNPs leading to coding variations, however, were identified in the Grp gene, although these amino acid substitutions are all located in the proGRP peptide, not the neuropeptide GRP

(which was thought to be the only bioactive form until recently). To determine whether these amino acid alterations occurred in conserved regions among species, B6 and CAST GRP precursor sequences were aligned against their homologues in rats and humans (Figure 2.8). The sequence alignment suggests that Arg80Ser might be the most interesting variation, as it is most dramatic amino acid change and is located in the center of a very conserved region. In addition to coding regions, SNPs located in the 5'- and 3'-untranslated regions (UTR) of the two genes were also found (Table 2.3). Alignment of Csnk1a1 mRNA sequences of several mammalian species suggested that all the UTR SNPs are located in conserved regions among mammals (alignment not shown). Among these SNPs, 75C and 1220T (e.g., the B6 alleles) while 1181T and 1334G (e.g. the CAST alleles) are the common alleles shared by mammalian species. None of the three 3'-UTR SNPs are located in potential microRNA target regions predicted by two independent internet-based resources, miRDB and miRanda/mirSVR (Betel et al., 2008; Wang, 2008; Wang & El Naqa, 2008). I also found a SNP in the 3'-UTR of Grp, although this SNP is not located in conserved regions among mammals or in any predicted microRNA target regions.
Discussion

Studies in rodents (Antoch et al., 1997; Kume et al., 1999; Lowrey et al., 2000; Shearman et al., 2000; Zheng et al., 2001) and humans (Toh et al., 2001; Xu et al., 2005) have demonstrated that the circadian clock is profoundly affected by genetic variability. A good deal of information has been obtained about the molecular underpinnings of the circadian clock from these genetic studies (Reppert & Weaver, 2001; Young & Kay, 2001; Lowrey & Takahashi, 2004), but information on the molecular mechanisms underlying phase control under entrained conditions is still lacking. The current report establishes a mouse model suitable for study of this poorly understood aspect of circadian rhythms. The early runner phenotype in CAST mice co-varies with a number of circadian endophenotypes, including strain differences in circadian period, altered timing of the PRC relative to circadian wheel running rhythms, and attenuated light masking of wheel running. Only a subset of these circadian endophenotypes is replicated in B6.CAST.18 congenic lines, and their phase angle of entrainment is intermediate between the parental strains. The congenic lines were not expected to fully replicate the early runner phenotype, as previous QTL study from the our lab on (B6 x CAST) x CAST backcross progeny indicated that the Era1 QTL on chromosome 18 is responsible for approximately 10% of the phenotypic variance (at least under the white lighting conditions used in that study). Nonetheless, an advanced phase angle of entrainment relative to B6 mice was indeed apparent in B6.CAST.18P and B6.CAST.18M strains and is thus conferred by CAST alleles on the mid-portions of chromosome 18.

CAST and B6 mice differ in circadian period, as shown previously (Wisor et al., 2007) and in this current study using more refined experimental conditions. The shorter circadian period in CAST might contribute to the early phase of activity onset in these
mice, as shorter circadian period dictates an earlier activity onset in entrained conditions, absent any other differences among experimental subjects (Pittendrigh & Daan, 1976b). However, in neither the genetically diverse B6 x CAST backcross (Wisor et al., 2007) or intercross (Jiang et al., 2008) populations, nor the genetically uniform CAST population studied here, did circadian period correlate significantly with phase angle of entrainment, as is often assumed. Furthermore, the coincidence of early phase angle of entrainment and longer circadian period in B6.CAST.18P and B6.CAST.18M mice relative to B6 was not expected from this theoretical standpoint. It is possible that circadian period is not lengthened intrinsically in B6.CAST.18P and B6.CAST.18M mice, but rather it is increased as a consequence of light exposure during the entrainment process. Indeed, in the CAST strain (Figure 2.4E) and other rodent species (Daan, 2000), increases in circadian period (tau) can occur in response to light exposure, as demonstrated by the “tau response curve” (Daan, 2000). I hypothesize that the lengthened circadian period in B6.CAST.18P and B6.CAST.18M mice relative to both parental strains may be due to the combination of (1) an intrinsic circadian period similar to that of B6 mice, conferred by loci distinct from the Era1 QTL, and (2) slowing of the clock due to repeated daily exposure to light at around the time of wheel running onset in LD, conferred in part by the Era1 locus.

The photic PRCs of CAST and B6 strains were virtually identical when the abscissa values were transformed to account for individual differences in timing of wheel running relative to light exposure in the LD12:12 cycle. It is clear from this observation that circadian resetting in response to photic stimulation is intact in CAST mice, and that the light-sensitive oscillator in the SCN is functional. In fact, the magnitude of maximal phase delays was somewhat greater in CAST than B6 mice, but did not quite reach significance, perhaps due to the limited number of phase delays collected within the 2-h
peak phase delay window of the PRC. Rather than the photic phase-shifting response being deficient in CAST mice, the timing of wheel running as an output of the circadian clock is coupled to the light-sensitive oscillator at a distinct phase in CAST when compared to B6 mice. In support of this model, the SCN oscillator itself, as defined by rhythms of *Period* gene expression, is not detectably phase advanced in early runner (B6 x CAST) x CAST mice relative to controls (Wisor *et al.*, 2007). Thus, alterations in activity rhythms may be a result of downstream mechanisms on the output side of the clock (Eskin, 1979), as appear likely in these CAST mice. The mammalian circadian system is believed to be organized in a hierarchy: the master clock located in the SCN controls the oscillations of downstream clocks, which in turn regulate rhythmic physiological processes including activity/rest and sleep/wake cycles. Studies also suggest the presence of SCN-independent circadian oscillations that can be induced by timed non-photic stimulation such as restricted food access (Stephan, 2002). Additionally, under a forced desynchrony protocol, separate compartments within the SCN can be shown to oscillate independently (de la Iglesia *et al.*, 2004). It is thus possible that the mechanisms linking the SCN oscillator to downstream oscillators, SCN-independent oscillators to SCN-oscillators, or independent oscillators within the SCN, are modulated by loci that are polymorphic between CAST and B6 mice, including *Era1*. It is also possible that output pathways such as those utilizing cardiotrophin-like cytokine, that normally inhibit activity late in the day (Kraves & Weitz, 2006), are deficient or altered in CAST mice.

The attenuated light masking of wheel running observed in CAST mice relative to B6 mice is likely to influence, in part, the early runner phenotype. Indeed, an early activity onset under normal LD conditions would not be observed at all if CAST mice were to exhibit negative masking to the same degree as B6 mice. Both attenuation of
masking and the advanced activity onset in LD were more robust in CAST mice when the light exposure was restricted to approximately 83 lux of green light. It is possible that the less-extreme early activity onset in CAST mice entrained under ~160 lux of white light resulted from modest negative masking, as activity density before lights-off was reduced under white light compared to green light. Alternatively, this parallel may suggest that the spectral sensitivity, and thus the relevant photoreceptors for phase determination and masking, are similar if not identical. Both melanopsin containing ganglion cells and classical photoreceptors are capable of performing non-image forming retinal irradiance detection (Panda et al., 2002; Hattar et al., 2003), and consequently, it cannot be determined whether any specific phototransduction pathway is deficient in CAST mice based on the current results. It is worth noting, however, that B6.CAST congenic lines exhibited intact negative masking, consistent with the fact that the advanced phase angle of entrainment in B6.CAST.18P and B6.CAST.18M mice did not lead to earlier activity onset under LD12:12 conditions, and that no known photopigment genes map to the portion of chromosome 18 shared by the B6.CAST.18P and B6.CAST.18M strains.

Collectively, these data begin to explain the early runner phenotype phenomenologically, if not mechanistically. For entrainment to occur, CAST mice and B6 mice alike require a phase-delaying effect of light to counteract the phase advancing effect of a circadian period less than 24 h. The advance of wheel running onset in CAST mice relative to B6 mice reflects, in part, the difference in the timing of light responses relative to wheel running in the two strains. Further, the attenuation of negative masking in CAST mice allows the early runner phenotype to be detected in LD12:12. Relationships between entrained phase and other circadian properties have been documented in many studies. In humans, subjects with similar endogenous period
length can still exhibit large variations in their time-of-day preference, as their clock
differs in oscillation amplitude and sensitivity to phase-shifting agents (Brown et al.,
2008). Despite an extremely long period, Clock mutant mice entrain to LD cycles at a
phase that coincides with dark onset, as in wild-type animals, because of a low-
amplitude circadian oscillation and consequently high-magnitude photic phase-shifting
responses produced by the Clock mutation (Vitaterna et al., 2006). The shorter
circadian period and slightly lower amplitude PRC in BALB/cJ mice leads to a phase
angle of entrainment that is similar to that of the CAST mice observed in the current
study, although they exhibit very little wheel running in the hours before dark onset in an
LD12:12 cycle due to masking (Schwartz & Zimmerman, 1990), even when similar green
light conditions are used (Shimomura et al., 2001). This current study differs from those
reports by suggesting a novel mechanism of circadian phase determination, which may
involve altered coupling between different oscillators of the mammalian circadian system,
or between the SCN and major output pathways. In addition, light-induced masking in
CAST mice was impaired in concert with the advanced phase angle of entrainment to
maintain robust activity in the preferred circadian phase. This suggests the possibility of
positive selection for altered chronotypes among wild populations of Mus musculus
castaneus from which CAST mice are derived. It is also conceivable that human
chronotype variation was and is under similar selection pressures, but as in mice, this
will have to await the identification of more specific gene alleles that influence
chronotype. Thus far, among common allelic variants already described in humans,
Per3 alleles are perhaps the best candidates (Ebisawa et al., 2001; Archer et al., 2003;
Dijk & Archer, 2009).

The phenotypic data from B6.CAST.18P and B6.CAST.18M provide confirmation
that the Era1 QTL on chromosome 18 identified in previous work from our lab influences
the unusual circadian traits in CAST mice. The refined Era1 locus is located in a 10 Mb region, within which two genes, Csnk1a1 and Grp, are of particular interest to us. The homologues of CSNK1A1, CSNK1D and CSNK1E phosphorate PER and regulate circadian rhythms (Lowrey et al., 2000; Vanselow et al., 2006; Xu et al., 2007). Animals bearing mutated CSNK1D and CSNK1E, which are deficient in PER phosphorylation, exhibited shortened circadian periods and therefore earlier behavioral phases relative to the LD cycles when entrained (Lowrey et al., 2000; Xu et al., 2005). However, I sequenced the Csnk1a1 cDNA in both CAST and B6 genotypes, but sequence variations similar to the mutations found in Csnk1d and Csnk1e were not found in Csnk1a1. The SNPs I found in Csnk1a1 cDNA did not indicate clear functional impact. In fact, Martin Striz in our lab has sequenced the genomic regions several hundred base pairs upstream from the transcription start site and several intron-exon boundaries of Csnk1a1 in both CAST and B6. These unpublished sequencing data also failed to reveal SNPs that appear to cause obvious functional consequences (Striz, unpublished). In addition, B6.CAST.18 congenic mice exhibited longer period along with the advanced phase angle of entrainment, indicating Era1 QTL mediates a early circadian phase through a mechanism other than shortening circadian period. Therefore, Csnk1a1 is less likely the candidate gene for Era1, or it plays a role distinct from Csnk1d and Csnk1e in the circadian system. The other candidate, Grp expressed in the ventrolateral subregion of the SCN (Okamura et al., 1986; Karatsoreos et al., 2004; Karatsoreos et al., 2006) with a circadian rhythmic pattern (Aida et al., 2002; Dardente et al., 2004). The mouse Grp gene encodes a 146-amino acid precursor propeptide, which after two cleavages and amidation gives rise to a 29-amino acid bioactive neuropeptide (GRP24-52). The circadian functions of this amidated GRP24-52 involves mediating light-like behavioral phase-shifts and induction of Per expression in the SCN (Aida et al., 2002; Gamble et al., 2007), as well as coupling and enhancing the circadian rhythms of
individual SCN neurons in a vasoactive intestinal polypeptide (VIP) null background (Brown et al., 2005; Maywood et al., 2006). However, the amplitude of light-induced phase-shifting responses were not different between CAST and B6 mice. Also, the sequencing data did not reveal functional variations between CAST and B6 in the GRP24-52 encoding region. Nevertheless, a amino acid substitution Arg80Ser was identified in a conserved region of proGRP peptide. Interestingly, recent studies in human cancer research have suggested that several nonamidated fragments (including the ones harboring Arg80) derived from the proGRP peptide is also bioactive through an unidentified receptor other than the canonic GRP receptor, and can be found in a range of tumors and even in several normal tissues [Figure 2.8; for a review, see (Ischia et al., 2009)]. It is not yet known whether these nonamidated peptides derived from proGRP also have circadian functions.

Nonetheless, my data demonstrated that QTL analysis has the potential to identify novel genetic loci that are involved in the regulation of circadian rhythms. In a QTL study of (B6 x BALB) F2 hybrid mice, 5 QTLs and 2 epistatic interactions, different from those loci that influence circadian period, were found to influence the phase angle of entrainment (Shimomura et al., 2001). This observation further strengthens the QTL approach for the genetic dissection of these poorly understood circadian traits. More generally, CAST and the congenic mice derived from them are likely to be a valuable resource for studies of the molecular mechanisms of phase relationships, and their regulation by light. The CAST strain may also provide a mouse model for human ASPS and other circadian disorders related to sleep and activity phase. Indeed, the complex and multifactorial nature of the unusual circadian phenotypes seen in CAST mice is similar to the majority of cases of ASPS in humans, as opposed to the very rare point mutations causing familial ASPS (Toh et al., 2001; Xu et al., 2005).
### Table 2.1. PRC primers for *Csnk1a1* and *Grp*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Csnk1a1</em></td>
<td>5'-CGACAGAGCCTTTGGTTTAG-3'</td>
<td>5'-AAACAAAAAGCTCCCATTCA-3'</td>
<td>1947 bp</td>
</tr>
<tr>
<td><em>Grp</em></td>
<td>5'-GTGGCAGAGCTCTGAGAAC-3'</td>
<td>5'-GTAGCAAATTGGAGCCCTGA-3'</td>
<td>822 bp</td>
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### Table 2.2. Sequencing primers for *Csnk1a1* and *Grp*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Start*</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Csnk1a1</em></td>
<td>forward</td>
<td>39</td>
<td>5'-CGACAGAGCCTTTGGTTTAG-3'</td>
</tr>
<tr>
<td></td>
<td>665</td>
<td></td>
<td>5'-GACAACAGGACAGCAAGGCAACA-3'</td>
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<tr>
<td></td>
<td>1008</td>
<td></td>
<td>5'-TATCCGATCTTTCAGG-3'</td>
</tr>
<tr>
<td></td>
<td>1549</td>
<td></td>
<td>5'-TCAGCCACAGTTGTGATG-3'</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>1985</td>
<td>5'-AAACAAAAAGCTCCCATTCA-3'</td>
</tr>
<tr>
<td></td>
<td>1544</td>
<td></td>
<td>5'-TGTTCTAGCCCTTGCTGTA-3'</td>
</tr>
<tr>
<td></td>
<td>930</td>
<td></td>
<td>5'-GCAGGAAACCCTACACAA-3'</td>
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<td></td>
<td>404</td>
<td></td>
<td>5'-ACCACCGTGATGGAGGATG-3'</td>
</tr>
<tr>
<td><em>Grp</em></td>
<td>forward</td>
<td>29</td>
<td>5'-CTGAGAACCCTCCTCGTC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>794</td>
<td>5'-GGAAAAAGCGTTCATGGAA-3'</td>
</tr>
</tbody>
</table>

*The start position is the position of the first 5’ nucleotide of the primer according to GenBank references sequences NM_146087 (*Csnk1a1*) and NM_175012.2 (*Grp*).*
Table 2.3. SNPs identified by sequencing of *Csnk1a1* and *Grp* cDNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position*</th>
<th>SNP</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B6</td>
<td>CAST</td>
</tr>
<tr>
<td><em>Csnk1a1</em></td>
<td>76</td>
<td>C</td>
<td>A</td>
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<tr>
<td></td>
<td>214</td>
<td>A</td>
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<td>C</td>
<td>T</td>
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<tr>
<td></td>
<td>829</td>
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<td>A</td>
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<tr>
<td></td>
<td>1181</td>
<td>C</td>
<td>T</td>
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<td></td>
<td>1220</td>
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<tr>
<td></td>
<td>1334</td>
<td>A</td>
<td>G</td>
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<tr>
<td><em>Grp</em></td>
<td>224</td>
<td>T</td>
<td>C</td>
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<tr>
<td></td>
<td>272</td>
<td>A</td>
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<td></td>
<td>351</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>759</td>
<td>C</td>
<td>G</td>
</tr>
</tbody>
</table>

* The positions of the SNPs are numbered according the GenBank references sequences NM_146087 (*Csnk1a1*) and NM_175012.2 (*Grp*);

