INTERPLAY OF NEURONAL NETWORKS MODULATES MAMMALIAN CIRCADIAN RHYTHMS

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OUTLINE

This thesis is a collection of investigations on the mammalian circadian system. The first chapter of this thesis is a review of the literature on the biological clock of the suprachiasmatic nuclei (SCN), and provides evidence for a role of synchronization of SCN neurons in health. The review summarizes the current state of knowledge on coupling mechanisms involved in (*i*) neuronal synchronization, (*ii*) the effects of light on the SCN and (*iii*) extra-SCN feedback systems. Additionally, it is discussed how these mechanisms are affected in circadian disorders that occur in aging and in disease.

The SCN's electrical activity ensemble follows a sinusoidal-like pattern that peaks during the day and is low during the night (1,2). The amplitude of the SCN's electrical activity rhythm is influenced by the external photoperiod, such that the amplitude increases in short days and decreases in long days (3-5). In chapter 2 of this thesis, the effect of the photoperiod on the ability of the SCN to synchronize to the external light-dark cycle by shifts of the rhythm (i.e.phase shifting capacity) is investigated by subjecting mice to a 4 hour phase advancing shift of the light-dark cycle after exposing them to a long or short photoperiod. Also, electrophysiological measurements are performed on SCN from mice following exposure to a 4 hour phase advancing shift of the light-dark cycle.

The electrical activity displayed on the SCN tissue level consists of electrical activity patterns of single SCN neurons (3,6). Single cells are active for a relatively short period (4 – 8 h), of which the majority of the neurons are active during the day and few are active during the night. Accumulating evidence indicates that plasticity of the phase distribution among single cell activity patterns underlies the seasonal adjustments in SCN electrical activity rhythm and waveform (4,7,8). In chapter 3 mathematical modelling approaches are utilized to determine whether SCN phase shifting characteristics are dependent on synchrony among the SCN neurons and how the proportion of stimuli-receiving neurons determines the magnitude of the SCN shift.

The major external stimulus affecting the SCN pacemaker is light. Light is detected in the outer retina by rod-and cone photoreceptors and in the inner retina by intrinsically photosensitive retinal ganglion cells (ipRGCs) containing melanopsin. Different types of photoreceptors are maximally sensitive for different wavelengths of light. For example, the retina of mice contains the short wavelength cone that has a maximum sensitivity in the UV light range (λ_{max} 360 nm) (9). The rod photoreceptor and the mid wavelength cone have a maximally sensitive to green light (λmax 508 nm) (10), whereas melanopsin is most sensitive to blue light (λmax 480 nm) (11,12). All light information detected by cones, rods and melanopsin are integrated at the level of the pRGCs, and transmitted to the SCN via the retinohypothalamic tract

(RTH). In the absence of rod-and cone photoreceptors ipRGCs can still respond to light and this light response is sufficient to entrain the SCN(13). The experiments of chapter 4 are aimed to explore the ability of rod- and cone photoreceptors to mediate the effects of light on SCN neuronal activity in the absence of melanopsin. Mice lacking melanopsin are able to entrain to light, indicating that rod and cones are also functional circadian photoreceptors (14,15). In chapter 5 we investigate to what extent cone photoreceptors contribute to photic entrainment of the SCN by performing SCN electrophysiological measurements in the SCN of mice that have only cones as functional photoreceptors.

While light is the main synchronizer of the SCN, SCN's rhythmicity is also influenced by non-photic factors such as the animal's own behavioral activity level. *In vivo* measurements of the SCN's electrical activity in freely-moving night-active rodents revealed that behavioral activity induces acute suppression of the SCN's firing rate (16-18). Because night-active animals display elevated levels of activity during the night the SCN firing rate during the night is suppressed even more. Consequently, SCN rhythm amplitude increases, which improves rhythmicity at the periphery. In chapter 6 the effect of behavioral activity on the amplitude of the circadian rhythm in SCN electrical discharge rate in a day-active animal will be assessed by performing long term *in vivo* electrophysiological measurements in the SCN of the freely-moving day-active grass rat (*Arvicanthis ansorgei*).

In nocturnal rodents an inverse relation exists between the SCN electrical activity rhythm and the temporal behavioral activity pattern (19,20). For example, in short days the SCN shows a compressed electrical activity rhythm (i.e. narrow peak-width) while the behavioral activity pattern is decompressed. On the other hand, in long days the SCN displays a more decompressed discharge rhythm (i.e. broad peak-width) while the behavioral activity pattern in compressed. In nocturnal animals the SCN electrical activity rhythm and the behavioral activity rhythm are anti-phasic, while in day-active animals these rhythms are in phase (21). The study presented in chapter 7 is aimed at the investigation of the regulation of temporal activity patterns by the SCN's electrical activity in a day-active rodent, by performing *in vivo* and *in vitro* recordings of SCN neuronal activity in day-active grass rats (*Arvicanthis ansorgei*).

Besides their role as a daily clock, the SCN function as a seasonal clock and convey day length information to the pineal gland and other parts of the central nervous system (22-25). The SCN encode photoperiod information by changing the phase distribution of activity patterns of single SCN cells. Underlying the seasonal adaptations are changes in neurotransmitter profiles and clock gene expression patterns (25-29). The literature study presented in chapter 8 discusses the unique light response properties of SCN neurons that are essential to measure the photoperiod. It will be shown that the SCN's heterogeneous organization is required

for storing photoperiod information, and that efferent pathways are involved in orchestrating seasonal responses in physiology.

The significance of the studies of this these, and future perspectives will be discussed in chapter 9.

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PART I

NEURONAL SYNCHRONIZATION

1

SYNCHRONIZATION OF BIOLOGICAL CLOCK NEURONS BY LIGHT AND PERIPHERAL FEEDBACK SYSTEMS PROMOTES CIRCADIAN RHYTHMS AND HEALTH

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Abstract

In mammals, the suprachiasmatic nucleus (SCN) functions as a circadian clock that drives 24-hour rhythms in both physiology and behavior. The SCN is a multicellular oscillator in which individual neurons function as cell-autonomous oscillators. The production of a coherent output rhythm is dependent upon mutual synchronization among single cells and requires both synaptic communication and gap junctions. Changes in phase synchronization between individual cells have consequences on the amplitude of the SCN's electrical activity rhythm, and these changes play a major role in the ability to adapt to seasonal changes. Both aging and sleep deprivation negatively affect the circadian amplitude of the SCN, whereas behavioral activity (i.e., exercise) has a positive effect on amplitude. Given that the amplitude of the SCN's electrical activity rhythm is essential for achieving robust rhythmicity in physiology and behavior, the mechanisms that underlie neuronal synchronization warrant further study. A growing body of evidence suggests that the functional integrity of the SCN contributes to health, well-being, cognitive performance, and alertness; in contrast, deterioration of the 24-hour rhythm is a risk factor for neurodegenerative disease, cancer, depression, and sleep disorders.

1. Introduction

The rotation of the Earth around its central axis causes a daily rhythm in environmental factors, including light intensity, temperature, and food availability. In order to anticipate these 24-hour changes in the environment, many species have evolved an internal clock. In mammals, this internal clock resides in the suprachiasmatic nucleus (SCN) in the ventral hypothalamus (1). The SCN is a bilateral structure containing 20,000 neurons that generate circadian rhythms. The SCN synchronizes its circadian rhythm to the external day-night cycle using light information that is projected via the retinohypothalamic tract (RHT). This information is then conveyed to other regions in the central nervous system (2,3). Based on neuropeptide expression, the SCN is sub-divided into the dorsal (i.e., shell) and ventral (i.e., core) SCN (4-7). The ventral SCN expresses gastrin-releasing peptide (GRP) and vasoactive intestinal polypeptide (VIP) (1,8), whereas the dorsal SCN contains the hormone vasopressin (9). Moreover, the dorsal SCN receives strong input from the ventral SCN (9), whereas the ventral SCN receives little input from the dorsal SCN (10).

Individual cells in the SCN generate a circadian rhythm via a series of interconnected positive and negative feedback loops; these feedback loops regulate the transcription and activity of clock genes and proteins, respectively (11,12). One feedback loop is regulated by the transcription factors Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-like protein 1 (Bmal1). These proteins drive the transcription of specific target genes in the Period (*Per1*, *Per2*, and *Per3*) and Cryptochrome (*Cry1* and *Cry2*) gene families; in turn, Per and Cry proteins inhibit CLOCK/Bmal1-mediated transcription. Another feedback loop consists of the nuclear receptors ROR (α, β, and γ), PPARα, and REV-ERB (α and β). Dissociated SCN cells retain a circadian rhythm in their electrical firing rate, with a relatively wide range of intrinsic periods (ranging from 22 hours to 28 hours) (13-15). This ability of isolated neurons to maintain their intrinsic rhythm indicates that individual SCN neurons function as autonomous single-cell oscillators driven by intrinsic molecular feedback loops (14). The key implication of this finding is that the SCN's multicellular structure depends upon cooperation among individual neurons in order to function effectively as a coherent pacemaker. In this review, we will discuss the mechanisms that underlie synchronization, and we will discuss the relevance and significance of synchronization for health and disease. Next, we will discuss the consequences of disrupted SCN synchronization on aging, sleep disorders, neurodegenerative diseases, and metabolic disorders. In addition, positive effects of physical exercise on SCN rhythm amplitude will be discussed. Finally, we will compare the organization of the SCN of nocturnal and diurnal species with special focus on potential differences in synchronization mechanisms.