# The positions of amino acid substitutions are numbered starting from the first amino acid of the GRP precursor peptide.
Figure 2.1. CAST/EiJ (CAST) mice and C57BL/6J (B6) mice differ in phase angle of entrainment. Wheel running data from one male B6 mouse (A) and one male CAST mouse (B) were plotted in percentile actogram format. The height of the vertical tick is proportional to the number of wheel revolutions in that interval, relative to the 24-h average. Data for 9 days in LD12:12 and 11 days in constant dark are shown. Time spent in darkness is indicated by gray shading. Individual values (circles for B6 and triangles for CAST) and group means (horizontal bars) are shown for activity onset derived from LD12:12 data (C) and phase angle of entrainment derived from DD data (D). Note: (1) Only one B6 mouse had a phase angle earlier than dark onset. This value is 3 SD from the group mean and was discarded as an outlier; (2) One CAST mouse died before release into DD, thus only 9 CAST values are plotted in panel D.
Figure 2.2. Early runner mice exhibit early daily awakening when tested on a piezoelectric system in the absence of a wheel. Data for 4 consecutive LD 12:12 cycles recorded from 4 (B6 x CAST) x CAST backcross mice are shown. Timing of light/dark is indicated both by gray shading and the white/black bar at the top of each panel. Dashed lines show the computer-defined onset for the major wake period within each LD cycle. Sleep/wake patterns and wake onset assessed by the piezoelectric system closely matched wheel-running patterns and onsets, as shown by these 4 examples: (A) A mouse with previous wheel running onset approximately 1 h before dark; (B) and (C) Mice with previous wheel running onsets 3 h prior to dark; (D) Mouse with wheel running onset 5 h before dark. In general, all mice examined had similar activity onsets whether assessed by wheel running, or, several weeks later, by piezoelectric defined sleep and wake.
Figure 2.3. Circadian period is shorter in CAST than in B6 mice and does not correlate with activity onset in LD12:12 or phase angle of entrainment within the CAST strain. (A) Individual values (circles for B6 and triangles for CAST) and group means (horizontal bars) are shown for circadian period in DD. Scatterplots of circadian period against activity onset derived from LD12:12 data (B) or phase angle of entrainment derived from DD data (C) show no correlation (P > 0.40) within the CAST strain.
Figure 2.4. Phase response and tau response curves differ between B6 and CAST mice. Phase and tau (i.e., circadian period) responses to green light were measured in B6 (A) and CAST (B) mice exposed to 30-min green light pulses at random circadian phases at 14-day intervals when animals were housed under DD. One light pulse for B6 and two light pulses for CAST are shown as gray dots. (C) A 2-harmonic Fourier fitted phase response curve was generated from n = 65 phase shifts measured in B6 mice (circles and solid line) and n = 59 phase shifts measured in CAST mice (triangles and dashed line). The fitted curves were re-plotted to the right for clarity. (D) Adjusting the definition of the circadian phase of light exposure for each CAST mouse according to its activity onset in LD alters the timing of the phase response curve in CAST mice (triangles). (E) The tau response curve demonstrates that light exposure does not influence circadian period in B6 (left) but has a slowing effect in CAST mice, as shown by the 2-harmonic Fourier fitted line (right).
Figure 2.5. Negative masking effects of light are strain and light dependent. (A) Seventy-two hours of wheel running data from B6 (top) and CAST (bottom) mice demonstrate the effects of green (left) and white (right) light exposure on wheel running activity. Animals were entrained under LD12:12 conditions using green light. The timing of the light pulse (ZT12.5-ZT13.5 on the third night shown) is illustrated by gray shading and carets at the base of each actogram. (B) The amount of wheel running during light pulse (LP) at ZT12.5-ZT13.5 and during the control dark condition is plotted individually for each B6 (upper panel) and CAST (lower panel) mouse. Baseline (BL) activity in the control dark condition is calculated as the mean wheel-running activity at the same Zeitgeber time (ZT) 2 days preceding the light pulse. P values from paired Student's t test are labeled at the top of each panel. n.s., not significant. (C) Group mean ± SEM values for wheel running during light exposure at ZT12.5-ZT13.5 are plotted as a percentage of baseline activity. *** P < 0.001, ** P < 0.01, B6 vs. CAST, Student's t test. # P < 0.05, green vs. white light within CAST mice, Student's t for paired measures.
Figure 2.6. Activity density before dark in CAST mice housed in LD12:12 is dependent on the type of light exposure. (A) Wheel-running data are shown from one male CAST mouse housed in white light (days 1-10) or green light (days 11-20). Mean ± SEM are plotted for mathematically defined and visually adjusted activity onsets (B) and activity intensity defined by number of wheel running revolutions during the interval between activity onset and dark onset (C) for CAST mice housed in white (white bars) and green light (grey bars). * P < 0.05 green vs. white light, Student's t for paired measures.
Figure 2.7. Congenic lines demonstrate an effect of the chromosome 18 genotype on wheel running phenotype. (A) Portions of chromosome 18 have been transferred from the CAST to the B6 background in congenic strains. The early runner phenotype was previously linked to a locus between marker D18Mit122 and D18Mit162 on chromosome 18 (Era1) by QTL analysis (upper panel reproduced from Wisor et al., 2007). Era1 is here aligned with the physical map of chromosome 18 and the genetic markers that have been genotyped as homozygous B6 (black in schematic
representations of chromosome 18) or homozygous CAST (white in schematic representation of chromosome 18) in B6.CAST.18P and B6.CAST.18M mice. Marker and genotype information were adopted from Davis et al., 2007 with modifications of the B6.CAST.18M genotypes according to my more detailed genotyping results. The current proximal flanking marker is D18Mit181 and the proximal internal marker is D18Mit51. Wheel running data from one male B6.CAST.18P mouse (B) and one male B6.CAST.18M mouse (C) were double-plotted in percentile actogram format over 20 days (10 days in LD12:12 using 83 lux green light and 10 days in constant dark). Time spent in darkness is indicated by gray shading. (D) Phase angle of entrainment is altered in both congenic strains. Group mean ± SEM values of phase angle of entrainment are derived from DD data. ANOVA, F = 28.69, P < 0.001. *** P < 0.001 vs. B6, Student’s t. (E) Free running period length is (surprisingly) longer in congenic mice relative to B6. Group mean ± SEM values are shown. ANOVA, F = 34.47, P < 0.001. ** P < 0.01; *** P < 0.001 vs. B6, Student’s t for unpaired measures. B6, B6.CAST.18P and B6.CAST.18M mice also differed significantly from CAST, P < 0.001, Student’s t for unpaired measures.
Figure 2.8. Sequence alignment of GRP precursor peptides of B6, CAST mice, rats, and three human isoforms. Peptide sequence alignment, conservation score, alignment quality score, and the structure of mouse GRP precursor peptide are shown. The amino acid variations between B6 and CAST mice are indicated by black arrows.
Recombinant and synthetic peptides (indicated by two red horizontal lines) of the human homologue of the mice proGRP\textsubscript{56-146} have been shown biologically functional on cellular proliferation via an unidentified receptor [(Patel et al., 2007), also reviewed in (Ischia et al., 2009)]. These two fragments are located in the regions of proGRP that are conserved among mammals. Note that Arg80Ser variation between B6 and CAST is located in one of these regions.
CHAPTER THREE

Distinct Phase Relationships between Suprachiasmatic Molecular Rhythms, Cerebral Cortex Molecular Rhythms and Behavioral Rhythms in Early Runner (CAST/EiJ) and Nocturnal (C57BL/6J) Mice

Introduction

The molecular machinery of the circadian clock consists of a series of interlocking feedback loops involving the transcription, translation and post-translational modifications of a small number of genes and proteins [reviewed in (Takahashi et al., 2008)]. The roles of discrete genetic loci in regulating circadian rhythms at the whole-organism level are demonstrated by the robust circadian phenotypes caused by point mutations (Lowrey et al., 2000) and gene knockout (van der Horst et al., 1999; Zheng et al., 2001) in animal models. Mutations with similarly robust effects are present but exceedingly rare in human populations (Jones et al., 1999; Toh et al., 2001; Xu et al., 2005). However, there is enormous variability in circadian phenotypes (also described as chronotypes) in human populations (Roenneberg et al., 2003), which are likely to be genetically complex. Studies of these phenotypes in animal models therefore serve as a useful complement to studies of single gene effects on circadian rhythms.

I described a genetically complex phenotype, the early runner phenotype, in hybrid offspring of the CAST/EiJ (CAST) and C57BL/6J (B6) strains of mice (Wisor et al., 2007). A (B6 X CAST) X CAST backcross population exhibited a wide range of variations in the onset of daily wheel running bouts in a light/dark 12:12 (LD12:12) cycle. Quantitative trait loci (QTL) analysis determined that a portion of mouse chromosome 18 conferred variability in phase angle of entrainment (Wisor et al., 2007). Transfer of the
QTL segment on chromosome 18 from the CAST background to the B6 background demonstrated conclusively that this chromosomal region confers circadian phenotypic variability (Jiang et al., 2011).

Circadian clock properties measured at the behavioral and molecular levels may provide information about the neurobiological basis of the phenotype. Variability in circadian period may contribute to variability in the phase angle of entrainment (Daan & Pittendrigh, 1976), but it did not do so in the (B6 X CAST) X CAST backcross population (Wisor et al., 2007) or within the CAST inbred line (Jiang et al., 2011). Variability in the shape of the photic phase response curve may also cause variability in the phase angle of entrainment (Pittendrigh & Daan, 1976a). The photic phase response curves of CAST and B6 mice were nearly identical in shape, but the strains differed in the timing of advancing and delaying responses to light relative to the behaviorally defined (i.e., wheel running) circadian phase (Jiang et al., 2011). This observation raised the possibility that the phase relationship of behavioral circadian rhythms to the SCN circadian clock differs between CAST and B6 mice. To address this possibility, I now describe measures of behavioral rhythmicity [piezoelectrically-defined sleep/wake cycles (Flores et al., 2007; Donohue et al., 2008)] and molecular rhythmicity in the SCN clock (Per1 and Per2 expression) in CAST and B6 mice. I show that while sleep/wake cycles are advanced even more robustly than wheel running rhythms in CAST mice relative to B6 mice, the SCN molecular clocks of the two strains are in phase. Daily rhythms of Per1 and Per2 expression in cerebral cortex are in synchrony with those of behavior and not the SCN clock. The early runner phenotype is therefore associated with differences downstream of, rather than within, the SCN clock.
Materials and Methods

Animals

Male mice of the CAST (strain # 000928) and B6 (strain # 000664) strains were purchased at 6 weeks of age from the Jackson Laboratory (Bar Harbor, ME). Distinct cohorts were used for piezoelectric measurement of sleep and wake and in situ hybridization studies. Sleep and wake recording also involved two strains of B6.CAST congenic mice (Iakoubova et al., 2001; Davis et al., 2007). These two strains carry CAST segments of proximal and middle portions of chromosome 18 introgressed on an otherwise 99% B6 background (B6.CAST.18P and B6.CAST.18M strains, respectively). Female and male B6.CAST congenic mice were obtained from the University of California, Los Angeles, bred at the University of Kentucky, and then transferred to Washington State University. Unless otherwise noted, animals were housed in individual cages under LD12:12 cycles of 80 lux white light and total darkness in light-tight chambers at a temperature set point of 24.5°C, with food and water provided ad libitum. All animal procedures were performed by Dr. Jonathan Wisor at Washington State University and were approved by the Institutional Animal Care and Use Committee and were consistent with the Institute of Laboratory Animal Resources Guide for Care and Use of Laboratory Animals.

Piezoelectrically-based sleep/wake assessment

Piezoelectrically-defined sleep was measured in CAST, B6 and B6.CAST congenic strains of mice using techniques described in detail elsewhere (Flores et al., 2007; Donohue et al., 2008; Wisor et al., 2009; Jiang et al., 2011). Mice were transferred from their wheel-running cages to the piezoelectric data collection environment for a two-day data collection session. Piezoelectric potential generated by
pressure on or distention of the cage floor was monitored continuously at 100 Hz throughout the duration of the multi-day recording sessions. Muscle activity was inferred based on piezoelectric waveforms. Custom software described elsewhere (Flores et al., 2007; Donohue et al., 2008) was used to discriminate between states of sleep (detection of respiratory muscle activity only) and wakefulness (detection of skeletal muscle activity). The piezoelectric device exhibits more than 90% sensitivity relative to electroencephalographically-defined sleep (Flores et al., 2007) but does not discriminate between non-rapid eye movement sleep and rapid eye movement sleep. Data were collected and processed algorithmically in 2-sec intervals. For state classification, data were collapsed into 10-sec epochs. Each epoch was classified as sleep if all 5 of the 2-sec sub-epochs were algorithmically scored as sleep, or as wake if one or more of the 5 2-sec sub-epochs was algorithmically scored as wake.

Measurement of daily cycles of Per1 and Per2 expression in the cerebral cortex and SCN

Mice of the B6 and CAST strains were housed in a 12-h light/12-h dark (LD12:12; lights-on at 4:00 and lights-off at 16:00, local time) cycle for at least ten days. As to avoid the effect of light-induced Per1 and Per2 expression interfering with their endogenous circadian oscillation, these mice were then kept in darkness beginning at 16:00 for 15, 18, 21, 24, 27, or 33 h (i.e., until local time 7:00, 10:00, 13:00, 16:00, 19:00 the next day, and 1:00 the day after). These times correspond to ZT3, ZT6, ZT9, ZT12, ZT15 and ZT21 in an LD12:12 cycle. Mice were euthanized by cervical dislocation and were kept in dim red light (<20 lux) until the optic nerves were severed. Immediately after dissection, brains were frozen on crushed dry ice and stored at -80°C. Frozen brains were shipped to the University of Kentucky, where coronal sections throughout the hypothalamus (14 microns thick) were prepared using a cryostat. The brain sections
were mounted on negatively-charged slides and stored at -80°C until in situ hybridization was conducted for Per1 and mPer2, as described below.

Measurement of circadian phase-dependence of light-induced Per1 and Per2 expression in the SCN by quantitative in situ hybridization histochemistry

Both CAST and B6 mice were housed in an LD12:12 cycle (lights-on at 4:00 and lights-off at 16:00, local time) for at least ten days, then kept in darkness beginning at 16:00 for 18, 21, 24 or 27 h (i.e., until local time 10:00, 13:00, 16:00 and 19:00 the next day). These times correspond to ZT6, ZT9, ZT12 and ZT15 in an LD12:12 cycle. Mice were then exposed to 80 lux white light. In preparation for this white light exposure, mice were moved in their cages to a different light-tight chamber in the same room but were not directly handled. Mice were euthanized by cervical dislocation at the end of the 30-min light exposure and were kept in dim red light (<20 lux) until the optic nerves were severed. Mice euthanized in constant darkness at the analogous time of day in the experiment described above served as control subjects. The brains were dissected at Washington State University and then prepared for in situ hybridization at the University of Kentucky, as described above.

In situ hybridization histochemistry

Two antisense DNA oligos for Per1 mRNA (5-TGG GTG GGG ATG GGCTCT GTG AGT TTG TAC TCT TGC TGC-3 and 5-TCG GGG TTT GGG GAC TGG CAG GGA AGG ACT TTG GCT TTG AA-3, corresponding to GenBank® ID NM_001159367.1 at 475-437 nt and 1958-1918 nt respectively, also to GenBank® ID NM_011065.4 at 490-452 nt and 1973-1933 nt, respectively) and for Per2 mRNA (5-GGT CCT TAT CAG TTC TTC GTG TGC GTC AGC TTT GGC-3 and 5-GCC TTT CTC CTC ACT CTC GCA GTA AAC ACA GCC TG-3, corresponding to 510-475 nt and 3831-3797 nt of GenBank® ID
NM_011066.3) were purchased from IDT® (Coralville, IA). Oligonucleotides were labeled by the addition of a 3' poly-\(^{33}\)P-dATP tail using terminal transferase (NEB®, Beverly, MA). The labeled oligo probes were then separated from unincorporated nucleotide through mini Quick-Spin oligo purification columns (Roche®, Indianapolis, IN).

Four brain sections encompassing the SCN from each animal were used in the \textit{in situ} hybridization experiment. Slide-mounted brain sections received a sequence of pre-hybridization treatments, including fixation in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) solution, several washes in 1X PBS and 1X PBS/27 mM glycine, acetylation using 0.25% acetic anhydride in 0.1 M triethanolamine solution (pH 8.0), and dehydration/delipidation using ethanol solutions and chloroform. Slides were then air dried and sections were hybridized to \(^{33}\)P-labeled oligo probes (> 1 x 10\(^6\) CPM per slide) diluted in a hybridization cocktail [final concentration of 37.5% formamide (deionized), 7.5% dextran sulfate, 0.75X Denhardt, 15 mM Tris-HCL (pH 7.4), 225 mM NaCl, 0.75 mM EDTA, 75 mM DTT, 250 µg/ml salmon sperm DNA, and 25 µg/ml yeast RNA] and covered by coverslips. After an overnight incubation at 37°C, slides were rinsed in 2X SSC/10 mM DTT/0.25% Na\(_2\)S\(_2\)O\(_3\) at room temperature to remove coverslips. Sections were then subjected to a series of post-hybridization washes, which consisted of one wash in 2X SSC/10 mM DTT/0.25% Na\(_2\)S\(_2\)O\(_3\) solution at room temperature, four washes in 1X SSC/10 mM Na\(_2\)S\(_2\)O\(_3\) at 55°C, and two additional washes in the same solution at room temperature. Slides were then quickly dipped in sterile water and then 95% ethanol, air dried, and placed against autoradiography films (Biomax MR™, Kodak, Rochester, NY) together with a set of \(^{14}\)C radioactive standards (Amersham Life Sciences, Piscataway, NJ) in X-ray cassettes. After exposure times ranging from 11 days to 23 days, the experimental films were developed.
Image analysis

The hybridization signal in each region of interest was determined using an MCID image analysis system (Imaging Research Inc., St. Catherines, Ontario, Canada). A standard curve was established using the optical densities of the $^{14}$C standards and curve fitting (3rd-degree-polynomial). The hybridization signal of each sample was determined from the standard curve. For the SCN, the hybridization signal for each mRNA species was first measured bilaterally within closed outlines around the entire SCN. Additionally, expression of Per1 and Per2 was also measured in SCN subregions by placing sampling tools (small circles) at the dorsomedial tip and the ventrolateral tip of the SCN. The exact same circle was used on all sections from all animals. For the cerebral cortex, Per1 and Per2 expression were measured bilaterally within a closed outline drawn around the dorsal cortex from the cingulate cortex to the dorsal boundary of the piriform cortex (thus containing cingulate/retrosplenial, motor, somatosensory, and insular cortices). Expression of Per1 and Per2 in the subparaventricular zone (sPVZ) was measured using a narrow rectangle box (of the same shape and size for all samples) placed dorsal to the SCN and ventral to the paraventricular nucleus (PVN) of the hypothalamus. Also, Per1 and Per2 expression was measured bilaterally in PVN using an outline drawn around each nucleus. Finally, the level of background signal was also measured from four areas with the lowest signals in each brain section, and its average value was subtracted from the hybridization signals of the corresponding regions of interest. Background-subtracted hybridization signals for each region of interest were averaged across all four sections used for each animal, and then subject to statistical analysis.
Statistical analysis

Statistical analyses were performed with Statistica 9.0. To measure strain-dependence of daily oscillations in sleep/wake timing, data were subjected to factorial analysis of variance (ANOVA) with strain (CAST vs. B6 vs. B6.CAST.18M vs. B6.CAST.18P) and time of day in 4-h bins (starting at 4:00 vs. 8:00 vs. 12:00 vs. 16:00 vs. 20:00 vs. 0:00, local time) as independent variables. In those cases when ANOVA indicated significant time of day X strain interaction, the data were subjected to post hoc contrasts across genotype groups within each time of day. To measure strain-dependence of daily oscillations in mRNA levels, data were subjected to factorial ANOVA with strain (CAST vs. B6) and time of day (7:00 vs. 10:00 vs. 13:00 vs. 16:00 vs. 19:00 vs. 1:00, local time) as independent variables. In those cases when ANOVA indicated significant time of day X strain interaction, the data were subjected to post hoc contrasts across genotype groups within each time of day. To measure effects of light exposure on SCN gene expression, dependent variables were subjected to factorial ANOVA with strain (CAST vs. B6), ambient condition in the 30-min interval before euthanasia (light vs. dark) and time of day (10:00 vs. 13:00 vs. 16:00 vs. 19:00, local time) as independent variables. In those cases when ANOVA indicated significant time of day X light exposure interaction, the data were subjected to post hoc contrasts of the light-exposed and dark control groups within each genotype/time of day condition. Post hoc contrasts were performed with Bonferroni corrected Student’s t. In all cases, results are presented as mean ± SEM.
Results

Piezoelectrically-defined sleep/wake assessment reveals strain differences in the phase angle of entrainment and total daily sleep time.

CAST and B6 mice exhibited sleep/wake cycles identified with piezoelectric recording (Table 3.1; Figure 3.1). There were significant main effects of time of day on sleep as a percentage of time whether the data were subjected to ANOVA in 1-h bins ($F_{23,1012}=26.01$, $P<0.001$) or 4-h bins aligned to the light/dark transitions ($F_{5,220}=58.73$, $P<0.001$). Subsequent analyses were performed on data in 4-h bins. Both CAST and B6 mice exhibited more time asleep during the light phase of the LD12:12 cycle than the dark phase (Table 3.1). ANOVA yielded a significant main effect of strain on sleep as a percentage of time ($F_{3,44}=7.14$, $P<0.001$). Amount of time spent asleep across the entire 24-h cycle was lower by one-fifth in CAST mice (33% of the time recorded) than in B6 mice (41% of the time recorded). The difference in time spent asleep by the two parental strains was due to CAST mice spending significantly less time asleep than B6 mice during both the light and dark phases of the cycle (Table 3.1). ANOVA also yielded a significant strain X time of day interaction for percent of time spent asleep in 4-h bins ($F_{15,220}=4.46$, $P<0.001$). This interaction was due to the fact that CAST and B6 mice initiated the major daily sleep and wake episodes at distinct phases of the LD12:12 cycle: Time spent asleep peaked during the first four hours of the light phase (i.e., 4:00-8:00, local time) in CAST mice and later (at 8:00-12:00) in B6 mice (Figure 3.1). CAST mice spent less time asleep than B6 mice in the last four hours of the light phase (i.e., 12:00-16:00; Figure 3.1), as the CAST mice initiate daily activity much earlier than B6 mice. The difference in the timing of sleep among strains was highly significant when it was measured as the ratio of sleep during the last four hours of the light phase relative to the first four hours of the light phase (Figure 3.1A insert; $F_{3,44}=7.01$, $P<0.001$). This
measure differed significantly between CAST (0.49±0.32) and B6 (1.96±0.28) mice (P = 0.006, Student’s t). Thus, the daily rhythm of sleep and wake was advanced in CAST mice relative to B6 mice under entrained conditions.

Piezoelectric sleep data were also collected from two congenic lines carrying portions of CAST chromosome 18 on an otherwise pure B6 background. The two strains exhibited a phenotype intermediate of the parental strains. Like the CAST strain, both B6.CAST.18P and B6.CAST.18M mice spent significantly less time asleep than B6 mice during the first four hours of the dark phase of the LD12:12 cycle (Figure 3.1A). Additionally, the amount of time spent asleep during the first four hours of the light phase was significantly greater in B6.CAST.18P mice than B6 mice (Figure 3.1A). However, percent of time spent asleep peaked later in B6, B6CE.18P and B6.CAST.18M mice than in CAST mice.

Per1 mRNA rhythms in extra-SCN brain regions but not in the SCN are affected by strain.

Expression of Per1 mRNA in the cerebral cortex was significantly affected by strain and time of day (Table 3.2A); there was also a significant strain X time interaction (Table 3.2A). Both the CAST and B6 strains exhibited circadian variations in the expression of Per1, which appeared to parallel the rhythms of wakefulness (Figure 3.2A-E). The peak of Per1 expression was advanced in CAST mice by three hours relative to B6 mice. In CAST mice, Per1 expression in the cerebral cortex, like wakefulness, increased dramatically from 10:00 to 13:00 (local time; Figure 3.2E). In B6 mice, Per1 expression in the cerebral cortex did not increase from its daytime low until 16:00. As a result of the discrepancy in the timing of Per1 mRNA elevation from the daily minimum, Per1 expression was significantly higher in CAST mice relative to B6 mice at 13:00. In
addition, the daily trough of Per1mRNA level also occurred at an earlier time of day in CAST mice (1:00) than in B6 mice (10:00). Therefore, the difference in the circadian timing of behavioral [i.e., wheel running (Jiang et al., 2011) and sleep] measures between CAST and B6 mice is accompanied by a difference in the circadian timing of Per1 expression in the cerebral cortex.

To further map the basis of the strain difference in circadian rhythmicity, I measured Per1 expression in the PVN (Figure 3.2F-J) and sPVZ (Figure 3.2K-O) of the hypothalamus. In the sPVZ, there were no significant main effects of time or strain on Per1 expression nor was there a significant interaction (all P > 0.07). In contrast to the sPVZ, Per1 expression in the PVN was significantly modulated by strain (Table 3.2A). Per1 expression was higher in CAST mice than B6 mice, by an average of 25% across all times (Figure 3.2J). Per1 expression in PVN was also modulated by time (Table 3.2A). Per1 mRNA levels peaked at 13:00 in CAST mice and 16:00 in B6 mice, although ANOVA did not yield a significant strain X time of day interaction (F_{5,36}=1.18, P=0.339).

The intensity of the Per1 mRNA signal in the SCN varied with time of day (Table 3.2A, Figure 3.3A-M). There was a trend toward an effect of strain and strain X time interaction (Table 3.2A). Circadian rhythms of Per1 expression were apparent in the SCN of both strains. Per1 mRNA was maximal at 10:00 and lowest at 19:00, irrespective of strain. The trend toward lower expression level in the SCN of CAST mice relative to B6 mice at 7:00 and 10:00 was not associated with any change in the timing of minimal or maximal Per1 expression.

To determine whether distinct subregions of the SCN exhibit distinct daily profiles of Per1 expression, Per1 hybridization signal was measured in the dorsomedial SCN
and the ventrolateral SCN, noted as dSCN and vSCN. In the vSCN, there were significant effects of strain, time, and strain X time interaction (Table 3.2A) on *Per1* expression (Figure 3.3, panel O). *Per1* expression was apparently lower in the vSCN of CAST mice relative to B6 mice during 7:00-10:00, a time interval analogous to early and middle of the light period of the prior LD 12:12 cycles. While peak expression in vSCN of CAST was lower than peak expression in B6, the peak was nonetheless at the same time of day (i.e., 10:00) in the two strains. In dSCN, there was a significant main effect for time of day only (Table 3.2A) and no strain effect nor strain X time interaction (P > 0.19). The phase of the rhythm was aligned across strains and peaked at a time corresponding to in the mid-portion of the previous light phase, as it did in the SCN as a whole and in vSCN (Figure 3.3M-O). These data demonstrate clearly that the SCN circadian clock of CAST mice is in phase with that of B6 mice. The early runner phenotype is apparent in the cerebral cortex but not in the SCN itself.

*Per2* mRNA rhythms are affected by strain in both SCN and extra-SCN brain regions.

*Per2* mRNA level in the cerebral cortex (Figure 3.4A-E) was significantly affected by strain, time, and strain X time interaction (Table 3.2A). *Per2* expression was higher in the cerebral cortex of CAST mice relative to B6 mice by an average of 45% across all times and was significantly higher in the cerebral cortex of CAST mice relative to B6 mice at 16:00. This difference was due to a difference between the strains in the timing of peak *Per2* expression in the cerebral cortex (Figure 3.4A-E): *Per2* expression peaked at 16:00 in CAST mice and 19:00 in B6 mice.

*Per2* expression exhibited circadian rhythmicity in the sPVZ (Table 3.2A, Figure 3.4K-O) and PVN (Table 3.2A, Figure 3.4F-J) of the hypothalamus. There was no significant main effect of strain on *Per2* expression in either the sPVZ or PVN (both P >
Per2 expression peaked at 13:00 in the sPVZ of both strains. Although peak Per2 expression appeared to occur at different times in the PVN of CAST mice (16:00) and B6 mice (19:00), there was not a statistically significant strain X time interaction in ANOVA ($F_{5,36}=1.79$, $P=0.139$).

Per2 expression in the SCN was significantly affected by time (Table 3.2A) but not strain ($P=0.899$). In both strains, daily peak Per2 expression in the SCN occurred at 13:00 and daily minimal Per2 expression occurred at 1:00 (Figure 3.5 A-M). There was, however, a significant strain X circadian phase interaction (Table 3.2A), which was manifest as a more precipitous decline in Per2 expression in CAST mice relative to B6 mice after peak expression: Per2 hybridization signal was significantly lower in the CAST SCN than the B6 SCN at 16:00. Both the dSCN (Table 3.2A, Figure 3.5N) and vSCN (Table 3.2A, Figure 3.5M) exhibited circadian rhythms of Per2 expression, and non-significant strain effects ($P > 0.500$). The vSCN (but not the dSCN) also exhibited a significant strain X time of day interaction (Table 3.2A). However, the strain difference in Per2 expression at 16:00 was not significant (Figure 3.5, panel O).

Light-induced Per1 expression in the SCN exhibits identical phase dependence patterns in CAST and B6 mice.

Per1 expression in the SCN was compared between mice euthanized at the end of 30-min light exposure starting at 10:00, 13:00, 16:00, or 19:00 (corresponding to ZT 6, 9, 12, or 15 of the prior LD cycles) and mice killed in darkness at these times. ANOVA indicated significant main effects of light, time of day, and light X time interaction (Table 3.2B). Light exposure resulted in upregulation of Per1 expression in the SCN in a time-dependent manner (Figure 3.6A-E, H-L): Per1 transcript level was significantly elevated in the SCN of light-exposed CAST mice relative to time of day controls at 16:00 and
19:00, and in the SCN of light-exposed B6 mice relative to time of day controls at 19:00. Time-dependent induction of *Per1* in the SCN by light was not significantly modulated by strain (strain X time X light interaction: F$_{3,40}$=0.79, P=0.769). In the dSCN, there was a significant effect of light (Figure 3.6F, M; Table 3.2B), which was not time-dependent (i.e., time X light interaction, F$_{3,40}$=2.21, P=0.102). In the vSCN, by contrast, there was a time-dependent effect of light on *Per1* expression (Table 3.2B, Figure 3.6G, N); no significant strain X time X light interaction was found for either subregion of the SCN (P>0.700). In both strains, *Per1* mRNA level was significantly elevated in the SCN of light-exposed mice relative to mice kept in dark at 16:00 and 19:00. These data demonstrate phase-dependence of light-induced *Per1* expression in the ventral portion of the SCN but fail to demonstrate any effect of strain background on this circadian clock property.

*Light-induced Per2 expression in the SCN exhibits strain-specific phase dependence patterns in CAST and B6 mice.*

SCN *Per2* expression was also affected by light. ANOVA yielded significant light X time interaction for *Per2* expression in the SCN, dSCN, and vSCN (Figure 3.7, Table 3.2B). The phase-dependence of light-induced *Per2* expression was further modulated by strain in vSCN (F$_{3,40}$=3.32, P=0.029), though not in SCN or dSCN (both P > 0.200). Because light-induced *Per2* expression is an indicator of clock responsiveness to light at the molecular level, strain background effects on vSCN clock responses to light may contribute to the circadian phenotype.
Discussion

CAST mice differ from B6 in a number of circadian phenotypes, including an advanced wheel-running activity rhythm, a slightly shorter endogenous period, altered timing of light-induced phase-shifting responses, and blunted light-masking (Wisor et al., 2007; Jiang et al., 2011). Comparisons of the circadian clock properties of these strains and genetic analyses of the circadian phenotypes of the intercross offspring of the strains may offer insights into the neurobiological basis for the well-known differences in circadian chronotype across human populations. I report here that additional measures of circadian rhythmicity differ dramatically between the CAST and B6 strains. The major daily waking bout occurs approximately 3-6 h earlier in CAST mice than in B6 mice in entrained (LD12:12) conditions. This behavioral difference is accompanied by differences in the expression of circadian clock-genes: Per1 and Per2 mRNA levels are elevated in the cerebral cortex of CAST mice relative to B6 mice in the hours before dark onset, when CAST mice spend the majority of time awake and B6 mice spend the majority of time asleep. The very robust strain differences in circadian behavior and the patterns of cortical Per expression contrast sharply with the relative lack of strain differences in Per1 and Per2 expression in the SCN, in which circadian rhythms of endogenous expression and photic induction were largely similar between CAST and B6 mice. Although a trend toward lower Per1 expression during the first several hours of the day (7:00-10:00) and a more steep decline of Per2 mRNA levels at 16:00 were apparent in the SCN of CAST mice, the timing of peak SCN Per1 and Per2 expression (that presumably helps determine clock time) was not different between strains. Thus, these fundamental properties of the SCN circadian clock are only affected by strain to a small extent, and are unlikely to underlie the differences between B6 and CAST in the timing of activity/rest and sleep/wake. Inasmuch as Per1 and Per2 mRNAs serve as
markers for SCN circadian clock function, the SCN clock itself appears to be quite similar, if not identical, between the two strains. Therefore, the early runner phenotype that have been described in CAST mice, and in many offspring from CAST x B6, is not a phenotype of the SCN, but rather appears to reflect differences in circadian mechanisms downstream of, rather than within, the master circadian pacemaker.