2. Synchronization of SCN neurons

2.1 Phase shifts in the SCN are driven by synchronized SCN neurons

Both *in vitro* and *in vivo* recordings of SCN firing frequency revealed that the SCN's electrical activity output has a sinusoidal-like waveform pattern that peaks during the subjective day and is low during the subjective night (16,17). In nocturnal animals, the trough of the SCN's electrical activity corresponds with the animal's behaviorally active phase (18-20). In diurnal animals, this relationship between electrical activity and behavioral activity is reversed; thus, the SCN's activity peaks in phase with the behaviorally active phase (21). Recordings in the SCN of freely moving mice revealed close correspondence between the SCN's pattern of electrical activity and the animal's behavioral activity. Specifically, the behavioral transitions from rest to activity—and vice versa—occur at the mid-point in the declining and increasing slopes in SCN activity, respectively (22). The most intense level of behavioral activity occurs during the trough in electrical activity rhythm, and silencing activity in the SCN by applying tetrodotoxin during the animal's resting phase induces behavioral activity (23).

A subpopulation of SCN neurons (comprising 32% and 38% of SCN neurons in rats and hamsters, respectively) exhibit light-induced changes in electrical activity (24-29). At low intensities (0.1 lux in rats and 1 lux in hamsters), light can suppress electrical activity, whereas high light intensity increases the rate of neuronal firing in an intensity-dependent manner (25). In response to external light, the clock genes *Per1* and *Per2* are induced in the SCN (30-34), and the duration of this induction is dependent upon the intensity of the light (35). Glutamate is the primary neurotransmitter used by the RHT to project light information to the SCN (36). The application of glutamate—or agonists of the glutamate receptor—to the SCN causes a phase shift in the SCN's electrical activity that mimics light-induced phase shifts in behavior (37-39) and alters the levels of *Per1* and *Per2* mRNA (34). The SCN's rhythm is synchronized to the daily light-dark cycle by the phase-shifting effects of light on the SCN. Early in the subjective night, light has a phase-delaying effect; in contrast, light in the late subjective night causes a phase advance (40-42). These phase-dependent responses are fundamental to the animal's ability to entrain to a new light cycle, and are present in all living organisms that exhibit circadian rhythmicity. Whether a light-induced phase delay or phase advance will occur depends upon intercellular signaling cascades and is therefore an intrinsic property of the SCN (43-46).

Following exposure to a shift in the light-dark cycle (for example, by crossing time zones), the SCN generally takes several cycles to resynchronize (47-52). Recordings of the SCN's firing rate in the rat revealed that delay-shifting the light-dark cycle by six hours induces a transient bimodal electrical activity rhythm in the SCN (47). One component of this bimodal activity pattern reflects the activity of a group of

neurons that synchronize immediately to the new light-dark cycle, whereas the other component reflects the activity of neurons that remain synchronized to the previous light-dark cycle. Separation of the ventral SCN from the dorsal SCN by surgical incision revealed a unimodal electrical activity pattern in both regions and revealed that the shifted and non-shifted components are generated by the ventral and dorsal SCN, respectively. Surprisingly, following a shift in the light-dark cycle, the electrical activity profile of the ventral SCN (i.e., the shifted component) is considerably more narrow than the electrical activity profile of the dorsal SCN (i.e., the non-shifted component). Curve-fitting analysis revealed that the narrow, shifted component is composed of the electrical output produced by only 20% of the entire SCN's neuronal population (53). Furthermore, simulations revealed that the ventral SCN's narrow electrical activity profile is not due to the low number of neurons that contribute to this component, but is actually the result of high synchrony among these neurons (54). The differences in light-induced phase shifts between the ventral and dorsal SCN could be the result of differential innervation by the optic nerve (5,55,56). In rats, the ventral SCN receives the majority of light input projected by the retina (3,6,7) and has more pronounced light-evoked changes in terms of electrical activity (57,58) and gene expression (59-64). In contrast, the dorsal SCN is only sparsely innervated by the retina (1).

2.2 Robustness of the SCN's output is correlated with synchrony among SCN neurons

In vivo recordings of SCN electrical activity revealed that the SCN's waveform pattern differs between long and short photoperiods (65). The changes in the SCN's electrical activity waveform in response to a change in photoperiod correspond with observable behavioral adaptations. In both long and short photoperiods, the onset and offset of behavioral activity occur at the mid-point threshold in the declining and rising slopes of electrical activity, respectively (65). Even in continuous darkness, the SCN retains its intrinsic photoperiod-induced waveform for several cycles, suggesting that the SCN has a photoperiodic memory. Furthermore, the photoperiod-induced waveform is preserved even after the SCN has been isolated *in vitro*. After entraining to a short photoperiod, the SCN's ensemble discharge rate has a waveform with a short duration of enhanced activity; in contrast, after entraining to a long photoperiod, the SCN's waveform has a long period of enhanced activity (65,66). Computational studies showed that changes in phase synchronization are extremely effective in terms of inducing a change in waveform, whereas changes in single-cell activity patterns have relative weak effects (67,68). Experimental studies have confirmed that nature indeed functions in this way. Both single-cell and subpopulation recordings revealed that after entraining to a short photoperiod, the activity of individual SCN neurons is more synchronized and clusters around subjective midday. On the other hand, after

entraining to a long photoperiod, the activity of SCN neurons is less synchronized (Figure 1).

In the dorsal SCN, the electrical activity patterns differ somewhat between short and long photoperiods; however, computational studies revealed that these differences in single-cell activity patterns in the dorsal SCN are not sufficient to explain the waveform changes that occur at the network level (67,69). However, the narrow phase distribution of subpopulation activity during short photoperiods does explain the narrow peak width in the SCN's waveform, whereas the broad phase distribution during long photoperiods explains the broad peak width in the SCN's waveform (65,66). These findings are supported by molecular studies that show similar single-cell *Per1* expression patterns after entrainment to either long or short photoperiods (70). The results from electrophysiological, computational, and molecular studies indicate that synchrony between individual SCN neurons—rather than a change in the activity pattern of those individual neurons—is important for adapting to a change in photoperiod.

2.3 The amplitude of the SCN's output correlates with the SCN's phase-shifting response

In the 1980s, Pittendrigh and colleagues reported that the photoperiod to which hamsters were entrained affected the resulting amplitude of the phase-response curve. Thus, hamsters that were entrained to a short photoperiod had a larger light-induced phase shift compared to hamsters that were entrained to a long photoperiod (71). More recent studies support these early findings (72-74). The difference in the light-induced behavioral phase shift between short and long photoperiods is not the result of a difference in retinal response; rather, the difference occurs at the level of the SCN. Bath application of the glutamate receptor agonist *N*-methyl-D-aspartate (NMDA) to an SCN entrained to a short photoperiod causes a significantly larger phase delay compared to an SCN that was entrained to a long photoperiod (74). Moreover, the acute effect of applying a pulse of NMDA is similar between a short photoperiod-entrained SCN and a long photoperiod-entrained SCN, suggests that the difference in the phase delay in the SCN is not caused by desensitization to glutamate in long photoperiods (74); thus, another mechanism must explain these results.

The amplitude of the SCN's electrical rhythm is high when the neurons in the SCN are more synchronized (i.e., when entrained to a short photoperiod), whereas the amplitude of the rhythm is low when the neurons are less synchronized (54,65,67,75). Results from both behavioral and *in vitro* studies revealed that an SCN with high amplitude exhibits a larger shift in response to a given perturbation compared to an SCN with a low rhythmic amplitude (i.e., from a long photoperiod) (74). These results are surprising, as they do not intuitively match predictions that arise from