The upregulation of SCN Per1 and Per2 mRNA levels as a consequence of light exposure is a circadian phase-dependent phenomenon [as shown here and elsewhere; (Shearman et al., 1997)]. In this sense, Per1 and Per2 expression is regulated in a manner akin to that of immediate early genes: the circadian clock gates the transcriptional response to light exposure within the SCN. And indeed, light-induced Per1 and Per2 expression is gated by circadian phase in a manner that is phenomenologically largely similar to light-induced immediate-early gene c-fos expression (Kornhauser et al., 1996). Light-induced expression of Per genes (Shearman et al., 1997) and c-fos (Kornhauser et al., 1996) is restricted to the circadian phase at which light triggers circadian phase shifts. Furthermore, the mechanisms by which light-activates Per gene expression in the SCN, including transcriptional activation by calcium/cyclic AMP regulatory element-binding protein [CREB; (Gau et al., 2002; Travnickova-Bendova et al., 2002; Tischkau et al., 2003)], are largely similar to the mechanism by which light-induces c-fos expression (Gau et al., 2002). In addition, SCN subregion-specific effects of light and other phase-shifting signals on Per1 and Per2 mRNA levels have been documented (Yan & Silver, 2002; Duncan et al., 2005). In the current study, I also observed subtle differences between B6 and CAST in the times at which the effect of light on Per expression reached statistical significance, despite a general trend of increase at times (16:00 and 19:00) analogous to the dark phase of prior LD cycles. The lack of statistical significance at a certain time-point may reflect a
statistical overcorrection of multiple-testing by Bonferroni correction; or, it may be due to the fact that light-exposed mice were euthanized 30 min later than mice kept in dark and thus there might be a reduction in the magnitude of light-induced increases in Per expression at 16:00 and 19:00 for both strains in the SCN and its subregions. Also, in contrast to previous studies which reported the effects of light exposure in reference to time of activity onset, this current study used local time. Nonetheless, the essentially similar pattern of light-induced Per gene expression in the CAST and B6 mice indicates that the clock resetting mechanisms, including light-input pathways and circadian gating, do not differ between CAST and B6 mice, and therefore cannot explain the circadian behavioral difference between the two strains.

Since the SCN clock oscillates at an equivalent phase and gates light-responsive gene expression in a similar manner in CAST and B6 mice, the mechanism responsible for the advanced phase of behavioral rhythms in CAST mice must be mediated by neural circuits downstream of the SCN in circadian regulatory pathways. As a first step in identifying the relevant loci, I measured rhythmic expression of Per1 and Per2 in other brain regions, e.g., the sPVZ, PVN, and cerebral cortex. The sPVZ, similar to the SCN, did not exhibit strain differences in the 24-h profiles of Per gene expression. Perhaps this is not surprising in view of the fact that the sPVZ receives heavy SCN input and innervates essentially the same areas as the SCN [reviewed in (Saper et al., 2005a; Saper et al., 2005b)]. In contrast to the sPVZ and SCN, the cortex exhibited strain differences in the 24-h profiles of Per gene expression. The CAST mice showed earlier peaks of Per1 and Per2 expression in the cortex. The daily highest levels of Per1 and Per2 mRNA in the PVN also appeared to occur at earlier times in the CAST mice than B6, although strain X time of day interaction was not significant as determined by ANOVA. It is interesting to note that the PVN, similar to the sPVZ, receives direct
projections from the SCN (Buijs et al., 1993; Vrang et al., 1995a; Vrang et al., 1995b), in contrast to the cortex, which has not been reported to receive direct SCN input. The PVN is also thought to receive indirect input from the SCN via projections from the sPVZ and dorsomedial nucleus of the hypothalamus. The PVN is part of the neural pathways regulating endocrine rhythms. For example, the PVN is participates in the regulation of the circadian secretion of corticosterone, via its CRH (i.e. corticotropin-releasing hormone)-containing neurons [reviewed in (Buijs et al., 2003)], and/or via an autonomic pathway, SCN-PVN-IML (i.e., intermediolateral column of the spinal cord)-adrenal gland, which regulates circadian changes in the sensitivities to adrenocorticotropic hormone [ACTH; (Ulrich-Lai et al., 2006), also reviewed in (Dickmeis, 2009)]. The PVN also regulates circadian melatonin secretion by relaying the SCN projections to the IML, which in turn projects to the pineal gland via the superior cervical ganglion [(Teclemariam-Mesbah et al., 1999), also reviewed in (Buijs et al., 2003)]. Thus, it will be interesting to examine in future studies whether the rhythmic secretion of these hormones is also phase-advanced in concert with behavioral rhythms in CAST mice.

The phase-advanced rhythms of Per1 and Per2 mRNA levels in the cerebral cortex of CAST mice relative to B6 mice are not necessarily a measure of circadian oscillator function within the cerebral cortex, but may be driven more by sleep and wake. Per gene expression in the cerebral cortex is regulated by sleep/wake cycles and is elevated during protracted wakefulness (Wisor et al., 2002; Cirelli et al., 2004; Franken et al., 2007; Liu et al., 2008; Wisor et al., 2008). This observation is compatible with a role for these genes in the homeostatic regulation of sleep, as are the many homeostatic sleep alterations in mice with deficient or altered clock gene expression (Wisor et al., 2002; O’Hara et al., 2007), although there is some debate about the homeostatic deficits in Per1,2 double knockout mice (Shiromani et al., 2004; O’Hara et al., 2007).
Regardless, the sleep/wake dependence of *Per1* and *Per2* expression in the cerebral cortex, similar to their induction in the SCN by light (see below), is likely to reflect their regulation as immediate early genes by the transcriptional activator CREB (Travnickova-Bendova et al., 2002; Doi et al., 2007). The induction of CREB-regulated immediate early genes (e.g. *c-fos*, *NGFI-A*, and *NGFI-B*) in the cerebral cortex is a reliable consequence of wakefulness (O'Hara et al., 1993; Pompeiano et al., 1994; Cirelli et al., 1996). The difference in the phase of *Per1* and *Per2* mRNA oscillations in the cerebral cortex of CAST and B6 mice is, therefore, unlikely to result from differences in molecular clock mechanisms in the cerebral cortex *per se*. Rather, it is driven by the sleep/wake cycle, the timing of which differs between the strains due to as yet undetermined differences in circadian output pathways downstream of the SCN.

Recent work suggests that circadian output of the SCN to peripheral tissues can be conveyed by changes in core body temperature (Buhr et al., 2010). Given the differences that exist between the CAST and B6 strains in metabolic parameters, including sensitivity to dietary obesity, body fat content, and many others (York et al., 1996; Mehrabian et al., 1998; Cervino et al., 2005), significant differences between the two strains in thermoregulatory properties, and thermoregulatory responses to SCN-driven rhythms, are possible as well.

Small differences between CAST and B6 in the rhythms of *Per1* and *Per2* mRNA expression in SCN subregions were observed in the current study. Although most of these differences were not coordinated with the strain differences in the timing of activity/rest and sleep/wake cycles, the vSCN and not the dSCN showed a strain X time of day difference in *Per2* expression such that the temporal pattern of *Per2* expression in the vSCN appears altered in the CAST mice (Figure 3.5, panel O). SCN subregions express distinct sets of neuropeptides. Neurons containing gastrin-releasing peptide
(GRP) and/or vasoactive intestinal peptide (VIP) are concentrated in the ventrolateral portion of the SCN, while arginine vasopressin (AVP)–positive cell bodies are predominantly found in the dorsomedial portion [reviewed in (Morin, 2007; Welsh et al., 2010)]. Although the current study is limited by lack of neurochemical references, the anatomically defined vSCN is likely to differ from dSCN due to a higher number of GRP- and VIP-positive neurons. This distinction may be relevant to the phenotypic difference between CAST and B6 mice, as the Grp gene is within the genomic region of the Chromosome 18 QTL that regulates the early runner phenotype (Wisor et al., 2007; Jiang et al., 2011). The fact that Per2 levels exhibited time of day X strain interaction in the vSCN and not dSCN raises the possibility that the early runner phenotype is driven by GRP-positive cells. Further support for this possibility derives from the fact that GRP regulates Per2 expression and the phase of oscillations in electrical activity within the mouse SCN (Gamble et al., 2007). Collectively, these data call for additional studies on the possible roles of GRP, and the cells that produce GRP within the ventrolateral subregion of the SCN in regulating the phase angle of entrainment.

Temporal profiles of Per expression in various brain regions have been compared between nocturnal and diurnal rodents. Generally, the circadian oscillations of Per1 and Per2 mRNA and/or protein levels in the SCN and sPVZ are very similar in diurnal and nocturnal grassrats (Ramanathan et al., 2006; Ramanathan et al., 2010). In contrast, rhythmic expression of Per mRNAs in the cerebral cortex of diurnal ground squirrels peaks during the light-phase, anti-phase to that in nocturnal hamsters (Mrosovsky et al., 2001). The differences between CAST and B6 in the expression of Per1 and Per2 in discrete brain regions are similar to those between these nocturnal and diurnal animals. It is thus possible that the neural pathways used by other species in determination of temporal niche are similar if not identical to those utilized in CAST mice.
to produce an advanced behavioral rhythms. A recent study found that long-term (5 weeks) exposure to "day-working" schedule in nocturnal laboratory rats leads to advanced timing of activity and food intake, which is accompanied by a hypothalamic desynchrony: rhythms of C-FOS and PER1 protein levels in the arcuate nucleus (ARC) and dorsomedial nucleus of the hypothalamus (DMH) were shifted due to day-working, while those rhythms in the SCN and interestingly PVN were unchanged (Salgado-Delgado et al., 2010). Despite the possible species difference, I hypothesize that the discrepancy between that study and the current study in the coupling of PVN gene expression and activity rhythms may reflect the difference between "endogenously driven" early timing (in CAST mice) and forced early timing for activity (in day-working rats).

Regardless of the mechanism that underlies the strain differences I describe here, my observations demonstrate that a circadian abnormality identified at the behavioral level in LD cycle-entrained mice is not necessarily indicative of an abnormality in the function of the SCN circadian clock or its photic inputs. The CAST B6 genetic model can be utilized at both the genetic and molecular/biochemical levels to identify circadian mechanisms downstream of the SCN oscillator that regulate behavioral rhythms. Such downstream mechanisms are poorly understood at present. The identification of the mechanism by which the cycling of a nocturnally-entrained oscillator is transduced into an early onset of sleep/wake and wheel running rhythms in CAST mice may also reveal novel targets for interventions to improve circadian alignment in shift-workers, jet lag, and advanced or delayed sleep phase syndromes.
Table 3.1. Piezoelectrically-defined sleep states (percent time spent asleep) in B6, CAST, and congenic B6.CAST genome-tagged strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Interval</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td>Dark</td>
<td>Light</td>
<td>24-h</td>
</tr>
<tr>
<td>B6</td>
<td>29±2</td>
<td>53±2</td>
<td>41±1</td>
</tr>
<tr>
<td>CAST</td>
<td>21±3*</td>
<td>45±7*</td>
<td>33±4*</td>
</tr>
<tr>
<td>B6.CAST.18M</td>
<td>33±2</td>
<td>57±1*</td>
<td>45±1*</td>
</tr>
<tr>
<td>B6.CAST.18P</td>
<td>27±2</td>
<td>57±1</td>
<td>42±1</td>
</tr>
</tbody>
</table>

* P< 0.05 vs. B6, same condition.
Table 3.2. Summary of ANOVA results of *Per1* and *Per2* expression experiments.

A. Constant Dark Experiment

<table>
<thead>
<tr>
<th>Structure</th>
<th><em>Per1</em></th>
<th></th>
<th></th>
<th></th>
<th><em>Per2</em></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Strain</td>
<td>Time of day</td>
<td>Interaction</td>
<td></td>
<td>Strain</td>
<td>Time of day</td>
<td>Interaction</td>
<td></td>
</tr>
<tr>
<td>SCN (Total)</td>
<td>F&lt;sub&gt;1,36&lt;/sub&gt;=3.96, P=0.054</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=75.67, P&lt;0.001</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=2.16, P=0.081</td>
<td>NS</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=89.94, P&lt;0.001</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=5.11, P=0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dSCN</td>
<td>NS</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=30.85, P&lt;0.001</td>
<td>NS</td>
<td>NS</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=74.78, P&lt;0.001</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vSCN</td>
<td>F&lt;sub&gt;1,36&lt;/sub&gt;=7.27, P=0.011</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=79.30, P&lt;0.001</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=2.62, P=0.040</td>
<td>NS</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=87.47, P&lt;0.001</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=2.89, P=0.027</td>
<td></td>
<td></td>
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<tr>
<td>sPVZ</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=20.10, P&lt;0.001</td>
<td>NS</td>
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<td></td>
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<tr>
<td>PVN</td>
<td>F&lt;sub&gt;1,36&lt;/sub&gt;=6.82, P=0.013</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=2.70, P=0.036</td>
<td>NS</td>
<td>NS</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=15.88, P&lt;0.001</td>
<td>NS</td>
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<tr>
<td>Cortex</td>
<td>F&lt;sub&gt;1,36&lt;/sub&gt;=26.69, P&lt;0.001</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=12.36, P&lt;0.001</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=9.34, P&lt;0.001</td>
<td>F&lt;sub&gt;1,36&lt;/sub&gt;=21.76, P&lt;0.001</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=33.27, P&lt;0.001</td>
<td>F&lt;sub&gt;5,36&lt;/sub&gt;=6.53, P&lt;0.001</td>
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B. Light Exposure Experiment*

<table>
<thead>
<tr>
<th>Structure</th>
<th><em>Per1</em></th>
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<th></th>
<th></th>
<th><em>Per2</em></th>
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<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Time of day</td>
<td>Interaction</td>
<td></td>
<td>Light</td>
<td>Time of day</td>
<td>Interaction</td>
<td></td>
</tr>
<tr>
<td>SCN (Total)</td>
<td>F&lt;sub&gt;1,40&lt;/sub&gt;=35.90, P&lt;0.001</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=73.89, P&lt;0.001</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=8.81, P&lt;0.001</td>
<td>F&lt;sub&gt;1,40&lt;/sub&gt;=4.70, P=0.036</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=37.31, P&lt;0.001</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=6.16, P=0.002</td>
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<tr>
<td>dSCN</td>
<td>F&lt;sub&gt;1,40&lt;/sub&gt;=15.29, P&lt;0.001</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=40.29, P&lt;0.001</td>
<td>NS</td>
<td>F&lt;sub&gt;1,40&lt;/sub&gt;=15.28, P&lt;0.001</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=37.08, P&lt;0.001</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=6.54, P&lt;0.001</td>
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<tr>
<td>vSCN§</td>
<td>F&lt;sub&gt;1,40&lt;/sub&gt;=46.50, P&lt;0.001</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=70.66, P&lt;0.001</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=11.08, P&lt;0.001</td>
<td>F&lt;sub&gt;1,40&lt;/sub&gt;=9.65, P=0.003</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=35.43, P&lt;0.001</td>
<td>F&lt;sub&gt;3,40&lt;/sub&gt;=8.08, P&lt;0.001</td>
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*As there was no significant strain effect, strain X light, or strain X time of day interactions in any case of the light exposure experiment, only F and P values of light, time of day effects, and their interactions are shown.

vSCN was the only structure in which a significant light X time of day X strain interaction was observed in the light exposure experiment, and this interaction was significant only for *Per2* (F<sub>3,40</sub>=3.32, P=0.029).
Figure 3.1. Piezoelectrically-defined sleep states differ among B6, CAST, and congenic B6.CAST genome-tagged strains. (A) Data are plotted in 4-h intervals throughout the LD12:12 cycle. The timing of the light and dark phases of the LD12:12 cycle are indicated, respectively, by the horizontal white and black bars at the top of the graph (lights-on at 4:00 and lights-off at 16:00, local time). INSERT: the ratio of the
amount of sleep that occurred in the last four hours of the light phase to that in the first
four hours of the light phase. *Significantly different from B6 in the same time interval (P
< 0.05, Student’s t with Bonferroni correction). Note that CAST mice have their
decrease in sleep (and thus greatest increase in wake) during the last four hours of the
light phase (12:00-16:00), consistent with their “early runner” behavior in circadian
wheel-running experiments (Jiang et al., 2011). (B) The advanced sleep/wake cycles in
CAST compared to B6 are more clearly demonstrated when the data from the two
parental strains are re-plotted with a line-graph and replicated for a second cycle. ** and
*, Significantly different from B6 in the same time interval (P < 0.01 and P <0.05,
respectively, Student’s t)
Figure 3.2. Differential effects of strain (CAST vs. B6) on Per1 expression in the extra-SCN brain regions.  LEFT: Photomicrographs depicting representative Per1 mRNA signal in the cerebral cortex (A-D), PVN (F-I) and sPVZ (K-N) of B6 (upper panels) and CAST (lower panels) strains at two times of day.  RIGHT: Daily profiles of Per1 mRNA levels in the cortex (E), PVN (J) and sPVZ (O) of B6 (open circles and solid lines) and CAST (filled squares and dashed lines) mice (N = 4 per group).  Lighting schedule (lights-on at 4:00 and lights-off at 16:00, local time) of the prior LD cycle is indicated by the gray and black bars at the top of each panel. *Significantly different from B6 at the same time point (P < 0.05, Student’s t with Bonferroni correction).
Figure 3.3. Similar patterns of Per1 expression in the SCN of CAST and B6 mice.

TOP: Photomicrographs showing representative Per1 mRNA signal in the SCN of the two strains (B6, upper panels; CAST, lower panels) at various times of day, as indicated.

BOTTOM: The daily profiles of Per1 mRNA levels in the SCN (M), dSCN (N) and vSCN (O) of B6 (open circles and solid lines) and CAST (filled squares and dashed lines) mice (N = 4 per group). Lighting schedule (lights-on at 4:00 and lights-off at 16:00, local time) of the prior LD cycle is indicated by the gray and black bars at the top of each panel.
Figure 3.4. Differential effects of strain (CAST vs. B6) on Per2 expression in extra-SCN brain regions. LEFT: Photomicrographs showing representative Per2 mRNA expression in the cerebral cortex (A-D), PVN (F-I) and sPVZ (K-N) of B6 (upper panels) and CAST (lower panels) strains. RIGHT: Daily profiles of Per2 mRNA levels in the cortex (E), PVN (J) and sPVZ (O) of B6 (open circles and solid lines) and CAST (filled squares and dashed lines) mice (N = 4 per group). Lighting schedule (lights-on at 4:00 and lights-off at 16:00, local time) of the prior LD cycle is indicated by the gray and black bars at the top of each panel. *Significantly different from B6 at the same time point (P < 0.05, Student’s t with Bonferroni correction).
Figure 3.5. Similar patterns of Per2 expression in the SCN of CAST and B6 mice. 

TOP: Photomicrographs showing representative Per2 mRNA signal in the SCN of the two strains (B6, upper panels; CAST, lower panels) at various times of day. BOTTOM: Daily profiles of Per2 mRNA levels in the SCN (M), dSCN (N) and vSCN (O) of B6 (open circles and solid lines) and CAST (filled squares and dashed lines) mice (N = 4 per group). Lighting schedule (lights-on at 4:00 and lights-off at 16:00, local time) of the prior LD cycle is indicated by the gray and black bars at the top of each panel.

*Significantly different from B6 at the same time point (P < 0.05, Student’s t with Bonferroni correction).
Figure 3.6. Similar light-induction of Per1 expression in the SCN of B6 and CAST mice. TOP: Photomicrographs showing representative Per1 mRNA signal in the SCN of B6 (A-D) and CAST (H-K) mice euthanized either during exposure to darkness at the times indicated or after 30-min of light exposure beginning at the times indicated.
BOTTOM: Quantitation of Per1 mRNA levels in various SCN regions of B6 (E, F, and G) and CAST (L, M, and N) killed as time of day controls in the dark (black bars; N = 4 per...
group) or after light exposure (white bars; N = 3 per group). The lighting conditions at these times under the prior LD cycle are indicated by the broken gray (light) and black (dark) bars at the bottom. *Significantly different from dark control (P < 0.05, Student’s t with Bonferroni correction).
Figure 3.7. Similar patterns of light-induced Per2 expression in the SCN of B6 and CAST mice. TOP: Photomicrographs showing representative light induction of Per2 in the SCN of B6 (A-D) and CAST (H-K) mice euthanized either during exposure to darkness at the times indicated or after 30-min of light exposure beginning at the times indicated. BOTTOM: Quantitation of Per2 mRNA levels in the SCN, dSCN, and vSCN of B6 (E, F, and G, respectively) and CAST (L, M, and N, respectively) killed as time of
day controls in the dark (black bars; N = 4 per group) or after light exposure (white bars; N = 3 per group). The lighting conditions at these times under the prior LD cycle are indicated by the broken gray (light) and black (dark) bars at the bottom. *Significantly different from dark control (P < 0.05, Student's t with Bonferroni correction).
CHAPTER FOUR

Sleep/Wake Dependent Cortical Expression of Clock-genes and Homer1a in SCN Lesioned and Intact Mice

Introduction

Sleep is regulated by both circadian and homeostatic processes. In mammals, circadian rhythms are generated in the suprachiasmatic nucleus (SCN) via transcriptional and translational feedback loops, which are comprised of transcription activators (CLOCK and BMAL1) as well as their inhibitors (PER1, 2, 3 and CRY1, 2) [for reviews, see (Reppert & Weaver, 2001; Lowrey & Takahashi, 2004)]. In the SCN, the transcript levels of these clock-genes (such as Bmal1, Per1, 2, 3 and Cry1, 2) exhibit ~24-h oscillations, which are crucial for the generation of circadian rhythms. However, in other areas of the brain, especially in the cerebral cortex where the "gold standard" assessment of sleep-- Electroencephalography (EEG) is measured, the expression of clock-genes Per1 and Per2 is elevated during sleep deprivation (SD) and goes down when recovery sleep is allowed, parallel to the process of homeostatic sleep pressure during these conditions (Wisor et al., 2002; Cirelli et al., 2004; Franken et al., 2006; Franken et al., 2007; Mongrain et al., 2010). Moreover, disruptions of clock-genes also alter sleep homeostasis, and interestingly, the altered homeostatic sleep drive in clock-gene mutant/knockout animals also appeared to positively correlate with changes in cortical Per1 and Per2 expression levels (for reviews see (O'Hara et al., 2007; Franken & Dijk, 2009)). Thus, while constituting the molecular circadian clock in the SCN, Per1 and Per2 may also function as a molecular hourglass keeping track of sleep pressure.
However, the relative contribution of circadian and sleep homeostatic drives on the cortical expression of *Per1* and *Per2* remains unclear. Under baseline conditions, cortical *Per1* and *Per2* transcript levels oscillate in nocturnal and diurnal animals with opposite phases: peaking during subjective night in nocturnal animals and subjective day in diurnal animals [i.e., the active periods in nocturnal and diurnal animals; (Abe et al., 2001a; Abe et al., 2001b; Mrosovsky et al., 2001; Reick et al., 2001)]. Thus, this oscillation could be driven by the circadian signal originated from the SCN, the homeostatic sleep pressure, or a combination of both. As a result, it is also difficult to quantitatively describe the dynamics of sleep-homeostatic expression of *Per1* and *Per2* in cerebral cortex under conditions of SD. A similar scenario exists in the cortical dynamics of a transcription factor gene, *Dbp* (*albumin D-binding protein*), whose expression is regulated by the molecular clock machinery but also appears to be influenced by sleep homeostasis. Under baseline conditions, cortical *Dbp* mRNA oscillates with a broad peak from late (subjective) day to early (subjective) night (Yan et al., 2000), while it decreases after SD (Wisor et al., 2002; Cirelli et al., 2004; Franken et al., 2007). Further, a putative core molecular correlate of sleep homeostasis, *Homer1a*, whose transcript level consistently parallels sleep loss in three distinct mouse strains with differing sleep phenotypes, is also expressed with a daily oscillation under baseline conditions (Maret et al., 2007). This oscillation was thought to be mostly the result of synchronized daily sleep/wake cycles, although further evidence is still needed.

In this study, I address this issue and separate the circadian and homeostatic processes by studying SCN lesioned (SCNx) mice, in which the circadian rhythmicity of sleep/wake is abolished while the homeostatic regulation remains intact (Ibuka et al., 1980; Easton et al., 2004; Bourgin et al., in preparation). By comparing SCNx and intact animals under conditions of SD and recovery sleep, I estimate the relative contributions
of circadian and homeostatic drives on cortical expression of *Per1, Per2, Dbp,* and *Homer1a*. My data suggest cortical *Per1* and *Per2* expression are likely to be regulated by both circadian rhythms and sleep homeostasis and thus may serve as a molecular bridge for the interaction of circadian and homeostatic sleep regulations.

**Materials and Methods**

**Animals and experimental treatments**

As this is a collaborative study, the animal work was performed by Dr. Patrice Bourgin at Stanford University [and now residing in Strasbourg, France]. Briefly, male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) of at least 6 weeks were either subject to SCNx by the radio frequency method (which uses a fine probe to deliver heat and destroy a small amount of tissue around the probe) or left intact. SCNx treated mice were then singly housed in cages equipped with infrared beams at the bottom to record their activity rhythms. Behavioral arrhythmia under constant dark conditions was taken as the standard for complete SCNx. Confirmed SCNx mice and intact controls were then housed under 12-hour light/12-hour dark conditions, and were subject to 4 different sleep treatments. Thus, a total of 8 groups resulted, and each group consists of at least 6 mice. 6 hour of SD (6SD) treatment was delivered at ZT0 (i.e. time of light-on) by transferring mice to new cages and gentle handling. A subset of these mice were sacrificed right after the SD treatment while other SD-treated mice were allowed to sleep for 2 hours (6SD2R) before sacrifice. Their corresponding control groups were left undisturbed for 6 or 8 hours (6C or 8C) respectively, also starting at ZT0. After euthanasia by cervical dislocation, mouse brains were removed, coarse-dissected into five pieces: the cerebellum, the pons-medulla region, left and right halves of the rostral
cortex (resulted from a coronal cut at approximately 1.5 mm rostral to the optic chiasm and then a sagittal cut to produce left and right halves), and finally the remaining large middle portion of the brain containing the rest of forebrain plus the diencephalon and midbrain. All brain tissues were then frozen on dry ice and shipped to the University of Kentucky, where they were kept at -80°C before further processing for in situ hybridization of quantitative real-time PCR.

**In situ hybridization histochemistry**

The middle portions of brains from 6SD- and 6C-treated SCNx and intact animals (a subset of randomly selected animals were used; 6 mice per group) were sectioned into 20 micron sections using a cryostat and mounted on slides at -20°C. Slides were then stored at -80°C until use. Sections encompassing the SCN (4 sections per animal) and caudal (roughly 0.9~1.2mm) to the SCN (4 sections per animal) were used in the in situ hybridization experiment. Brain sections were fixed in 4% paraformaldehyde/0.1 M phosphate buffer solution (pH 7.4), washed in 1X PBS and 1X PBS/27 mM glycine, acetylated in 0.25% acetic anhydride/0.1 M triethanilamine (pH 8.0), dehydrated and delipidated in ethanol solutions and chloroform, and then air dried. After pretreatment, sections were hybridized overnight at 60°C in a hybridization solution [50% formamide (deionized), 10% dextran sulfate, 1X Denhardt, 20 mM Tris-HCL (pH 7.4), 300 mM NaCl, 1 mM EDTA, 100 mM DTT, 0.1% SDS, 0.1% Na₂S₂O₃, 250 μg/ml salmon sperm DNA, and 625 μg/ml yeast RNA] with ³⁵S-labeled anti-sense Per1 or Per2 riboprobe, which was in vitro transcribed using Maxiscript™ (Ambion, Foster City, CA) from linearized mouse cDNA clones in pBlueprint (KS-; Stratagene, La Jolla, CA) or pCRII (Invitrogen, Carlsbad, CA) plasmids, respectively. During the hybridization, sections with hybridization mix were covered by siliconized coverslips. After hybridization, coverslips were rinsed in 2X SSC/10 mM DTT/0.25% Na₂S₂O₃ at room temperature. Sections
were then washed twice in 2X SSC/10 mM DTT/0.25% Na$_2$S$_2$O$_3$, rinsed in RNaseA buffer [400 mM NaCl, 10 mM Tris-HCL (pH 7.5), 5 mM EDTA], and incubated at 37°C in RNase A buffer with 40 mg/ml RNAse A, followed by a 15-min wash in 1X SSC/0.25% Na$_2$S$_2$O$_3$ at room temperature, two 30-min washes in 0.1X SSC/0.25% Na$_2$S$_2$O$_3$ at 63°C, and finally a rinse in 0.1X SSC/0.25% Na$_2$S$_2$O$_3$ at room temperature. Slides were dehydrated, air dried, and placed in X-ray cassettes and against autoradiography films (Biomax MR™, Kodak, Rochester, NY) with a set of $^{14}$C radioactive standards (Amersham Life Sciences, Piscataway, NJ). A series of test sections were also processed along with the experimental sections but were placed with films in separate cassettes for the purpose of determining the optimal exposure time. After the exposure time determined by these test exposures, experimental films were developed and the autoradiograms were subject to analysis using the MCID image analysis system (Imaging Research Inc., St. Catherines, Ontario, Canada). The image analysis was performed similarly as described in the previous chapter. Per1 and Per2 mRNA levels (i.e., the gene specific radioactivity) in the region of interest were determined using a standard curve established by the optical densities of a series of $^{14}$C standards.

The in situ hybridization experiments were performed in collaboration with a former Masters-student, Ling Liu, in our lab. Ling participated in the sectioning of brain samples and performed Per1 in situ hybridization assay and image analysis, while I also sectioned some of the brain samples, prepared the linearized Per1 and Per2 cDNA clones for the synthesis of radiolabeled-probes, and performed Per2 in situ hybridization assay and image analysis.
**Quantitative real-time PCR**

The rostral cortices from all animals in every experimental group (≥ 6 animals per group, 8 groups in total) were subject to quantification of *Per1, Per2, Dbp, and Homer1a* mRNA levels using real-time PCR. Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA) and was treated with Turbo DNA-free™ (Ambion, Foster City, CA) for the removal of genomic DNA contamination. cDNA synthesis was then performed using 3 μg of purified total RNA, Oligo(dT)₁₂₋₁₈, and Superscript II™ (Invitrogen, Carlsbad, CA). 2 μl of 1:4 diluted cDNA from each sample or from serial dilutions of pooled cDNA (stock, 1:5, 1:10, 1:100, and 1:1000) were mixed with 25 μl of iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA) and 0.2 μM each of forward and reverse gene-specific primers (Table 4.1) in a 50 μl reaction volume. Reaction mix was placed in duplicates in 96-well thin-wall PCR plates (Bio-Rad, Hercules, CA), covered with optical-quality sealing tape (Bio-Rad, Hercules, CA). The PCR and real-time recording were performed in an iCycler iQ™ real-time system (Bio-Rad, Carlsbad, CA). Melting curves for each amplification product were constructed after the PCR to examine the specificity of the amplification. Relative abundance of each gene was determined by the automated program of iCycler iQ™ real-time system (Bio-Rad, Carlsbad, CA) using the standard curve method, and normalized to the averaged relative abundance of *Gapdh* and *Actb*.

**Statistical analysis**

As limited by the 96-well real-time system, samples collected at ZT6 (i.e., 6SD and 6C) and ZT8 (i.e., 6SD2R and 8C) had to be processed on two separate 96-well plates. Thus, direct comparison between the two time points was not available, and the two time points were analyzed separately. For both *in situ* hybridization and real time
PCR assays, two-way analysis of variance (ANOVA) was applied to determine the effects of sleep treatment, SCNx, and their interaction (if any), and was followed by Student's t tests for independent measures to compare between two groups of interest. For *Per1* and *Per2* expression in the SCN under 6SD vs. 6C conditions, Student's t tests for independent measures were used. Statistical analyses were performed using R software (version 2.12.1).

**Results**

*Sleep/wake independent expression of Per1 and Per2 in the SCN of intact animals*

I first investigated the effect of 6SD on *Per2* expression in the SCN of intact animals (Figure 4.1A). The SCN *Per2* transcript level in 6SD animals were not different from time-control (e.g., 6C) animals (P = 0.1750), as assessed by 35S-labeled *in situ* hybridization. Similarly, *Per1* expression was not affected by the treatment of 6SD [P = 0.8988; Figure 4.1D, data adopted from (Liu *et al.*., 2008)].

*Sleep/wake dependent cortical expression of Per1 and Per2 in both SCNx and intact animals*

In the cerebral cortex, radio-labeled quantitative *in situ* hybridization revealed interesting changes in clock-gene expression. Both SCNx and SD treatments significantly affected *Per2* expression (F = 37.599, P < 0.0001 for SD treatment; F = 8.847, P = 0.0078 for SCNx treatment), as measured in the dorsal cortex (contains cingulate/retrosplenial, motor, somatosensory, and insular cortices) in the SCN-encompassing brain sections from the intact animals and equivalent brain sections from the SCNx animals (Figure 4.1B). As expected from previous studies from our lab, 6SD
induced a 2.2-fold increase of Per2 mRNA level in the dorsal cortex of intact animals (P < 0.0001 vs. 6C). Further, a suggestive change (P = 0.071) in dorsal cortical Per2 expression was also observed in the 6SD-treated SCNx animals as compared to the 6C SCNx mice. Interestingly, although dorsal cortical Per2 level exhibited no difference between SCNx and intact mice under 6SD conditions, the baseline (e.g., under 6C conditions) Per2 expression at ZT6 (i.e., 6 hours after lights-on) was significantly higher (by 1.75 folds, P = 0.0187) in SCNx than intact animals. Only a statistically suggestive but not significant SD X SCNx interaction was found to influence Per2 expression in the dorsal cortex (F = 3.7465, P = 0.068).