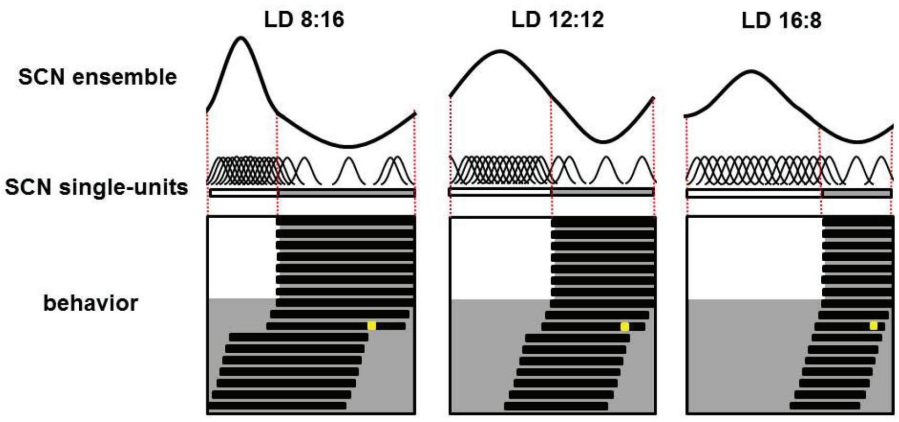

Figure 1. Plasticity of the SCN. Schematic overview of the SCN's ensemble rhythm (top), single-cell activity in individual SCN neurons (middle), and the animal's behavioral activity pattern (bottom) in a nocturnal rodent entrained to a short (LD 8:16; left column), medium (LD 12:12; middle column), or long (LD 16:8; right column) photoperiod. In the lower panels, days are depicted on successive lines, and light and dark periods are represented by white and grey backgrounds, respectively. Following a transition to continuous darkness, aftereffects of the photoperiod on behavioral activity can be detected. Following a short photoperiod, the animal's active phase longer; in contrast, following a long photoperiod, the active phase is shorter. A light pulse applied late in the subjective night in the third cycle of continuous darkness (the yellow square) results in a large-magnitude phase advance in animals entrained to a short photoperiod, a smaller phase advance in animals entrained to a medium photoperiod, and virtually no phase advance in animals entrained to a long photoperiod (phase advance is measured as the difference in activity onset before and following the light pulse). For each photoperiod, the SCN's ensemble electrical activity rhythm is depicted in the top row. The relative amplitude of the SCN ensemble rhythm is large in short photoperiods and small in long photoperiods; this difference is due to different levels of synchronization among the electrical activity patterns of the individual neurons. The single-unit activity traces are highly synchronized in animals entrained to a short photoperiod, and less synchronized in animals entrained to a long photoperiod (middle row).

limit cycle oscillator theory, a theory that is often used to model the phase-shifting behavior of oscillators. The limit cycle model predicts that following a perturbation of a given magnitude, oscillators that oscillate with a high amplitude will shift to a lesser degree than oscillators that oscillate with a lower amplitude (71,76,77). This prediction holds true for primitive organisms such as *Gonyaulax* (78) and *Neurospora* (79,80). However, the experimental finding that an SCN with a high-amplitude oscillation shifts to a greater extent than an SCN with a low-amplitude oscillation is not consistent with the predictions of limit cycle theory. This apparent discrepancy between theory and practice may be due to the SCN's functioning at the level of a network. In a population of highly synchronized neurons, each individual neuron will be more in phase, and an external perturbation of the system will cause a similar

phase-shifting response in the individual cells, thereby driving a large net shift in the SCN network. In a relatively desynchronized SCN, the individual neurons will be out of phase, and an external perturbation will induce different phase-shifting responses among the individual neurons, thereby causing a relatively small net shift in the SCN network (81). Simulations have confirmed this prediction with surprisingly high accuracy (62). In Afh/Afh mice the amplitude of the SCN ensemble is reduced by a reduction of the amplitude of single cell oscillations (82). In accordance to the limit cycle theory, the Afh/Afh mice show high amplitude resetting to light. To explain light-resetting by the SCN both the amplitude of single cell oscillations as well as phase-synchronization among single cells should be taken into consideration.

Under certain conditions, the SCN—as a network—can behave as a limit cycle oscillator. Simulation studies showed that the phase-shifting response of the SCN is opposite to the predictions of a limit cycle oscillator if just a fraction of the network is directly influenced by the perturbation (e.g., if light affects only 20% of the population). On the other hand, if all of the neurons in the SCN network are affected by the perturbation (for example, a change in temperature, which would affect 100% of the neurons in the population), the SCN can exhibit the behavior predicted by limit cycle oscillator theory (83). Thus, although limit cycle theory accurately predicts the behavior of an *individual* oscillator, the phase-shifting behavior of an entire *network* of oscillators is more difficult to predict, as such behavior is dependent upon the degree of synchrony among the individual oscillators and the percentage of neurons that will respond to the perturbation (i.e., a 100% response rate to temperature versus a 20% response rate to external light).

2.4 The role of chemical coupling in SCN neuronal synchronization

Several neurotransmitters play a role in the phase synchronization of SCN neurons (Figure 2). For example, γ-aminobutyric acid (GABA) is the most prevalent neurotransmitter in the SCN. In the adult SCN, activation of $GABA_A$ receptors causes an inhibitory response (84). This inhibitory effect of GABAergic signaling often plays a role in synchronizing neuronal networks within the brain (13,85-87). Examining synchronization in SCN slices in the presence or absence of a $GABA_A$ signaling blocker revealed that GABA plays a role in synchronizing SCN neurons. When slices prepared from a desynchronized SCN were treated with a $GABA_A$ signaling blocker, the SCN neurons remained desynchronized; in contrast, in the absence of the blocker, the neurons became synchronized again (88). Although GABA acts predominantly as an inhibitory transmitter in the adult brain, it can play an excitatory role when coupled with the activity of the NKCC1 chloride pump (89,90). GABAergic transmission is also excitatory in the dorsal SCN (47,90,91), and this excitation may play a role in communication between the ventral and dorsal SCN (47). Recently, we reported that GABAergic excitatory transmission is more prevalent in a desynchronized

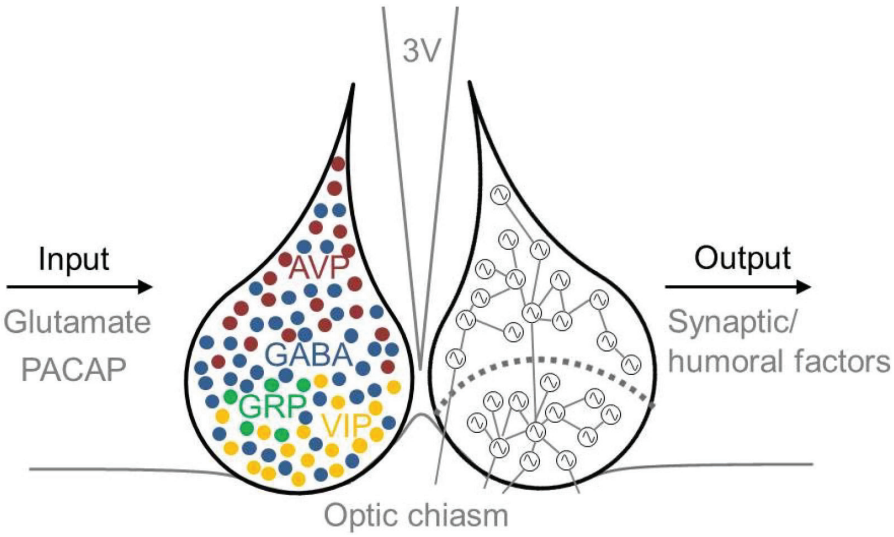

Figure 2. Schematic illustration of the mammalian SCN, including inputs and outputs. Input to the SCN is mediated by the neurotransmitters glutamate and PACAP, and output from the SCN is mediated by synaptic and humoral factors. The approximate locations of specific neurotransmitter-expressing neurons are indicated in the left nucleus. GABA (blue) is co-expressed in other neuronal cell types, including VIP- (yellow), AVP- (red), and GRP- (green) expressing neurons. AVP-expressing cells are located primarily in the dorsal SCN, and VIP-expressing cells are located primarily in the ventral SCN. The SCN depicted on the right schematically shows the ventral and dorsal SCN subdivision (the curved dotted line), and coupling between rhythmic SCN neurons is indicated by lines connecting the cells. Many neurons in the ventral SCN are directly innervated by afferent fibers arising from the optic chiasm, whereas far fewer neurons in the dorsal SCN receive direct light information input. The ventral SCN has direct input to the dorsal SCN, while the dorsal SCN is only sparsely innervated by the ventral aspect. Each neuron is depicted schematically as a cell-autonomous single-cell oscillator. Abbreviations: 3V, third ventricle; AVP, vasopressin; GABA, γ-aminobutyric acid; GRP, gastrin-releasing peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; SCN, suprachiasmatic nucleus; VIP, vasoactive intestinal peptide.

SCN than in a synchronized SCN (40% vs. 28%, respectively) (92), which suggests that the inhibitory/excitatory ratio of GABAergic activity plays a role in the phase synchronization of individual SCN neurons.

The primary neurotransmitter in the ventral SCN is vasoactive intestinal peptide (VIP). Intrinsically photoreceptive retinal ganglion cells (ipRGCs) contain the photopigment melanopsin and convey light information to the SCN via the RHT (93) (94,95). VIP-containing neurons process light information received from the RHT and then transfer this information to the dorsal SCN (45,96). The RHT contains both glutamate and PACAP (pituitary adenylate cyclase-activating polypeptide). The application of glutamate has an excitatory effect on SCN neurons, whereas

glutamate receptor antagonists inhibit light-induced responses both *in vivo* and *in vitro* (97). Eliminating glutamate from ipRGCs impairs photo-entrainment of behavioral rhythm in mice (98); similarly, eliminating PACAP or its receptor also impairs photo-entrainment (99,100). Although eliminating VIP reduces the light-induced upregulation of the clock gene *Per1*, photic induction of *Per1* is unimpaired in PACAP-deficient mice (99). Thus, VIP-expressing cells are critically important for relaying externally received light information to the SCN network. Eliminating VIP or the VIP receptor (VIP2R, also known as VPAC2) reduces SCN electrical activity (101), molecular rhythms (102,103), and behavioral rhythms (104-107). In contrast, application of VIP mimics light-induced responses in the SCN (108,109), drives long-lasting increased electrical activity in dorsal SCN neurons (110), and restores synchrony among SCN neurons in VIP-knockout mice (102-104). Finally, the SCN in VIP-knockout mice does not exhibit photoperiod adaptation. Taken together, these compelling findings indicate that VIP is important both for SCN neuronal synchronization and for the ability of the SCN to encode photoperiod-related information (111).

A subpopulation of neurons in the ventral SCN express gastrin-releasing peptide (GRP), and these neurons are important for conveying information regarding external light throughout the SCN (5,8,102,103). In Syrian hamsters, *in vivo* microinjections of GRP into the third ventricle induces the expression of *c-fos*, *Per1*, and *Per2* in the dorsal SCN (8). GRP receptor–knockout mice have reduced light-induced phase shifts and reduced induction of *Per* and *c-fos* expression in the dorsal SCN (112). The *in vitro* application of GRP to SCN slices induces a light-like phase shift in the SCN (113); moreover, applying GRP to SCN slices from VIP receptor–knockout mice increases synchrony among SCN neurons (5,114).

The majority of neurons in the dorsal SCN express the neuropeptide vasopressin (AVP). This expression is rhythmic and is driven by the intrinsic molecular feedback loop in the core clock machinery. The *in vitro* application of AVP to SCN neurons isolated from VIP-deficient mice restores the rhythmicity and synchrony of the neurons (103). Furthermore, the expression pattern of AVP is different after entraining to a long photoperiod (i.e., in a desynchronized network) than after entraining to a short photoperiod (i.e., in a more synchronized network) (115,116).

Connectivity within the SCN network is surprisingly plastic. In addition to seasonal plasticity, the SCN also exhibits a daily rhythm of synaptic connectivity. Measuring the firing rates of individual SCN neurons in the presence or absence of $GABA_A$ receptor antagonists revealed that SCN connectivity is dependent upon GABAergic communication, and the strength of this connectivity can change in a matter of days or even hours (117). Recently, confocal microscopy studies revealed that synaptic changes occur in VIP-expressing neurons in the SCN's retinorecipient region, but not in AVP-expressing neurons in the non-retinorecipient region (118). The authors

hypothesized that remodeling of the synaptic connectivity in VIP-expressing neurons over a 24-hour cycle might contribute to the ability of these neurons to adapt to light, thereby increasing the efficacy of photic transmission (118). In addition, during the subjective night, the neuron-glia network in the SCN undergoes morphological rearrangements (119), and during the day, axon terminal coverage of VIP-expressing neurons increases (119). These findings indicate that the SCN is a remarkably plastic structure that can efficiently adapt its network structure in response to changes in functional needs.

2.5 The role of electrical coupling in synchronization of SCN neurons

The SCN network contains a large number of gap junctions that mediate electrical synchronization among SCN neurons (19,120-123). Gap junction–mediated coupling improves the connectivity of neuronal networks (124,125) and plays a role in the synchronization of several brain areas (121,126-131). In the absence of chemical synaptic transmission, 24-26% of SCN neurons in rodents exhibit synchronous electrical activity (28,121,132); moreover, tracer-coupling experiments showed that 30% of SCN neurons are coupled via gap junctions (28,120). In mice, gap junctions couple neurons in both the ventral and dorsal SCN, and clusters of coupled cells are restricted to each subdivision (120). In the mammalian CNS, the protein connexin-36 (Cx36) is a key component of gap junctions (131), and Cx36-knockout mice have significantly reduced electrical coupling in the SCN (121). Moreover, Cx36-knockout mice that were housed in continuous darkness had significantly reduced wheel-running activity compared to wild-type mice housed under the same conditions, suggesting that gap junctions play a role in the circadian organization of locomotor activity rhythms (121). Although immunofluorescence microscopy and immunogold labeling experiments confirmed that Cx36 is expressed abundantly in the postnatal SCN, tracer coupling and electrical coupling (i.e., coupled spiking) is relatively weak between SCN neurons in adult animals (122) compared to young animals (28,120,121). Interestingly, recent findings suggest that VIP increases gap junction–mediated coupling, as the application of VIP increased coupling efficiency between SCN neurons in which chemical coupling was blocked (123). This recent result supports the notion that the balance between chemical and electrical communication in the SCN is important for modulating synchronous rhythms, and disruption of only one form of communication can disrupt the circadian rhythmicity of the SCN and the periphery.

3. SCN neuronal synchrony is disrupted in aging and disease

3.1 Aging and the SCN

With aging, many species—including humans—experience changes in circadian timing. These changes are manifested as a reduction in the behavioral activity rhythm and in disruptions of the sleep-wake cycle (133-136). Given that the output of the SCN drives rhythms in behavior and physiology, these age-related disturbances in circadian rhythmicity could be caused by age-related deficits in the SCN. In support of this notion, transplanting fetal SCN tissue into the anterior hypothalamus of aged animals improves circadian rhythmicity in both hamsters (137,138) and rats (139). *In vivo* recordings of electrical activity revealed reduced circadian amplitude in the SCN of middle-aged mice compared to young mice. Moreover, the amplitude of electrical activity rhythm in the subparaventricular zone—which receives input from the SCN—is substantially reduced in aged animals (140). Specifically, the amplitude of the SCN's electrical activity rhythm in aged animals is approximately half of the amplitude in young animals (136) (Figure 3). This reduced amplitude cannot be explained simply by a loss of SCN neurons in aged mice (141) or rats (142), and experimental data suggest that age-related deficits in coupling between SCN neurons underlies the decrease in amplitude (136,143).

Experiments have also revealed that SCN neurons in aged animals have an altered pattern of electrophysiological activity (136,143-147). For example, the activity patterns of individual cells are less synchronized in the SCN of aged mice compared to young mice, and SCN neurons in aged mice even have anti-phasic activity (136). Computational studies found that decreased coupling in the aged SCN can lead to reduced synchrony among SCN neurons, thereby reducing the amplitude of the SCN's network output (148). Physiologically, aging causes a clear change in the expression of neurotransmitters in the SCN. For example, the number of VIP- and AVP-expressing neurons is decreased in the SCN of aged rats (142,149). In addition, the amplitudes of the circadian expression levels of *VIP* mRNA (150) and *VPAC2* mRNA (151) decline with aging. Functionally, *in vitro* patch-clamp recordings of SCN neurons from aged mice revealed that postsynaptic GABAergic currents are lower in both frequency (143) and amplitude (136) compared to recordings from young SCN neurons. Furthermore, the number of GABAergic synaptic terminals in the SCN of aged mice is reduced by 26% compared to young animals (152). These findings are relevant to humans as well, as neurotransmission decreases with aging. In elderly people, the number of VIP-expressing SCN neurons is reduced (153), and vasopressin levels are reduced in the SCN of elderly people from the age of 80 years (154). This age-associated decline in neurotransmission in the SCN could affect both the quality and degree of synchronization among SCN neurons.

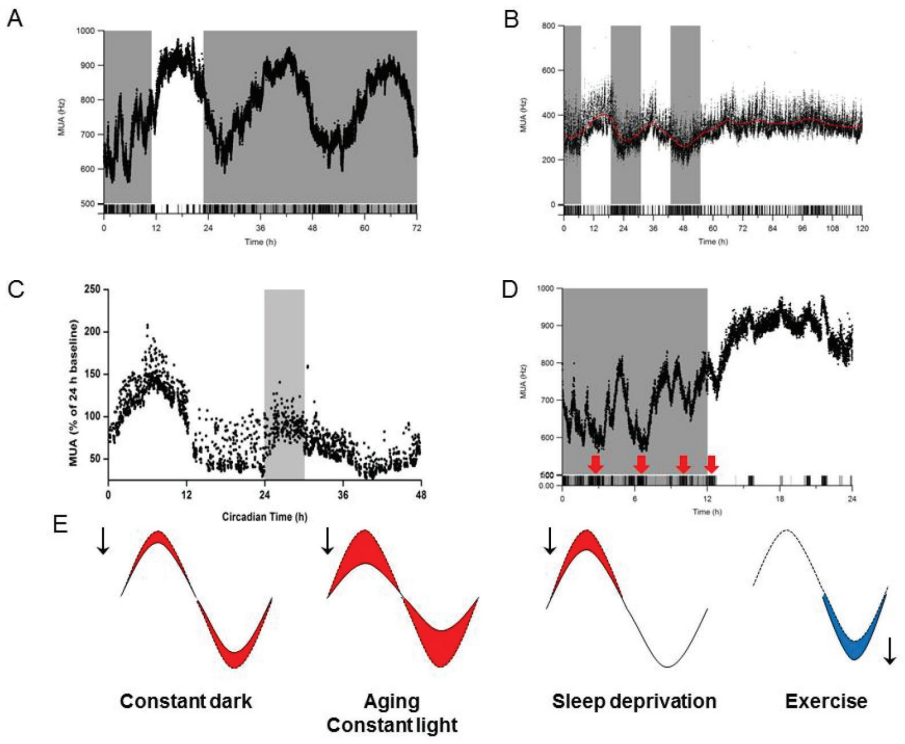

Figure 3. Influence of environmental conditions on SCN amplitude. Effects of (**A**) continuous darkness (212), (**B**) continuous light (212), (**C**) sleep deprivation (170), and (**D**) behavioral activity/physical exercise (254) on SCN neuronal activity measured using long-term *in vivo* recording of the SCN in freely moving mice. In **A-D**, the *x*-axis indicates (circadian) time (in hours), and the *y*-axis shows the electrical activity of the SCN ensemble (MUA, or multiunit activity) recording. (**E**) Schematic summary of the effects of continuous darkness, continuous light, sleep deprivation, and behavioral activity (exercise) on SCN neuronal activity. The red and blue areas under the curves represent a decrease or increase, respectively, in the SCN's rhythm amplitude.