As for the lower cortical region, I measured clock-gene expression in the piriform cortex, a region primarily involved in olfactory perception. Only SD treatment was found to significantly affect Per2 expression in the piriform cortex of SCN-encompassing brain sections (F = 51.539, P < 0.0001; Figure 4.1C). 6SD strongly increased Per2 mRNA level in the piriform cortex by ~2 folds in intact animals (P < 0.0001 vs. 6C) and by 34% in SCNx animals (P = 0.016 vs. 6C). Although the mean value of Per2 level appeared to be higher in SCNx mice under baseline (e.g., 6C) conditions as compared to intact mice, the difference was not statistically significant (P = 0.1869). No SD X SCNx interaction was found to impact Per2 expression in the piriform cortex (F = 1.1845, P = 0.2900).

Similar to Per2, cortical expression of Per1 in the SCN-encompassing brain sections was also affected by experimental treatments [Figure 4.1E and F, data adopted from (Liu et al., 2008)]. In the dorsal cortex of the SCN-encompassing sections, 6SD but not SCNx treatment strongly influenced Per1 mRNA levels at ZT6 (F = 15.780, P = 0.0008 for SD treatment; F = 2.274, P = 0.1480 for SCNx treatment; Figure 4.1E). 6SD produced a significant increase of dorsal cortical Per1 level by 1.5 folds in the intact mice (P = 0.0077 vs. 6C) and a suggestive elevation in the SCNx mice (P = 0.0840 vs. 6C).
However, in contrast to Per2 expression pattern, the difference of baseline (e.g., under 6C conditions) Per1 expression between SCNx and intact animals in the dorsal cortex was not statistically significant, although a general trend of higher levels in SCNx mice persisted. Moreover, Per1 levels in the piriform cortex were only suggestively affected by SD treatment ($F = 3.693, P = 0.070$) and not affected by SCNx treatment ($F = 0.0208, P = 0.88690$). Last but not least, similar to Per2, SD X SCNx interaction was not found to affect Per1 expression in either dorsal ($F = 0.5164, P = 0.4811$) or piriform cortex ($F = 0.1319, P = 0.72044$).

In addition, Per1 and Per2 mRNA levels were also measured in brain sections approximately 0.9 - 1.2 mm caudal to the SCN [Figure 4.1B', C', E', F'; Per1 data adopted from (Liu et al., 2008)]. Very similar results were obtained as compared to the brain sections encompassing the SCN, although the dorsal cortex in these caudal sections are composed of different sub-cortical regions (including, retrosplenial, visual, somatosensory, auditory, temporal association, and rhinal cortices). The only exception was that 6SD induced a significant elevation of Per2 expression (~1.6 folds, $P = 0.0190$ vs. 6C) in the dorsal cortex in the caudal sections, as opposed to a suggestive change in the SCN-encompassing sections.

I also assessed behavioral-state-dependent changes in clock-gene expression in the rostral cortex (i.e., left or right half of the rostral cerebrum resulted from a coronal cut at ~1.5 mm rostral to the optic chiasm) using quantitative real-time PCR (Figure 4.2A-D). Consistent with the in situ hybridization data, real-time PCR reveal that rostral cortical Per2 expression at ZT6 were significantly influenced by both 6SD ($F = 7.938, P = 0.0100$) and SCNx ($F = 16.3732, P = 0.0005$) but not the interaction of the two treatments ($F = 0.0555, P = 0.8159$). 6SD increased rostral cortical Per2 level by 1.8 folds in the intact animals ($P = 0.0390$) but insignificantly in the SCNx animals ($P =$
Meanwhile, *Per2* expression at ZT6 in the rostral cortex of the SCNx animals was also significantly increased by 2 folds under baseline conditions (e.g., 6C; \(P = 0.0009\)) and suggestively increased under 6SD conditions (\(P = 0.0696\)), compared to the intact mice. On the other hand, *Per1* expression at ZT6 in the rostral cortex was also strongly affected by 6SD (\(F = 7.4588\), \(P = 0.0122\)) and SCNx (\(F = 20.2891\), \(P = 0.0002\)), and no SD x SCNx interaction was found (\(F = 0.0013\), \(P = 0.9720\)). 6SD only produced a suggestive change in *Per1* expression in the intact animals (\(P = 0.0661\)), but no significant change in the SCNx animals (\(P = 0.1595\)) despite a consistent tendency of higher levels. SCNx, however, significantly elevated *Per1* expression in the rostral cortex under either 6SD (\(P = 0.0164\)) or undisturbed 6C conditions (\(P = 0.0054\)).

Finally, when recovery sleep was allowed, cortical mRNA levels of *Per1* and *Per2* responded accordingly. At ZT8, neither the sleep treatment (e.g., 6SD2R; \(F = 0.0402\), \(P = 0.8427\)) nor the SCNx treatment (\(F = 0.3266\), \(P = 0.5725\)) affected *Per2* expression in the rostral cortex. Also, no effect from the interaction between sleep treatment and SCN treatment was found (\(F = 0.0003\), \(P = 0.9869\)). In fact, no difference was observed between any pairs of groups. Interestingly, *Per1* cortical expression at ZT8, however, was significantly affected by 6SD2R (\(F = 10.1111\), \(P = 0.0038\)). In contrast to 6SD, 6SD2R lowered *Per1* level in rostral cortex significantly in SCNx mice (by ~28% \(P = 0.0460\)) and suggestively in intact mice (\(P = 0.0595\)). SCNx treatment and the interaction between SCNx and sleep treatments, however, had no effect on cortical *Per1* expression at ZT8 (\(F = 0.0226\), \(P = 0.8816\) for SCNx; and \(F = 0.0482\), \(P = 0.8280\) for sleep x SCNx interaction).
Sleep/wake dependent expression of clock-controlled gene Dbp in the rostral cortex of both SCNx and intact animals

Like clock-genes Per1 and Per2, cortical expression of Dbp, a clock-controlled gene, was also dependent on sleep and wake in both SCNx and intact mice as assessed by quantitative real-time PCR (Figure 4.2E, F). At ZT6, 6SD (F = 21.3788, P = 0.0001) and SCNx (F = 5.5333, P = 0.02802) but not their interaction (F = 0.6032, P = 0.4456) significantly affected Dbp mRNA levels in the rostral cortex. In contrast to Per1 and Per2, 6SD lowered Dbp expression in the rostral cortex by ~30% in the intact animals (P = 0.0359) and by ~50% in the SCNx animals (P = 0.0014). In addition, 6SD-treated SCNx mice showed ~36% lower cortical Dbp levels compared to the intact mice (P = 0.02229), while no difference between SCNx and intact mice under baseline 6C conditions was found (P = 0.3079).

Recovery sleep also largely reversed the effect of 6SD on cortical Dbp expression. Although ANOVA indicated a significant influence by the sleep treatment at ZT8 (e.g., 6SD2R; F = 7.5081, P = 0.01095), cortical Dbp mRNA levels under 6SD2R conditions were only suggestively lower than 8C conditions in both intact (by ~20%; P = 0.0825) and SCNx animals (by ~25%; P = 0.0790). Meanwhile, SCNx did not affect Dbp levels at ZT8 (F = 0.4735, P = 0.49748). There was no difference between SCNx and intact animals under either 6SD2R or 8C conditions. In addition, no interaction between sleep treatment and SCN treatment was found to influence cortical Dbp expression at ZT8 (F = 0.1714, P = 0.6823).
Sleep/wake dependent Homer1a expression in the rostral cortex of both SCNx and intact animals

Homer1a is a short splicing variant of the Homer1 gene and is a dominant-negative regulator of the longer isoforms. While the long isoforms are constitutively expressed, Homer1a expression can be acutely induced by neuronal activity (Bottai et al., 2002) and is sleep-and-wake dependent (Maret et al., 2007). I also measured Homer1a expression in the rostral cortex of the same animals described above, using real-time PCR (Figure 4.2G, H). At ZT6, 6SD significantly affected cortical Homer1a mRNA levels (F = 50.6980, P < 0.0001), but SCNx did not (F = 0.8069, P = 0.3770). In both intact and SCNx animals, 6SD induced a significant increase in cortical Homer1a levels 3.5 fold (P = 0.0002) and 2.7 fold (P = 0.0035), respectively. No difference was found between the intact and the SCNx animals that underwent either 6SD or 6C conditions. When recovery sleep was allowed, however, cortical Homer1a levels remained strongly affected by the sleep treatment (e.g., 6SD2R; F = 47.0460, P <0.0001). Compared to 8C animals, 6SD2R-treated intact and SCNx animals both exhibited significantly higher cortical Homer1a mRNA levels, by 2.5 fold (P = 0.0007) and 2.8 fold (P = 0.0023), respectively. Although ANOVA also indicated SCNx as a factor influencing cortical Homer1a level at ZT8 (F = 5.7672, P = 0.0238), only suggestive differences between SCNx and intact animals were found under both 6SD2R (P = 0.0809) and 8C conditions (P = 0.1070). Lastly, no interaction between sleep treatment and SCN treatment was found at either ZT6 (F = 1.3378, P = 0.2575) or ZT8 (F = 2.1447, P = 0.1550).
Discussion

A number of studies have illustrated that clock-gene expression in rodent forebrain, especially the cerebral cortex, is sleep/wake dependent: SD consistently increases Per1 and Per2 expression (Wisor et al., 2002; Cirelli et al., 2004; Franken et al., 2006; Franken et al., 2007; Mongrain et al., 2010), while this effect is largely reduced or diminished when recovery sleep is allowed (Wisor et al., 2002; Franken et al., 2007). These studies suggested a sleep/wake homeostatic process in the regulation of cortical clock-gene expression. However, it is unclear whether this sleep homeostatic regulation of cortical Per1 and Per2 expression is independent of the circadian influence originated from the SCN. Also, under normal conditions, the timing of sleep and wake are driven or influenced by the SCN based circadian system, and the baseline Per1 and Per2 expression in the cortex oscillates with a peak during the time of day when the animal is mostly awake (Abe et al., 2001a; Abe et al., 2001b; Mrosovsky et al., 2001; Reick et al., 2001). Thus, it is difficult to determine whether the SCN clock also directly influences cortical Per expression, and (if so) how much circadian and homeostatic processes each contribute to the regulation of cortical clock-gene expression. In the current study I showed that sleep/wake dependent changes in cortical expression of clock-genes Per1 and Per2, clock-controlled gene Dbp, and a putative molecular sleep correlate Homer1a, largely persisted in the absence of direct circadian influence from the SCN. In addition, no statistically significant 6SD-by-SCNx or 6SD2R-by-SCNx interaction was found under any of the experimental settings, as suggested by two-way ANOVA. These data suggest that the sleep homeostatic drive of cortical gene expression is independent of the circadian process originating in the SCN.

Consistently, Per1 and Per2 expression in the SCN was not affected by 6SD. Thus, the SD-induced changes in cortical clock-gene expression is not a result of
circadian phase shifts in the SCN. Two studies in hamsters have suggested that SD in constant lighting conditions produced circadian phase-shifts of wheel-running activities and a correlated change in the immunoactivity of an immediate early gene, c-fos, in the SCN (Antle & Mistlberger, 2000; Mistlberger et al., 2002). These differences may reflect that light/dark cycles (i.e., the lighting conditions used in this study), as the predominant synchronizing cue, overrides the impact of SD on the phase of the SCN clock. Other possible sources for these differences may include the amount of activity involved in the SD procedures and circadian timing of the SD treatment. Nonetheless, under experimental conditions of SD used in this study as well as other conditions such as circadian splitting (Abe et al., 2001a; Abe et al., 2001b), methamphetamine treatment (Masubuchi et al., 2000), and restricted feeding (Wakamatsu et al., 2001), cortical Per1 and Per2 expression changed in concert with the alteration of activity/rest or sleep/wake, while their expression in the SCN remained unaffected.

SCNx also affected cortical expression of Per1 and Per2, particularly at ZT6, as suggested by both in situ hybridization and real-time PCR experiments. Most notably, under baseline conditions SCNx mice exhibited higher Per1 and Per2 levels compared to intact animals at ZT6 but not ZT8. The effects of SCNx on sleep and wake have been studied extensively in rats [for a review, see (Mistlberger, 2005)] and two strains of mice (Ibuka et al., 1980; Easton et al., 2004; Bourgin et al., in preparation), including C57BL/6J (i.e., the same strain used here). In C57BL/6J, after SCNx, homeostatic sleep pressure as determined by EEG slow-wave activity (SWA) was constantly at a relatively low level, and the amount of non-rapid eye movement (NREM) sleep was higher but much more evenly distributed across the day compared to the intact animals (Easton et al., 2004; Bourgin et al., in preparation). Interestingly, the EEG SWA at ZT6 in intact animals, although dissipating from the high levels during the night, was still slightly
higher than in the SCNx animals, and it continued going down and reached the same level as in the SCNx animals at ZT8. Thus, there is a contrast between cortical $Per$ expression and the EEG SWA in the intact animals at ZT6 when both are compared to the SCNx animals. In addition, although direct comparison was not available due to technical limitations, I would expect $Per$ levels at ZT6 and ZT8 to be the same in SCNx animals, as SCNx eliminates the circadian rhythmicity of sleep/wake. Thus, the cortical $Per$ levels at ZT6 should be lower than ZT8 in the intact animals, as was shown in previous studies from our lab using three mouse strains (Franken et al., 2007). Taken together, these data indicate that there may also be a circadian process, presumably originated from the SCN and independent from the homeostatic process, that lowers the cortical $Per1$ and $Per2$ expression at ZT6. Consistent with this assertion, my data also indicated that cortical $Per$ levels in the 6SD-treated SCNx animals were the same or even higher than 6SD-treated intact animals, while the EEG SWA in the SCNx mice was in fact slightly lower than intact controls when both groups underwent the same 6SD treatment as described in this study (Bourgin et al., in preparation). This observation could also have resulted from a circadian process which lowered the cortical $Per$ levels at ZT6 preventing further elevation after the SD treatment from ZT0 to ZT6. Furthermore, an independent circadian process regulating the cortical $Per$ expression is also consistent with a previous finding that a "round-the-clock" 6SD treatment starting at ZT0, ZT6, ZT12, and ZT18 attenuated the daily oscillation of many brain transcripts (including $Homer1a$) and computationally simulated sleep pressure but not forebrain $Per2$ transcript levels (Maret et al., 2007). Therefore, instead of being driven by a circadian or a homeostatic process alone, cortical $Per1$ and $Per2$ are more likely to be driven by an integration of the two.
Along with elevated baseline expression, the amplitude of 6SD-induced Per expression was relatively smaller in the SCNx animals as compared to the intact animals. The already high levels of baseline Per expression together with the non-linear relationship between the homeostatic sleep pressure and the duration of wakefulness may have limited the amplitude of 6SD-induced elevation of Per levels. Alternatively, because of the arrhythmia, SCNx mice under normal conditions slept less during ZT0-ZT6 compared to intact mice (Easton et al., 2004; Bourgin et al., in preparation). Thus, the smaller amplitude of 6SD-induced elevation of Per levels in the SCNx animals could simply reflect the fact that their relative sleep loss (6SD vs. 6C) was smaller than in the intact animals.

Last but not least, the overall pattern of cortical clock-gene expression at ZT6 in all experimental groups as assessed by in situ hybridization were similar with the data obtained by real-time PCR, indicating a general reproducibility of the current study by independent methods. In addition, the sleep/wake and SCNx dependent Per expression was also similar in all cortical regions examined, indicating an interesting generalizability across the cortex and perhaps much of the brain. However, a few exceptions to the general similarity were also found. Most notably, Per1 expression in the piriform cortex only slightly responded to the 6SD treatment, different from that in the dorsal cortical region as assessed by in situ hybridization or in the rostral cortex as assessed by real-time PCR. Also, Per expression in the rostral cortex as assessed by real-time PCR appeared more sensitive to the SCNx treatment compared to that in dorsal or piriform cortices in the SCN-encompassing and caudal-to-SCN sections. The baseline Per expression level in the rostal cortex of SCNx animals was even as high as 6SD-treated intact animals. These differences may suggest regional-specific details in the circadian and homeostatic regulations of Per expression as well as sleep and wake. Indeed, in
studies of humans and model organisms, regional-specific EEG patterns/properties during sleep (Werth et al., 1996a; Werth et al., 1997; Zavada et al., 2009), activity dependent local sleep/wake in the brain (Rector et al., 2005; Krueger et al., 2008), regional-specific neuronal activity during sleep (Sherin et al., 1996; Gerashchenko et al., 2008), and spatial differences in sleep/wake dependent gene expression (Thompson et al., 2010) have been documented. Thus, more detailed studies and analyses are needed to understand these interesting regional differences and whether these correlations represent important causal relationships.