Theoretically, a decline in the amplitude in the SCN's rhythm can result from a decrease in synchronization and/or from a decrease in the amplitude of individual neurons(148). In mice, an age-related decrease in the SCN ensemble rhythms of *Per2* (155), *Clock*, and *Bmal1* expression (156-158) have been reported. SIRT1 is an NAD-dependent protein deacetylase that activates transcription of the *Clock* and *Bmal1* genes, thereby regulating circadian rhythms in tissues. In mice, an age-related decrease in SIRT1 levels in the brain corresponds with decreased levels of BMAL1 and PER2 and is associated with a longer intrinsic period, disrupted behavioral activity patterns, and impaired light entrainment (158). Several studies

reported robust rhythms in *Per1* and *Per2* expression in the SCN of aged animals (159), and an increased amplitude of *Per2* expression has been reported in the SCN of aged mice (160).

3.2 Sleep deprivation and the SCN

The sleep-wake cycle is regulated by both the circadian clock and a homeostatic mechanism that regulates sleep homeostasis (161). Homeostatic sleep pressure is reflected on an electroencephalogram (EEG) as slow-wave activity (SWA) during non-rapid eye movement (NREM) sleep. This homeostatic process can be manipulated by sleep deprivation; for example, sleep deprivation causes an increase in SWA in rats, hamsters, birds and humans (162-168). During NREM sleep, SWA is negatively correlated with electrical activity in the SCN. (169). Moreover, spontaneous transitions between NREM and REM sleep occur simultaneously with changes in SCN electrical activity (169), suggesting that sleep deprivation can directly affect the amplitude of the SCN's electrical activity rhythm. This hypothesis was tested by performing simultaneous *in vivo* measurements of EEG and SCN firing rate in sleep-deprived rats. After six hours of sleep deprivation, both SWA NREM sleep and REM sleep were significantly increased, and the SCN's electrical activity was significantly decreased (170). These effects of sleep deprivation on SCN activity were long-lasting and could be measured at least six hours after sleep deprivation (170) (Figure 3). In humans, disruptions in the quality and/or timing of sleep are common among the elderly and among individuals with neurodegenerative disorders (see below).

3.3 Neurodegenerative disorders and the SCN

Patients with neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD) often exhibit perturbations in their 24-hour activity patterns and in their sleep-wake cycles. Moreover, the expression patterns of clock genes are altered in the brains of patients with these neurodegenerative disorders compared to healthy subjects (171). In patients with AD, the loss of AVP is generally correlated with the severity of symptoms, suggesting a causal relationship (172). The 3xTg-AD mouse model of AD has disrupted circadian behavior and a decrease in VIP- and AVP-expressing neurons in the SCN (173,174). Another model of AD, the APPxPS1 mouse, has disrupted sleep-wake rhythms compared to wild-type mice, but only a modest change in *Per2* expression in the SCN (175). R6/2 mice, a model of HD, develops progressively severe disruptions in behavior rhythms as the disease progresses (176), despite normal electrical output from the SCN (177). Two mouse models of HD and PD (the BACHD and ASO mouse lines, respectively) have disrupted circadian rhythms in locomotor activity, heart rate, and body temperature (178,179). These two mouse lines also have reduced

single-cell electrical activity in the SCN, although *Per2* expression is unaffected (178,179).

Fragile X syndrome is the most common form of inherited mental retardation. Fragile X syndrome is caused by silencing of the *FMR1* gene and the subsequent loss of FMRP (fragile X mental retardation protein, also known as FXR1P) (180). Patients with fragile X syndrome suffer from sleep disorders (181,182), and mice that lack either FMRP or FXR2P show a complete lack of behavioral rhythmicity (183). Interestingly, the SCN in Fmr1/Fxr2 double-knockout mice have normal expression rhythms of the *Per1*, *Per2*, *Bmal1*, and *Cry1* genes, and the SCN has high-amplitude electrical activity output (183). Molecular and electrophysiological data show that the SCN in the various mouse models of AD, HD, PD, and fragile X syndrome is functional, suggesting that the cause of the disease-associated perturbations in behavioral rhythmicity lie downstream of the SCN.

3.4 Metabolic disorders and the SCN

Exposure to light during the subjective night alters the circadian rhythm of melatonin and cortisol levels (184,185) and can alter sleep patterns (186,187). Shift workers often develop severe circadian disruptions due to irregular timing of light exposure, which can increase the risk of developing type 2 diabetes or other conditions (188-191). Animal studies revealed that disrupting the circadian system by exposing animals to a shifted light-dark cycle or continuous light causes a range of symptoms that are similar to the effects of aging, including sleep disorders (192,193), cardiovascular disorders (194-196), cognitive difficulties (197), and metabolic deficits (198-200). CLOCK-knockout mice have a dampened feeding rhythm and develop obesity and a host of other symptoms that are associated with metabolic disorder (199). The SCN plays a role in the regulation of energy homeostasis in both mice (201) and rats (202), and controls rhythms in energy metabolism by modulating rhythms in plasma glucose levels (203,204), lipogenesis and lipolysis (205-207), and plasma leptin levels (208). Thermal ablation of the SCN in wild-type mice disrupts the circadian rhythmicity of energy intake, activity, and energy expenditure (209), as well as a loss of rhythm in plasma leptin levels (208). Lesioning the SCN induces mild obesity compared to sham-operated mice; however, hepatic insulin sensitivity decreases considerably, and basal glucose levels increase (209).

Repeated exposure to light during the subjective night causes increased body weight in mice (210,211), and continuous exposure to light results in the complete loss of circadian rhythmicity in both energy metabolism and insulin sensitivity (212). Continuous exposure to light also reduces the amplitude of the SCN's rhythm via desynchronization among the SCN neurons (213,214). *In vivo* recordings of electrical activity in the SCN of freely moving mice that were continuously exposed to light revealed a 50% reduction in amplitude (212) (Figure 3). Remarkably, mice that are

exposed continuously to light and fed a regular diet gain more weight than mice that are exposed to a standard light-dark cycle (12h light:12h dark) and fed a high-fat diet (212). A mixed-model analysis revealed that the reduction in the amplitude of the SCN's output has a more severe effect on body weight than consuming a high-fat diet, thus underscoring the importance of robust SCN output for maintaining health. Disruptions in circadian rhythm and obesity are interrelated, and each disorder exacerbates the other, as a high-fat diet has a severely negative effect on the SCN's ability to synchronize in response to light (215). Mice that genetically lack leptin (*ob/ob* mice) develop obesity and have altered phase-delaying capacity compared to heterozygous (*ob/+*) mice (216). Injecting *ob/ob* mice with leptin normalizes the photic response of the SCN, suggesting that leptin modifies light-induced responses in the SCN via an indirect pathway (216).

4. Behavioral activity and the SCN

In addition to external light, several other stimuli provide input to the clock, and these inputs are known as non-photic stimuli. Examples include wheel-running activity (217), social interactions (218), dark pulses (219), and sleep deprivation (220). In general, non-photic stimuli induce behavioral activity, suggesting either that behavioral activity actually induces phase-shifting responses in the SCN (221) or that behavioral activity activates the same pathways. Interestingly, substances that are known to induce behavioral activity and/or influence neurotransmitter pathways involved in non-photic resetting—such as neuropeptide Y (NPY) (222), serotonin agonists (223), opioids (224), and short-acting benzodiazepines (225,226)—induce a phase shift in the SCN's rhythm.

The SCN receives its major inputs from two afferent pathways; the serotonergic tract provides input from the raphe nuclei, and the geniculohypothalamic tract provides input from the intergeniculate leaflet (IGL). Ablation of the serotonergic afferent SCN pathways attenuates the phase-shifting effects of several non-photic cues (227-230); moreover, increased levels of behavioral activity increase the levels of serotonin in both the SCN and the IGL (231,232). In addition to behavioral activity, a phase shift can also be induced by arousal, and serotonin appears to play an important role in arousal-induced phase-shifting (230,233). The geniculohypothalamic tract provides input to the SCN via the neurotransmitters NPY, GABA, enkephalin, and neurotensin (234-237). In rodents, wheel-running activity can induce the release of NPY in the SCN (238). The delivery of anti-NPY antibodies to the SCN attenuates the phase shift induced by novel wheel-running activity (239) and increases light-induced phase shifts (240). This finding suggests that NPY plays a role in both photic and non-photic resetting in the SCN (240).