Although the decrease of $Dbp$ transcript after SD has been repeatedly demonstrated previously (Wisor et al., 2002; Cirelli et al., 2004; Franken et al., 2007), this effect of SD remains difficult to explain. $Dbp$ is a transcription factor, whose own transcription is under the control of a cis-regulatory element called $E$-box and is thus induced by CLOCK-BMAL1 and shut-down by PER-CRY (Ripperger et al., 2000; Yamaguchi et al., 2000). The transcription of $Per1$ and $Per2$ genes are also controlled, in part, by $E$-box (Gekakis et al., 1998; Hogenesch et al., 1998; Jin et al., 1999). Thus, it is puzzling that SD exerts opposite effects on cortical $Per$ and $Dbp$ expression. A plausible explanation is that the expression of $Per1$ and $Per2$ are also driven by other cis-regulatory elements, such as the cAMP response element [CRE; (Travnickova-Bendova et al., 2002)] and the glucocorticoid response element [GRE; (Yamamoto et al., 2005; So et al., 2009)]. SD elevates the activity of CRE-binding protein [CREB; (Cirelli & Tononi, 2000; Graves et al., 2003)] as well as the plasma corticosterone levels (Gip et al., 2004; Tartar et al., 2009; Mongrain et al., 2010). Assuming that PER protein levels follow the increased transcript levels in a timely manner (e.g., within the 6 hours of SD), it is thus possible that 6SD induces $Per$ expression mainly through CRE and GRE, while the decline of $Dbp$ expression during SD results from suppressed CLOCK-BMAL1
transcriptional activity as PER increases. Indeed, my data suggested that cortical mRNA levels of Dbp at ZT6 in each individual animal of all experimental groups was negatively correlated with Per1 (Pearson r = -0.6012, P = 0.0012) and Per2 levels (Pearson r = -0.5688, P = 0.0024). In addition, previous studies also found that the most dramatic decrease in Dbp levels occurred in the strain with the most prominent increase in Per expression during 6SD (Franken et al., 2007). In contrast with this scenario, however, the same previous study also found Dbp and Per2 levels are positively correlated after 6SD (Franken et al., 2007), and no significant correlation between Dbp and Per was found at ZT8 in the current study. Further, under baseline conditions, Dbp transcripts in the cortex and peripheral tissues peak at ZT8 proceeding the peak of Per expression by ~4 hours (Yan et al., 2000; Yamamoto et al., 2004), although this time-course of Dbp oscillation may be explained by the contribution of another cis-regulatory element RORE in the Dbp gene (Yamamoto et al., 2004).

The sleep homeostatic responses of cortical Homer1a expression in both intact and SCNx mice described in this study largely resembles the sleep/wake pattern and EEG SWA dynamics in the same strain of mice that underwent similar SCNx and SD treatments (Easton et al., 2004; Bourgin et al., in preparation). Thus, it is likely that cortical Homer1a is driven mostly by sleep and wake, and contribution from the circadian process is minimal if any. In line with this idea, the daily oscillation of forebrain Homer1a expression in three mouse strains was greatly reduced when the animals were subject to an "around-the-clock" treatment of 6SD (Maret et al., 2007). In addition, the SCNx treatment in this study had no effect on cortical expression of Homer1a at ZT6, and only a weak effect at ZT8. This small effect may also be explained by prior sleep and wake. Under 8C conditions, the observation that intact animals exhibited suggestively lower Homer1a than the SCNx animals may result from a continuous decay of sleep pressure.
in the intact animals as they sleep the most during the first 8 hours of the light period. In addition, 6SD2R intact animals showed suggestively lower Homer1a expression compared to SCNx animals. This may be due to a faster decline of Homer1a levels during the 2 hours of recovery sleep in the intact animals (from 3.5-fold difference, 6SD vs. 6C, to 2.5-fold difference, 6SD2R vs. 8C) compared to the SCNx animals (from 2.7-fold difference, 6SD vs. 6C, to 2.8-fold difference, 6SD2R vs. 8C). Thus, this difference may reflect the fact that intact mice exhibited more NREM rebound after SD (Easton et al., 2004; Bourgin et al., in preparation). However, substantial dissipation of EEG SWA was found in both intact and SCNx mice (Bourgin et al., in preparation), inconsistent with this explanation.

Collectively, data from this study suggest a circadian and a sleep homeostatic process that independently regulate or influence the cortical expression of clock-genes Per1 and Per2. Instead of being the molecular correlate of either individual process, Per1 and Per2 expression outside of the SCN may provide a molecular mechanism for the interaction of circadian and homeostatic processes proposed by the classic two-process model (Borbely, 1982; Daan et al., 1984). Therefore, via Per expression, the clock machinery in the cortex (and perhaps also in other regions of the brain) may integrate the information of the circadian timing originated from the SCN as well as the sleep pressure driven by behavioral state, and thereby perform coordinated functions of circadian rhythms and sleep. Such functions of the clock machinery may include the regulation of cellular metabolism and correctly sensing its feedback [for a review, see (Green et al., 2008)]. However, there is a time-course issue potentially complicating this integration model. The increase in PER1 and PER2 protein levels typically lag the increase in mRNA transcripts in the SCN and peripheral tissues by up to six hours (Reppert & Weaver, 2001; Yoo et al., 2004; Segall et al., 2006). It would therefore take
many hours for the change in downstream gene expression and the functional effects to occur in response to the changes in sleep homeostat and/or circadian rhythms, especially when the immediate downstream gene is also a transcription factor, such as Dbp, which requires another round of transcriptional and translational regulation. Thus, this integrated model may not be suitable or relevant for the changes of sleep within short periods of time (e.g., a few hours), but may be critical to longer term regulation. Lastly, the sleep homeostat may utilize other molecular machinery independent of the circadian process, such as those involving Homer1a, which may regulate sleep and wake via regulation of glutamate neurotransmission and calcium homeostasis (Xiao et al., 2000; Worley et al., 2007).
Table 4.1. Gene specific primers used in the quantitative real time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank ID</th>
<th>Direction</th>
<th>Primer sequence</th>
<th>Start</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per1</td>
<td>NM_011065.4*</td>
<td>Forward</td>
<td>5'-CTTGATGTGATGGCGTGTTGTG-3'</td>
<td>3772</td>
<td>404 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-AGCTGGGCGAATTCTATT-3'</td>
<td>4175</td>
<td></td>
</tr>
<tr>
<td>Per2</td>
<td>NM_011066.3</td>
<td>Forward</td>
<td>5'-ATCTCCAGCGGTCGTTGAAG-3'</td>
<td>3650</td>
<td>303 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-AGGGTTACGTCTGGCCCTCT-3'</td>
<td>3952</td>
<td></td>
</tr>
<tr>
<td>Dbp</td>
<td>NM_016974.3</td>
<td>Forward</td>
<td>5'-AAGGCAAGGAAGTCCAGGT-3'</td>
<td>1104</td>
<td>324 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-TGGGACAAGGAAGTCAG-3'</td>
<td>1427</td>
<td></td>
</tr>
<tr>
<td>Homer1a</td>
<td>NM_011982.2</td>
<td>Forward</td>
<td>5'-GCAGGGAGGATGGAGCTGA-3'</td>
<td>1154</td>
<td>305 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-CAATAAATTAAGGAGTCTGACCTGA-3*</td>
<td>1458</td>
<td></td>
</tr>
<tr>
<td>Gapdh</td>
<td>NM_008084.2</td>
<td>Forward</td>
<td>5'-CCATCAACGACCCCTCATT-3'</td>
<td>139</td>
<td>321 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-TCTCGTGTTGACCCCATC-3'</td>
<td>459</td>
<td></td>
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<tr>
<td>Actb</td>
<td>NM_007393.3</td>
<td>Forward</td>
<td>5'-GCCGGGACCTGACAGACTAC-3'</td>
<td>624</td>
<td>430 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5'-ATGGTGCTAGGACGAGGACG-3'</td>
<td>1053</td>
<td></td>
</tr>
</tbody>
</table>

* The mouse *Per1* has two transcript variants, GenBank ID NM_011065.4 and NM_001159367.1. The primers are designed to amplify both variants without any discrimination, although only one reference ID was listed in the table.

# The mouse *Homer1* gene has three transcript variants. While the forward primer can be used for all three variants, the reverse primer is specific to the short variant, *Homer1a*. 
Figure 4.1. *Per2* and *Per1* mRNA levels are sleep/wake dependent in the cerebral cortex but not SCN as assessed by radioactive *in situ* hybridization. Gene-specific hybridization signals (mean ± SEM) measured from 6SD-treated mice (light gray bars) and their time-controls (6C, black bars) are shown, as measured in the SCN (A, D), dorsal (B, E) and piriform (C, F) cortices of the SCN-encompassing brain sections, as well as in the dorsal (B', E) and piriform (C', F) cortices of the brain sections ∼0.9 - 1.2 mm caudal to the SCN. * P < 0.05, ** P < 0.01, *** P < 0.001, 6SD vs. 6C; # P < 0.05, SCNx vs. intact. Note: Data used in Panels D, E, E', F, F' were adopted from (Liu et al., 2008).
Figure 4.2. Sleep-and-wake dependent gene expression in the rostral cortex as assessed by quantitative real-time PCR. Relative mRNA levels (mean ± SEM) of Per2, Per1, Dbp, and Homer1a were measured at ZT6 (A, C, E, G, respectively) and ZT8 (B, D, F, H, respectively). Data from animals underwent 6C (light gray), 6SD (black), 8C (white) and 6SD2R (dark gray) conditions are shown. * P < 0.05, ** P < 0.01, *** P < 0.001, sleep treatment (e.g., 6SD and 6SD2R) vs. control (e.g., 6C and 8C respectively); # P < 0.05, ## P < 0.01, ### P < 0.001, SCNx vs. intact.
Solving the health, social and economic problems related to inadequate sleep and sleep disorders presents a major challenge, especially in more industrialized societies. It requires continuing growth of our knowledge on the functions and regulations of sleep. With this aim, this dissertation provides insights into the circadian and homeostatic mechanisms of sleep regulation. I showed that CAST inbred mice exhibited advanced activity/rest and sleep/wake rhythms when compared to normal B6 mice. This circadian-sleep variant is similar to human advanced sleep phase syndrome. Genetic tools were utilized to confirm and refine a previously identified QTL, Era1, which contributes to the advanced circadian phase of sleep in CAST mice. In addition, I identified several circadian behaviors in CAST mice that co-varied with the advanced activity and sleep rhythms, including altered phase alignment between behavioral rhythms and the timing of photic phase-shifting responses as well as blunted light-induced suppression of activity (i.e., masking). At the molecular level, the peaks of clock-gene Per1 and Per2 expression in the cortex and PVH were advanced in coordination with the advanced behavioral rhythms in CAST mice, while the circadian oscillations of Per1 and Per2 in the SCN were not affected. These data revealed a previously unappreciated mechanism of circadian phase determination, which is located downstream of the master circadian clock. Meanwhile, I described more generally the behavioral-state dependent expression of Per1 and Per2 in the cerebral cortex. Sleep-and-wake affected cortical Per expression independent of the SCN: SCN expression of Per1 and Per2 remained unchanged after sleep deprivation, and sleep-and-wake
induced changes of cortical *Per* expression in SCN-lesioned (SCNx) animals were similar to intact controls, although SCNx elevated baseline *Per* expression and thus appeared to attenuate SD-induced increases in cortical *Per* expression. This interesting difference between SCNx mice and controls suggests that an additional component, which is likely to originate from the circadian outputs of the SCN, influences cortical Per expression in the intact animals. I thereby hypothesize that cortical Per expression integrates circadian and homeostatic sleep drive and allows for coordinated functions of sleep. Finally, I also described the dynamics of the cortical expression of a clock-controlled gene, *Dbp*, and a putative molecular component of the sleep homeostat, *Homer1a*, during sleep deprivation and subsequent recovery sleep in both SCNx and intact animals.

**CAST/EiJ: a novel animal model for circadian phase determination**

Circadian rhythms regulate sleep to occur at an adaptive time of the day. In humans, sleep predominantly occurs during the night and activity initiates around dawn. However, a wide range of variations in the timing of sleep and activity can be observed (Horne & Ostberg, 1976; 1977; Roenneberg *et al.*, 2003; Roenneberg *et al.*, 2007). These variations, when extreme, can be pathological, such as seen in the advanced and delayed sleep phase syndromes (ASPS and DSPS), hindering performance and health (Baker & Zee, 2000; Zisapel, 2001; Ando *et al.*, 2002; Sack *et al.*, 2007). Similar problems also occur with jet-lag and shift work (Arendt *et al.*, 2000; Monk, 2000; Davis & Mirick, 2006). These difficulties result from a conflict between the phases of endogenous circadian rhythms and the work/social demands.
Previous studies on circadian phase determination focused on mechanisms involving alterations in the circadian period. Activity rhythms in animals and humans with shorter endogenous circadian periods tend to entrain to the external light/dark cycle at an earlier phase (Pittendrigh & Daan, 1976b; Duffy et al., 2001). Particularly, animals and humans bearing a mutation that conveys a longer or shorter circadian period also lead to entrainment of the circadian rhythms at a delayed or advanced phase, respectively (Ralph & Menaker, 1988; Hamblen-Coyle et al., 1992; Toh et al., 2001; Xu et al., 2005). However, humans also exhibit variations in the preferences of morningness and eveningness that cannot be fully explained by the differences of individual circadian periods (Brown et al., 2008). In addition, the phase of behavioral rhythms failed to correlate with the length of endogenous period in this current study of the inbred CAST mice, previous study of (B6 x CAST) x CAST backcross mice (Wisor et al., 2007), or studies of B6 x CAST F2 progenies and wild-derived outbred castaneus mice (Kazu Shimomura, personal communication). Thus, mechanisms other than variations in circadian period must also affect circadian phase determination.

Indeed, a novel mechanism for circadian phase determination, located downstream of the master circadian clock, was found in inbred CAST mice. Despite an advanced phase of activity and sleep, CAST mice exhibited intact light-induced responses of behavioral phase-shifting and increase of Per1 and Per2 expression in the SCN. In addition, the circadian oscillations of Per1 and Per2 transcript levels in the SCN of CAST mice were essentially identical to B6 mice. Therefore, the cause of advanced behavioral rhythms in CAST mice cannot be located at the light input pathways or the core oscillatory mechanisms of the master clock. However, the timing of light-induced phase-shifting responses lagged in relation to the activity rhythm, indicating an altered coupling between the master circadian clock and its output. Thus, CAST mice provide a
unique model to study the circadian downstream mechanisms that regulates the phases of overt behavioral and physiological rhythms.

Although studies have revealed anatomical targets of SCN projections [for reviews, see (Mistlberger, 2005; Saper et al., 2005b)] and three candidates (e.g., TGFα, Prokineticin 2 and Cardiotrophin-Like Cytokine) for the diffusible SCN output molecules (Kramer et al., 2001; Cheng et al., 2002; Kraves & Weitz, 2006), a clear picture of how the timing information is transduced to and utilized by downstream "effectors" to produce overt behavioral and physiological rhythms is unfortunately not available. This is partly due to lack of genetic models suitable to searches for molecular components of circadian downstream mechanisms. With a pronounced early circadian phase rooted downstream of the clock and all the powerful genetic tools available in mice, CAST mice provide a unique model for these kinds of studies. As such an attempt, previous work from our lab identified a QTL, Era1, which accounted for 10% of the variance in the timing of activity in the (B6 x CAST) x CAST backcross population (Wisor et al., 2007).

In this current work, I verified this QTL and fine-mapped it within a 10 Mb genomic region, using B6.CAST congenic strains. Two genes within this region, Casein kinase 1-alpha 1 (Csnk1a1) and Gastrin-releasing peptide (Grp), are especially good candidates. Csnk1a1 is the homologue of two other casein kinase genes Csnk1d and Csnk1e, mutations of which have been shown to produce advanced activity phase by shortening the circadian period (Lowrey et al., 2000; Xu et al., 2005). However, my data and unpublished data from our lab have not found sequence variations between CAST and B6 that appear to have a functional impact in the coding sequence, intron-exon junctions, or <1000 bp region upstream of the transcription start site of the Csnk1a1 gene. Also, B6.CAST congenic mice exhibited a surprising longer circadian period along with the advanced circadian phase. Thus, should Csnk1a1 be the gene underlying Era1 QTL, it
may affect entrained circadian phase in a way distinct from that of its homologues.

Another candidate gene, \textit{Grp} is rhythmically expressed in the SCN (Aida \textit{et al.}, 2002). It encodes a 146-amino acid precursor peptide, which give rise to the amidated bioactive neuropeptide GRP$_{24-52}$. The functions of GRP (i.e., the amidated GRP$_{24-52}$, unless otherwise mentioned) in circadian biology have been extensively studied. Although GRP is thought to be mostly involved in the resetting of the master circadian clock by light (Aida \textit{et al.}, 2002; Gamble \textit{et al.}, 2007) while CAST mice exhibited intact photic resetting responses, studies also suggested that, in the absence of vasoactive intestinal polypeptide (VIP) signaling, GRP acutely synchronized the oscillations of individual SCN neurons and even promoted circadian rhythmic firing of single SCN neurons (Brown \textit{et al.}, 2005; Maywood \textit{et al.}, 2006). In addition, binding of GRP to its receptor was detected not only in the SCN but in brain regions outside of the SCN, such as the lateral hypothalamus and the amygdala (Karatsoreos \textit{et al.}, 2006). These findings thus may imply new roles of GRP within or even downstream of the circadian pacemaker. I sequenced the cDNA of \textit{Grp}, but did not find nonsynonymous variations between CAST and B6 in the region coding for the mature peptide GRP$_{24-52}$. However, I have not assessed the possibility of expression differences of GRP between CAST and B6 mice, and interestingly enough, one study found distinct phases of GRP mRNA oscillation in the SCN of nocturnal mice and a diurnal rodent (Dardente \textit{et al.}, 2004). In addition, I found a SNP leading to an amino acid substitution Arg80Ser located in a region of the propeptide that appears to be conserved from rodents to humans. Recent studies have shown that nonamidated peptides derived from proGRP may be also biologically active through a pathway independent of the classic GRP receptor [for a review, see (Ischia \textit{et al.}, 2009)]. Furthermore, \textit{Grp} expression in the SCN is restricted in the ventrolateral subregion (Okamura \textit{et al.}, 1986; Karatsoreos \textit{et al.}, 2004; Karatsoreos \textit{et al.}, 2006). Interestingly, I found \textit{Per2} expression in the ventrolateral tip (a region presumably
contains more Grp-positive neurons) but not other subregions of the SCN is affected by the interaction of strain (B6 vs. CAST) and time of day, an observation that further implies a possible role of Grp underlying the phenotypic differences between B6 and CAST. Taken together, it will be interesting to further investigate the candidacy of Grp for the Era1 QTL and to study whether nonamidated fragments of GRP propeptide have circadian functions.

Genetic architectures that underlies the circadian downstream mechanisms and the determination of circadian phase are likely to be complicated. The Era1 QTL accounts for 10% of the phenotypic variance in the backcross population. Although this is a relative large effect size for QTLs, it is likely that the timing of activity/rest and sleep/wake is affected by other genetic underpinnings, which probably consists of many loci with small individual effects, epistasis, and gene-environment interactions. In support of this, epistasis was indeed found in the previous study of (B6 xCAST) x CAST backcross mice between loci on chromosome 1 and 16 (Wisor et al., 2007). In addition, the (B6 xCAST) x CAST backcross mapping population works for loci at which CAST alleles are additive, but has no power to detect loci at which CAST alleles are dominant. Nonetheless, these issues could be overcome in future QTL studies by using more comprehensive experimental designs, better statistical models, and more powerful mapping panels such as the Collaborative Cross mice, in which CAST mice were also used as a founder strain (The-Complex-Trait-Consortium, 2004).

Last but not least, I also found impaired light-masking in CAST mice, in concert with their advanced activity. This change ensures normal (e.g., full amplitude) activity under light during the day, which is not possible for normal nocturnal mice. This may suggest an adaptation for the temporal niche in the Mus musculus castaneus subspecies, from which the inbred CAST mice were derived. In addition, I found Per1
and Per2 levels oscillated with an advanced peak in the cerebral cortex and PVN but not in the SCN and sPVZ of CAST mice, compared to B6 mice. These findings resemble those observed when comparing diurnal and nocturnal animals (Abe et al., 2001a; Mrosovsky et al., 2001; Takahashi et al., 2001; Meza et al., 2008; Ramanathan et al., 2010; Dzirbíková et al., 2011), but exhibit interesting detailed differences with those seen in nocturnal rats undergo "day-working" schedule (Salgado-Delgado et al., 2010). Most notably, the oscillation of PER1 protein levels in the PVN were not changed despite advanced timing of activity and food intake after continuous exposure to "day-working" for 5 weeks. I hypothesize that the difference between "day-working" rats and early runner CAST mice may represent the difference between the long-term consequences of forced activity (potentially related to the health problems of shift workers) and adaptation (presumably a healthy state). Even if CAST mice represent a "normal" adaptation, they nonetheless provide us a wonderful animal model for the study of circadian phase determination and circadian pathways downstream of the master clock, and may thereby lead to novel innervations relevant to ASPS/DSPS, jet lag, and shift work.

**Per1 and Per2 expression outside of the SCN: a bridge between circadian and homeostatic regulation of sleep?**

In early runner CAST mice, cortical expression of Per1 and Per2 oscillated with an advanced phase, which is correlated with their advanced activity/rest and sleep/wake rhythms while dissociated from the phase of clock-gene oscillations in the SCN. This finding is not necessarily surprising. As discussed before, cortical Per expression peaks during the (subjective) day in diurnal animals and during (subjective) night in nocturnal
animals, while in the SCN of all animals studied so far the peak of Per oscillations occurs in the middle of the (subjective) day (Abe et al., 2001a; Mrosovsky et al., 2001). In addition, daily activity patterns in mice can be altered under conditions such as circadian splitting, restricted feeding, and amphetamine administration. In such cases, cortical Per expression changes coordinately with the alterations in activity/rest and sleep/wake, while the expression of Per in the SCN remains unchanged (Masubuchi et al., 2000; Abe et al., 2001a; Wakamatsu et al., 2001). Last but probably most intriguingly, sleep deprivation elevates Per1 and Per2 transcript levels in the forebrain, especially in the cerebral cortex (Wisor et al., 2002; Cirelli et al., 2004; Franken et al., 2006; Franken et al., 2007; Mongrain et al., 2010). In this dissertation, I also gave direct evidence that waking-induced changes in Per expression is SCN-independent: Per expression in the SCN was not acutely affected by sleep deprivation, and sleep deprivation increases cortical Per expression in the absence of the SCN. Therefore, the advanced cortical Per oscillation in CAST mice, and perhaps more generally the daily rhythmic Per expression in the cortex of all mammals, may in fact largely reflect the cycles of activity/rest and sleep/wake, rather than represent direct SCN effector mechanisms.

Interestingly, detailed comparisons between SCNx and intact mice may also suggest a circadian component (likely to be originated from the SCN) for the regulation of cortical Per expression in the intact animals. Most notably, SCNx animals exhibited higher baseline expression of Per1 and Per2 at ZT6 compared to intact controls, which did not correlate with the behavioral and EEG indicators of sleep pressure (Easton et al., 2004; Bourgin et al., in preparation). It has long been assumed that the clock-genes in brain regions outside of the SCN as well as in the peripheral tissues represent the "downstream" circadian oscillators, which are influenced by the output signals from the
master pacemaker and impose circadian rhythmicities on physiological functions. However, as the circadian downstream mechanisms are not yet clear, evidence in favor of this notion is still lacking. My data might be the first piece of such evidence by showing a portion of cortical Per expression that did not correlate with sleep pressure in SCNx and intact animals. I hypothesize that cortical Per expression, as influenced by both sleep/wake status and the SCN outputs, integrates homeostatic and circadian sleep drives and regulates downstream gene expression in order to perform coordinated cellular functions of sleep in cortical neurons.

Waking/activity-dependent Per expression is not specific to the cortex. Unpublished data from our lab found that Per expression in the cerebellum and pons-medulla was also increased during prolonged waking (Passafiume and Liu, unpublished). Also, a comprehensive in situ hybridization study suggested that Per1 and Per2 expression during sleep deprivation was elevated in a similar fashion across the entire brain [(Thompson et al., 2010), also see (Franken et al., 2007)]. Therefore, I hypothesize that Per and presumably the entire the clock machinery outside the SCN may function "globally" as an integrator for homeostatic and circadian drive to allow coordinated sleep function. In the brain regions that are mostly active during sleep, such as the VLPO and nNOS-GABA positive cortical neurons (Sherin et al., 1996; Gerashchenko et al., 2008), Per expression may also correlate with neuronal activity—high during sleep and low during waking. It would be interesting for future studies to test these hypotheses.

The molecular mechanisms for sleep/wake dependent expression of Per1 and Per2 may be complicated. One of the possible mechanisms may rely on the interaction between the clock machinery and intracellular metabolism [for reviews, see (Rutter et al., 2002; Tu & McKnight, 2006; Green et al., 2008; Bass & Takeshita, 2010)]. Cellular metabolism is generally believed to vary across at distinct states of sleep and wake, and
restoring/regulating the balance of cellular metabolism may be a key function of sleep. In vitro studies have suggested that the formation and DNA-binding activities of CLOCK-BMAL1 and NPAS2-BMAL1 heterodimers are enhanced by the reduced form of the redox cofactors (i.e., NADH and NADPH) and suppressed by the oxidated forms [i.e., NAD$^+$ and NADP$^+$; (Rutter et al., 2001)]. In addition, a NAD$^+$-dependent protein deacetylase Sirtuin 1 (SIRT1) regulates the activity of the clock machinery via deacetylating PER2 and modulating CLOCK-mediated chromatin remodeling (Asher et al., 2008; Nakahata et al., 2008). On the other hand, clock machinery also influences cellular metabolism by controlling the expression of a rate-limiting enzyme, nicotinamide phosphoribosyltransferase (NAMPT), in NAD$^+$ biosynthesis (Ramsey et al., 2009). CLOCK-BMAL1 and NPAS2-BMAL1 may also regulate energy metabolism and the NADH/NAD$^+$ ratio via transcriptional regulation of an enzyme, lactate dehydrogenase-A (LDHA), whose metabolites, such as lactate, in turn affects redox state (Rutter et al., 2001; Rutter et al., 2002). Thus, under this mechanism, sleep/wake dependent expression of Per1 and Per2 may represent a compelling scenario for feedback the status of a sleep function to its regulation. However, if sleep/wake dependent Per expression is purely a consequence of sensing the metabolism state by the clock machinery, one would expect other clock-controlled genes to exhibit similar dynamics during sleep deprivation as Per1 and Per2. Inconsistent with this hypothesis, Dbp transcript levels are decreased after sleep deprivation, as shown here and previously (Wisor et al., 2002; Franken et al., 2007), and Ldha levels are not changed (Franken et al., 2007), although both genes are under transcriptional control of CLOCK-BMAL1 (Ripperger et al., 2000; Yamaguchi et al., 2000; Rutter et al., 2001). In addition to being regulated by CLOCK-BMAL1 via E-box motifs, the transcription of Per1 and Per2 is also regulated by glucocorticoid response elements [GREs; (Yamamoto et al., 2005; So et al., 2009)] and Ca$^{2+}$/cAMP response elements [CREs; (Travnickova-Bendova et al., 2002)]
located at their 5' upstream regions. Sleep deprivation induces a surge of plasma glucocorticoids (i.e., corticosterone) in mice and rats (Gip et al., 2004; Tartar et al., 2009; Mongrain et al., 2010), and adrenalectomy indeed reduced, but does not eliminate the increase in Per expression induced by sleep deprivation (Mongrain et al., 2010). Furthermore, sleep deprivation increases the brain level of activated CRE-binding protein [CREB; (Cirelli & Tononi, 2000; Graves et al., 2003)], which can activate transcription of CRE-controlled genes, including Per1 and Per2. Consistently, a comprehensive computational analysis of all the publicly available microarray data across sleep and wake identified CRE rather than E-box motifs as the central transcriptional regulatory element for sleep/wake dependent gene expression (Wang et al., 2010).

**Insights into molecular mechanisms of the sleep homeostat**

In addition to Per1 and Per2, hundreds of other genes have been shown to change during sleep deprivation (Cirelli et al., 2004; Terao et al., 2006; Mackiewicz et al., 2007; Maret et al., 2007; Mongrain et al., 2010; Thompson et al., 2010). However, most of these changes are likely to be responses of a change in sleep/wake state, not driving it. Thus, although gene expression studies, especially those utilizing microarrays, had great successes in forming testable hypothesis about the functions of sleep, they are much less powerful in the search for the molecular components of the sleep homeostat, which not only keeps track of the sleep pressure but more importantly regulates sleep and wake accordingly. Therefore, more convincing evidence for a gene to be involved in the sleep homeostat should come from genetic studies, such as the case for clock-genes. Disruption of Clock, Npas2, Bmal1, Dbp and Cry1,2 all lead to alterations in
sleep structure and/or homeostasis, and these alterations also appear to correlate with changes in *Per* expression in the brain [for reviews, see (O'Hara et al., 2007; Franken & Dijk, 2009)]. *Per1* and *Per2* double knockout mice only show small effects on sleep homeostasis [such as a prolonged process of recovery from elevated EEG SWA after sleep deprivation; (Shiromani et al., 2004; O'Hara et al., 2007)]. However, human *Per3* polymorphisms are associated with variations in multiple parameters of sleep homeostasis, including the amplitude of EEG SWA immediately after prolonged waking (Viola et al., 2007). Thus, it is possible that *Per* homologues have detailed functional differences and/or the absence of *Per1* and *Per2* was (in part) compensated for by *Per3*. Nonetheless, in my view, one piece of key evidence for *Per* as a part of the sleep homeostat is still missing: overexpression or knockdown of *Per* in vivo should quantitatively mimic the animal's responses as they were subjected to prolonged waking or sleep, respectively. It would be interesting for future studies to pursue this hypothesis.

Compared to the above-mentioned reverse genetic approaches, forward genetics screens provide much-less biased search for the machinery of the sleep homeostat. In fact, mutagenesis screens in *Drosophila* have found 2 genes, *shaker* and *sleepless*. Null-mutations of either gene cause extremely low amounts of daily sleep without affecting circadian rhythms (Cirelli et al., 2005; Koh et al., 2008). *shaker* encodes the alpha-subunit of a tetrameric potassium channel involved in the control of repolarization and release of neurotransmitters. *shaker* mutants exhibit intact sleep homeostatic responses to sleep deprivation, and thus *shaker* is likely to function as an effector of the sleep homeostat but not the homeostat itself. However, null-mutant of *sleepless*, which encodes a glycosylphosphatidylinositol-anchored protein with unknown function and no known vertebrate homologue, exhibit blunted homeostatic responses after prolonged waking. In mammals, a QTL that influences EEG SWA rebound induced by sleep...
deprivation was mapped to a 15 cM region on mouse chromosome 13, using C57BL/6J x DBA/2J recombinant inbred strains (Franken et al., 2001). The most likely candidate gene for this QTL is Homer1a (Maret et al., 2007; Mackiewicz et al., 2008b). Homer1a encodes a truncated negative regulator of the HOMER1b,c long isoforms, whose function may involve glutamate signaling and intracellular calcium homeostasis (Xiao et al., 2000; Worley et al., 2007). Homer1a transcript level in the brain is also elevated by sleep deprivation (Maret et al., 2007). In my study, I also compared cortical Homer1a expression during sleep deprivation in SCNx and intact animals, and found minimal impact from the SCN clock, at least at the two time points assessed. These data indicate that cortical Homer1a expression is mostly (if not all) driven by sleep and wake, and thus further support a role of Homer1 as a component of the sleep homeostat. Interestingly, study in rat somatosensory cortex (Nelson et al., 2004) and unpublished data from our lab using mice forebrain (Thomason, unpublished) suggest that the transcript levels of Homer1 long isoforms did not change much during sleep deprivation despite a pronounced increase of the short, negative transcript variant. Therefore, post-transcriptional mechanisms, such as activity-dependent alternative splicing, may play an important role in keeping track of the sleep pressure. This, again, is a fascinating direction for future study.
APPENDIX

LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADX</td>
<td>adrenalectomy</td>
</tr>
<tr>
<td>ASPS</td>
<td>advanced sleep phase syndrome</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>CAST</td>
<td>CAST/EiJ</td>
</tr>
<tr>
<td>CRE</td>
<td>calcium/cyclic AMP regulatory element</td>
</tr>
<tr>
<td>CREB</td>
<td>calcium/cyclic AMP regulatory element -binding protein</td>
</tr>
<tr>
<td>DD</td>
<td>constant dark</td>
</tr>
<tr>
<td>DSPS</td>
<td>delayed sleep phase syndrome</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalography</td>
</tr>
<tr>
<td>FASPS</td>
<td>familial advanced sleep phase syndrome</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early genes</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LD</td>
<td>light/dark</td>
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<tr>
<td>NREM</td>
<td>non-rapid eye movement (a sleep stage)</td>
</tr>
<tr>
<td>PRC</td>
<td>phase response curve</td>
</tr>
<tr>
<td>QTL</td>
<td>quantitative trait locus</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>REM</td>
<td>rapid eye movement (a sleep stage)</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>dSCN</td>
<td>dorsomedial SCN</td>
</tr>
<tr>
<td>vSCN</td>
<td>ventrolateral SCN</td>
</tr>
<tr>
<td>SCNx</td>
<td>SCN lesion (or SCN lesioned)</td>
</tr>
<tr>
<td>SD</td>
<td>sleep deprivation</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>sPVZ</td>
<td>subparaventricular zone</td>
</tr>
<tr>
<td>SWA</td>
<td>slow-wave activity (EEG spectra power density in the &lt;4 Hz band)</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VLPO</td>
<td>ventrolateral preoptic area</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable-number tandem-repeat</td>
</tr>
<tr>
<td>ZT</td>
<td>Zeitgeber time (ZT0 = lights-on; ZT12 = lights-off)</td>
</tr>
<tr>
<td>6SD</td>
<td>6-hour sleep deprivation</td>
</tr>
<tr>
<td>6C</td>
<td>ZT6 time-of-day control</td>
</tr>
<tr>
<td>6SD2R</td>
<td>2-hour recovery sleep after 6-hour sleep deprivation</td>
</tr>
<tr>
<td>8C</td>
<td>ZT8 time-of-day control</td>
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REFERENCES


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