Some non-photic stimuli suppress expression of *Per1* and/or *Per2* during the subjective day (241,241-245), and this suppression may underlie the SCN's phase-shifting response to non-photic stimuli at the molecular level. Applying NPY (246-249) or the serotonin receptor agonist 8-OH-DPAT (250,251) to SCN neurons *in vitro* decreases the neurons' firing rate. At the *in vivo* level, recordings in the SCN of freely moving hamsters (252), rats (253), and mice (254) revealed that behavioral activity induces an immediate suppression of the SCN's firing rate.

In mice, behaviorally induced suppression of SCN electrical activity is superimposed on the SCN's circadian rhythm and occurs at all phases of the circadian cycle (254). The magnitude and duration of the suppression depend upon the intensity and duration of the behavioral activity, respectively. Even an ultra-short (i.e., briefer than one minute) bout of behavioral activity can suppress the SCN's firing rate to a rate similar to the rate achieved with longer bouts of activity. Less intense behaviors such as grooming, eating, drinking, and rearing can suppress electrical activity in the SCN by ~30%, and more intense activity such as locomotor activity can decrease electrical activity by up to 60% (254). The suppression in firing rate remains stable throughout the entire bout of behavioral activity, and switching between types of behavior (for example, from less intense activity to more intense activity) causes a change in the level of suppression. Because mice are nocturnal animals, their behavioral activity increases during the night; consequently, behaviorally induced suppressed firing decreases the trough of the SCN rhythm even further. On the other hand, during the day mice are relatively inactive; consequently, the firing rate of the SCN is only barely suppressed during the peak of the rhythm. When a nocturnal animal is active during its resting phase, the peak in the SCN's rhythm is lower, thereby resulting in a lower amplitude SCN rhythm. These studies suggest that scheduled behavioral activity can increase the SCN's rhythmic amplitude, thereby improving peripheral rhythmicity. In support of this notion, voluntary exercise in aged mice increases the amplitude of the SCN's firing rate *in vitro* and improves resynchronization of the SCN and peripheral systems to the light-dark cycle (255). Furthermore, mice lacking VIP or the VIP receptor show improved behavioral activity in response to scheduled locomotor activity (256,257), and voluntary exercise improves circadian behavioral rhythmicity in a mouse model of HD (258). In humans, physical exercise accelerates the synchronization of sleep-wake rhythms to the external light-dark cycle (259) and improves health, mood, and performance (260-266).

Comparison of the SCN between nocturnal and diurnal animals

A long-standing—and inadequately addressed—question is whether the circadian clock is organized similarly or differently between nocturnal and diurnal animals. A comparison of the neuronal networks in the SCN of diurnal (*Acomys russatus*) and nocturnal (*Acomys cahirinus*) spiny mice revealed no differences with respect to

neurotransmitter localization, SCN subdivisions, input fibers, or output fibers (267). Similar to nocturnal species, diurnal species have circadian rhythmicity in clock gene expression and electrical activity profiles in the SCN (268-278). On the other hand, diurnal and nocturnal species differ with respect to photic responses and photoperiod encoding. In the SCN of nocturnal species, approximately 25% of neurons are excited by light, whereas a smaller percentage of neurons are inhibited or silenced completely (29,57,279). In the SCN of diurnal species such as ground squirrels (280), and degus (281), the percentage of light-responsive cells is lower (~10%), and within the group of light-responsive cells, the proportion of light-suppressed cells is higher. Under LD 12:12 the overall rhythm in Fos expression is similar between the nocturnal rat and the diurnal grass rat, however the spatial distribution of the Fos-expressing neurons differs. Whereas Fos-expression occurs in 40% of GRP containing SCN neurons in nocturnal rats (282), less than 1% of the GRP neurons in diurnal grass rats show Fos-expresion (283). On the other hand, in grass rats approximately 30% of AVP containing SCN neurons express Fos, while Fos expression in AVP neurons in nocturnal rats is uncommon (284-286). Photoperiod studies suggest that light has a synchronizing effect on clock gene profiles and neuronal electrical activity in the SCN of mice, rats, and hamsters (65,66,287-291). However, the expression profiles of *Per1* and *Per2* in the SCN of diurnal grass rats do not adapt to short photoperiods (292). The SCN of diurnal and nocturnal species show a differential phase window for serotonin-mediated phase resetting (293). In nocturnal species, injections of serotonin receptor agonists 8-OH-DPAT or (+)8-OH-DPAT cause large phase advances of behavioral activity only during subjective midday (241,294,295) while in the diurnal Arvicanthis injections of (+)8-OH-DPAT induce small phase advances during the subjective night (293). Importantly, in diurnal Arvicanthis, serotonin agonists strengthen, instead of oppose, the effects of light on the circadian system, which could be clinically relevant (293).

The SCN of nocturnal mice and diurnal grass rats respond differently to GABA. Activating $GABA_A$ receptors during the subjective day causes a phase advance in the SCN of mice, whereas the SCN of grass rats exhibits a phase delay (296). In the SCN of mice, the rhythms in GRP and VIP are strengthened following continuous exposure to darkness. In contrast, in the SCN of diurnal grass rats, these rhythms do not become stronger; rather, they show a shift in their peak time of expression (297). Moreover, in the SCN of three-striped South Indian squirrels (*Funambulas palmarum*, a diurnal species), the phase relation of daily VIP and AVP rhythms differ from those found in nocturnal species (298). Recently, a study of the SCN in capuchin monkeys confirmed the presence of circadian oscillations inPER2 in the SCN of primates (299). Moreover, VIP-containing cells are present in the ventral SCN of capuchin monkeys (299), similar to VIP expression in nocturnal rodents (1,3,300,301). In addition, AVP is present in both the ventral and dorsal SCN in capuchin monkeys (299), which

is strikingly different than the expression patterns in the SCN of most nocturnal species studied to date (1,3,301-303).

Thus, the molecular clock is an evolutionarily preserved structure, and the SCN has both structural and functional similarities between nocturnal and diurnal species. On the other hand, the SCN of nocturnal and diurnal species differ with respect to their responsiveness to light, photoperiod, and neurotransmitters. These differences suggest fundamental differences between nocturnal and diurnal animals with respect to neuronal coupling in the SCN and synchronization mechanisms. These key differences should be considered carefully when making recommendations to improve the well-being of humans based largely on results obtained from studying nocturnal species.

Summary

The functioning of the SCN depends on its intrinsic molecular machinery, as well as on its organization at the network level. Communication and synchronization among SCN neurons is essential for generating a robust rhythm at the SCN's tissue level which is transmitted to other brain structures, thereby impacting many of our bodily functions. The SCN is influenced by environmental factors, the most important of which is light. In a short photoperiod, the SCN's electrical activity rhythm is robust due to highly synchronised single-cell activity patterns, while in a long photoperiod, the SCN's electrical output is dampened by reduced synchrony among individual cells. Photoperiod-induced changes in the expression of clock genes generally coincide with photoperiod-induced changes in the SCN's electrical rhythm. Despite this similarity, spatial differences exist with respect to SCN clock gene expression, while the electrical activity rhythm has only small spatial differences among various regions within the SCN. With aging, the amplitudes of both single-cell rhythms and ensemble rhythms decline; however, reports of the amplitude of clock gene expression patterns in the aged SCN have been inconclusive. These points lead to the fundamental question of how the molecular feedback loop is correlated with the SCN's electrical output.

The SCN clock is part of a larger brain network that includes areas involved in the sleep-wake cycle, energy metabolism, and behavioral activity. A dampened SCN rhythm is associated with reduced amplitude of the behavioral activity rhythm and can lead to metabolic disorders; vice versa, a disrupted behavioral rhythm (for example, induced by shift work or by food intake during the resting phase) can adversely affect the amplitude of the SCN's rhythm. Increased levels of behavioral activity and voluntary exercise have been identified as potential strategies to boost the amplitude of the SCN's rhythm, presumably by increasing cellular synchronisation at the ensemble level. To be therapeutically effective for humans it is important to investigate the effects of exercise on the SCN in diurnal species, particularly given

that some differences have been found between nocturnal and diurnal animals. Whether – and how – the peripheral systems involved in behavioural activity and food intake influence the SCN in diurnal mammals remains largely unknown. Increasing our knowledge of the interplay between the SCN and the periphery in diurnal animals will increase our understanding of circadian-related disorders in humans and might lead to novel, effective recommendations for improving lifestyle patterns.

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2

ENHANCED PHASE RESETTING IN THE SYNCHRONIZED SCN NETWORK

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Abstract

The suprachiasmatic nucleus (SCN) adapts to both the external light-dark cycle and seasonal changes in day length. In short photoperiods, single-cell activity patterns are tightly synchronized (i.e., in phase); in long photoperiods, these patterns are relatively dispersed, causing lower-amplitude rhythms. The limit cycle oscillator has been used to describe the SCN's circadian rhythmicity and predicts that following a given perturbation, high-amplitude SCN rhythms will shift less than low-amplitude rhythms. Some studies reported, however, that phase delays are larger when animals are entrained to a short photoperiod. Because phase advances and delays are mediated by partially distinct (i.e., non-overlapping) biochemical pathways, we investigated the effect of a 4-hour phase advance of the light-dark (LD) cycle in mice housed in either short (LD 8:16) or long (LD 16:8) photoperiods. In vitro recordings revealed a significantly larger phase advance in the SCN of mice entrained to short as compared to long photoperiod (4.2 ± 0.3 h vs. 1.4 ± 0.9 h, respectively). Surprisingly, in mice from long photoperiod the behavioral phase shift was larger than the phase shift of the SCN (3.7 ± 0.4 h vs. 1.4 ± 0.9 h, respectively). To exclude a confounding influence of running wheel activity on the magnitude of the shifts of the SCN we repeated the experiments in the absence of running wheels, and found similar shifts in the SCN in vitro in short and long days (3.0 ± 0.5 h and 0.4 ± 0.9 h respectively). Interestingly, removal of the running wheel reduced the phase shifting capacity of mice in long days, leading to similar behavioral shifts in short and long photoperiods (1.0 ± 0.1 h vs. 1.0 ± 0.4 h). As the behavioral shifts in the presence of wheels were larger than the shift of the SCN it is suggested that additional, non-SCN neuronal networks in the brain are involved in regulating the timing of behavioral activity. On the basis of the phase shifts observed in vitro, we conclude that highly synchronized SCN networks with high amplitude rhythms show a larger phase shifting capacity than desynchronized networks of low amplitude.

Introduction

Circadian rhythms are intrinsic to most organisms and are controlled by an endogenous pacemaker or clock. In mammals, the circadian clock is located in the suprachiasmatic nuclei (SCN) within the anterior hypothalamus (Ralph et al., 1990). Individual SCN neurons are genetically encoded for rhythm generation (Welsh et al., 2010) and are considered to function as autonomous single-cell oscillators. To achieve robust oscillations and high output amplitude, these oscillators in the SCN are mutually coupled and require sufficient phase synchrony (Welsh et al., 2010). Entrainment to the external light-dark (LD) cycle is mediated by the retinohypothalamic tract (RHT), which projects directly from the retina to the SCN via glutamatergic and PACAP-containing fibers (Morin and Allen, 2006). The influence of light on the clock depends on the clock's phase—light pulses applied at the beginning of the night produce a phase delay, whereas light pulses applied at the end of the night produce a phase advance (Johnson, 1999). After a phase shift, the rhythm resumes with the previous period (i.e., before a light pulse was applied). Because this behavior is typical of a limit cycle oscillator, the circadian clock has been modeled extensively as such an oscillator (Kronauer et al., 1982; Goldbeter, 1995; Winfree, 2001; Forger and Peskin, 2003; Abraham et al., 2010; Phillips et al., 2013). The phase-shifting capacity of a limit cycle oscillator depends on the amplitude; high-amplitude oscillators have less phase-shifting capacity than low-amplitude oscillators. This limit cycle behavior has been shown in unicellular organisms (Johnson and Hastings, 1989) and for example, in cyanobacterium *Synechococcus elongates*, high-amplitude rhythms have weak phase-shifting capacity, whereas low-amplitude rhythms show large-magnitude shifts (Hosokawa et al., 2013).

The amplitude of the phase-response curve (i.e., the phase-shifting capacity) is not fixed, but is dependent on the photoperiodic history of the organism. After adaptation to a short photoperiod, the amplitude of the phase response curve (PRC) is larger than in long photoperiods (Refinetti, 2002; Pittendrigh et al., 2008; vanderLeest et al., 2009). The phase shifting capacity in long and short days can be mimicked *in vitro* by application of the glutamate receptor agonist NMDA and thus it seems to be determined at the level of the SCN (vanderLeest et al., 2009). In short photoperiod, the neurons of the SCN become more synchronized, leading to high amplitude rhythms, while in long photoperiod, the neurons are more desynchronized, leading to low amplitude rhythms (Schaap et al., 2003; Brown et al., 2005; Rohling et al., 2006; Inagaki et al., 2007). Thus, based on limit cycles, one would have expected to obtain large phase shifts in the SCN from long photoperiod which is in contrast with the experimental result. These findings suggest that networks of single-cell limit cycle oscillators may have emergent properties that are not present at the single cell level.

To date, however, studies have been limited to behavioral experiments, and the response to phase-advancing pulses has not been tested at the level of the SCN (Pittendrigh et al., 1991; Refinetti, 2002; Refinetti, 2003). This lack of data regarding phase advances in the SCN has hampered our ability to generalize the results regarding limit cycle oscillator behavior to the SCN network. Testing SCN phase-advancing behavior is particularly relevant, as the intracellular signaling cascade that is triggered by glutamate release differs between delays and advances (Ding et al., 1998; Gillette and Mitchell, 2002; Schwartz et al., 2011).

Here, we investigated the advancing responses of the SCN and behavior of mice from long and short photoperiods. As C57-mice show only small advancing phase shifts in response to a light pulse we subjected the mice to an advance of the light-dark cycle to induce an advanced rhythm (vanderLeest et al. 2009). *In vitro* recordings of SCN electrical activity revealed that the SCN in mice from short photoperiod phase-advanced significantly more than the SCN from mice entrained to long photoperiod. Surprisingly, the *behavioral* phase advances observed when a running wheel was available to the mice were significantly larger in long photoperiods as compared to short photoperiods. When the running wheel was removed, the behavioral phase-advancing capacity of the long photoperiod-entrained mice was significantly reduced but the shifts in the SCN were unaltered, indicating that the occurrence of intense physical activity (e.g., wheel-running activity) can influence the magnitude of behavioral phase advances without affecting the SCN. Based on its phase-response properties, we conclude that unlike typical limit cycle oscillators, the SCN network does not have an inverse phase relationship between amplitude and phase-shifting capacity.

Methods

Ethics Statement

All experiments were performed in accordance with national animal welfare laws and were approved by the Animal Experiment Ethics Committee of Leiden University Medical Center.

Behavioral Experiments

C57BL/6 mice were entrained to either a long (LD 16:8) or short (LD 8:16) photoperiod for at least 30 days prior to experimentation. The mice were housed in clear plastic cages, and locomotor activity was monitored by either passive infrared detectors or a running wheel, and recorded using Actimetrics software. The animal cabinets contained 9 cages each and were light-tight and illuminated using white fluorescent tubes. The light intensity at the bottom of each cage was approximately 85 $\mu W/cm^2$. After 30 days of entrainment, the mice were subjected to a phase advancing light stimulus. Because C57-mice show only small advancing phase shifts in response to a light pulse, the mice were subjected to a phase advance of the LD regime. The advance was accomplished by advancing the time of lights onset. We chose to apply a 4-h phase advance in the LD cycle (rather than a 6-h advance) to ensure that the advanced light cycle would exclusively cover the advance part of the phase-response curve (vanderLeest et al. 2009). This 4-h phase advance in the LD regime was then followed by one fully shifted LD cycle. After receiving this phase-advancing protocol, the mice were housed in continuous darkness (DD) for at least 10 days. The behavioral phase shift was calculated as the time difference between the activity onset (e.g., wheel-running activity) before the advance in the LD regime and the time of activity onset in DD. Activity onset was determined using ClockLab analysis software.

In Vitro Experiments

Mice were entrained to either long or short photoperiod days as described above. SCN slices were prepared just before the light offset in the second cycle following the 4-h advance in the LD cycle, at external time (ExT, with Ext 12 defined as middle of the day (Daan et al., 2002)) 15.5 h and 19.5 h, for the short and long photoperiod groups, respectively. Control experiments were performed using mice that were not subjected to a 4-h phase advance in the LD regime. Brain slices (~450 microns thick) were prepared using a tissue chopper, and the slice containing the SCN was transferred to a laminar flow chamber within six minutes after decapitation (Schaap et al., 2003). The tissue was bathed in bicarbonate-buffered ACSF that was gassed by continuously blowing a warmed, humidified mixture of O_2 (95%) and CO_2 (5%) over the solution. The slice was submerged in the solution and stabilized using

an insulated tungsten fork. The slice settled in the recording chamber for ~1 h before the electrodes were placed in the center of the SCN. Of note, there are no consistent phase differences between SCN regions in electrical activity rhythms. In the intact SCN, electrical activity patterns are integrated, as opposed to patterns in gene expression (Meijer et al. 2010). Action potentials were recorded using 50-µm or 75-µm 90% platinum/10% iridium electrodes. The signals were amplified 10 k time and bandpass-filtered (0.3 Hz low-pass, 3 kHz high-pass). The action potentials that exceeded a predetermined threshold well above noise (~5 µV) were counted in 10-second bins using a custom-made automated computer program.

Data Analysis

The electrophysiological data were analyzed using a custom-made program in MATLAB as described previously (VanderLeest et al., 2007). The time of maximum activity was used as a marker of the phase of the SCN and was determined as the first peak in multiunit activity (both for the control and phase-advanced groups). Multiunit recordings of at least 24 hours in duration that expressed a clear peak in multiunit activity were moderately smoothed using a least-squares algorithm (Eilers, 2003). Subsequently, the SCN peak time, the peak width, and the relative peak amplitude (peak-to-trough ratio) of the first cycle *in vitro* were determined. Statistical analyses were performed using Origin 7 (OriginLab Corporation). All summary data are reported as the mean ± the standard error of the mean (s.e.m.). P-values were calculated using the two-tailed Student's *t*-test, and differences with $p<0.05$ were considered to be statistically significant.

Model

We examined the difference in amplitude and phase-shifting capacity between mice entrained to long and short photoperiod using a mathematical model based on limit cycle oscillator theory. We refer to high- and low-amplitude *rhythms* when we discuss the biological system, whereas we refer to high- and low-amplitude *oscillations* when we discuss the mathematical oscillator. The Poincaré model is a model for describing limit cycle oscillators and is used to describe the 24-h oscillations in the SCN (Abraham et al., 2010). This model is a generic model with two variables (x and y) in Cartesian coordinates and is described as follows:

$$\frac{dx}{dt} = \gamma x(A-r) - \frac{2\pi}{\tau} y + L$$
$$\frac{dy}{dt} = \gamma y(A-r) + \frac{2\pi}{\tau} x \qquad (1)$$

where γ represents the relaxation parameter, A and τ represent the amplitude and free running period of the SCN, respectively, L is the light term, and r is the amplitude of the SCN oscillator. r is defined as follows:

$$r = \sqrt{x^2 + y^2}$$

Experimental data showed that the amplitude of the SCN rhythm is higher in short photoperiod than in long photoperiod (vanderLeest et al., 2009; Ramkisoensing et al., unpublished data). Without loss of generality, the amplitude A is defined as $A_s = 2$ for short days and $A_L = 1$ for long days. The light term L also differs between photoperiods; L_s represents the light term under short photoperiod, and L_L represents the light term under long photoperiod. L_s and L_L are defined as follows:

$$L_s = \begin{cases} \text{if } \bmod(t, 24) < 8, & K_f \\ \text{if } \bmod(t, 24) \geq 8, & 0 \end{cases}, \quad L_L = \begin{cases} \text{if } \bmod(t, 24) < 16, & K_f \\ \text{if } \bmod(t, 24) \geq 16, & 0 \end{cases}$$

where K_f represents the light intensity. The other parameters are the same for the short and long photoperiods and are defined as follows:

$$\gamma = 0.1, \tau = 23.5, K_f = 0.2$$

We used the fourth-order Runge-Kutta method with a time increment of 0.01 h to numerically simulate this system. The initial 100,000 time increments were excluded from the analysis in order to avoid the effect of transients. The initial conditions were selected randomly from a uniform distribution in the range 0-1 for the variables x and y in the Poincaré model.

The simulations produced a 3.0-h phase advance for long photoperiod and a 1.5-h phase advance for short photoperiod following a 4-h advance in the light-dark cycle (Fig. S1). The limit cycle oscillator theory predicts that –in line with the outcomes of the simulation- the phase shift will be larger for small amplitude rhythms than for large amplitude rhythms. Therefore, we consider our protocol to be valid for testing the phase-advancing properties of the SCN for short and long photoperiods, and we consider that deviations from the predicted outcome cannot be attributed to our protocol and should therefore be considered meaningful.

Results

Large advances in the SCN in short days

The SCN of mice that were entrained to either a short or long photoperiod with access to a running wheel were prepared following a 4-h phase advance in the LD regime. Electrical recordings (Figure 1A and 1B) revealed a narrow waveform in the SCN in mice entrained to short photoperiods (8.91 ± 0.64 h, n = 5 mice) and a significantly broader peak in the SCN from mice entrained to long photoperiods (15.53 ± 1.93 h, n = 6 mice; $p<0.01$). The circadian phase of the SCN was defined as the maximum SCN activity (SCN_{Tmax}), and the phase advance was defined as the difference between the subjective time of the SCN_{Tmax} in the phase-advanced mice and the SCN_{Tmax} of the control (i.e., non-phase-advanced) mice. In the short-day and long-day control SCN groups, SCN_{Tmax} occurred at ExT 11.7 and 12.5, respectively (i.e., midday). Mice entrained to short days showed a 4.2 ± 0.3 h (n = 8) phase advance in SCN_{Tmax} after a 4-h advance in the LD regime (Figure 1A and 1C). In contrast, the mice from long days showed a 1.4 ± 0.9 h (n = 7) advance in SCN_{Tmax} (Figure 1B and 1C). The SCN phase-advancing capacity of mice that were entrained to a short day in the presence of a running wheel was significantly larger than the SCN phase-advancing capacity of mice entrained to a long day ($p <0.001$) (Figure 1C, Table 1).

In the presence of a running wheel, the behavioral phase advance is larger in mice from long days

In both the short and long day groups, the mice were physically active almost exclusively during the dark phase (Figure 2A and 2B). The behavioral phase advance following a 4-h advance in the LD regime was measured using wheel-running onset in continuous darkness. Mice from short days had a 1.3 ± 0.2 h (n = 18) behavioral phase advance (Figure 2A and 2C) and the mice from long days had a 3.7 ± 0.4 h (n = 18) phase advance (Figure 2B and 2C). Thus, when given free access to a running wheel, the phase-advancing capacity of mice in long photoperiod was significantly larger than in mice from short photoperiod ($p<0.0001$, Table 1) (Figure 2C).

Phase advance in the SCN in vitro is not affected by running-wheel activity

To exclude any potential effect of wheel-running activity on the behavioral advances, we subjected mice to a 4-h phase advance in the absence of a running wheel; the SCN slices were then prepared immediately following the phase advance and recorded. The electrical recordings revealed a narrow waveform for the SCN of mice from short days (9.15 ± 0.77 h, n = 6 mice) and a significantly broader waveform for the SCN of mice from long days (12.84 ± 1.44 h, n = 5; $p<0.05$). Following the phase advance, the SCN of mice from short days had a 3.0 ± 0.5 h (n = 6) phase advance in SCN_{Tmax}

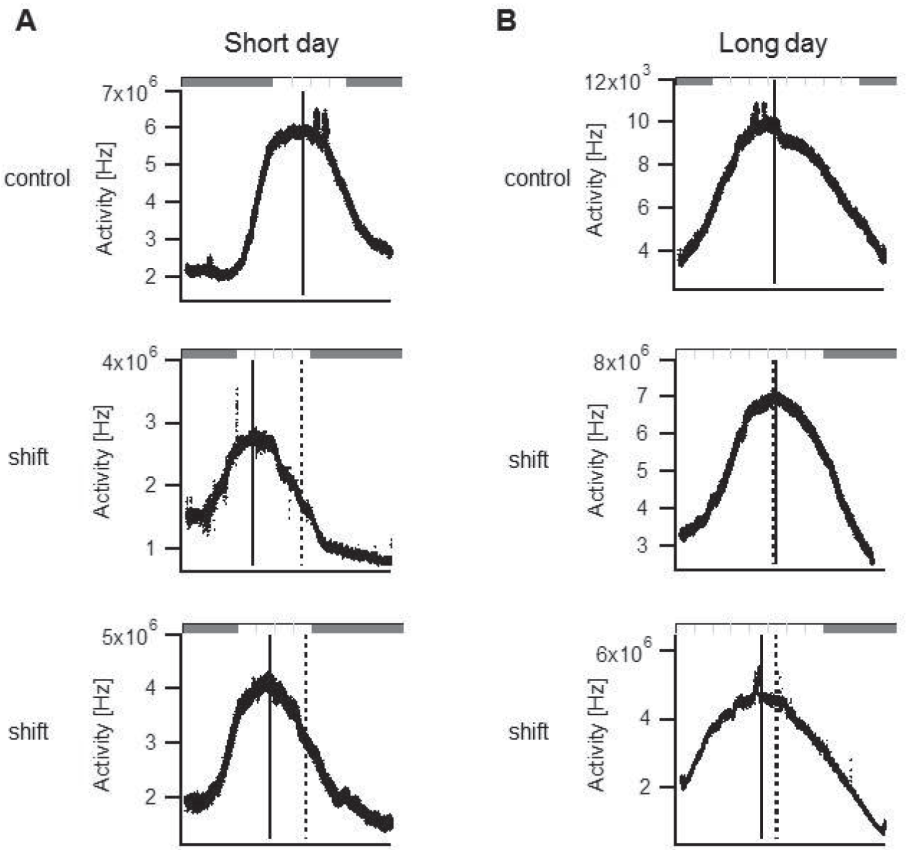

Figure 1. Phase advances in electrical activity rhythms recorded in SCN isolated from mice entrained to a short or long photoperiod in the presence of a running wheel. (**A and B**) Examples of electrical activity recorded from the SCN of mice entrained to either a short day (LD 8:16) protocol (**A**) or a long day (LD 16:8) protocol (**B**). The plotted LD regimes were extrapolated from the LD schedules to which the mice were entrained prior to the preparation of the SCN slices. The upper panels show representative electrical activity measured from SCN isolated from mice that did not experience a 4-h advance in the LD regime (control). The middle and lower panels show the electrical activity recorded from the SCN of mice from short and long days following a 4-h phase advance in the LD regime. The dashed vertical lines indicate the mean SCN_{Tmax} of the control groups, and the solid vertical lines indicate the SCN_{Tmax} of each example recording. The magnitude of the phase advance of the SCN was calculated as the difference between the mean control SCN_{Tmax} and the shifted SCN_{Tmax}. (**C**) Summary of the advance in the activity rhythm of SCN prepared from mice from short days (n = 8) and long days (n = 7) mice.*, $p<0.05$.

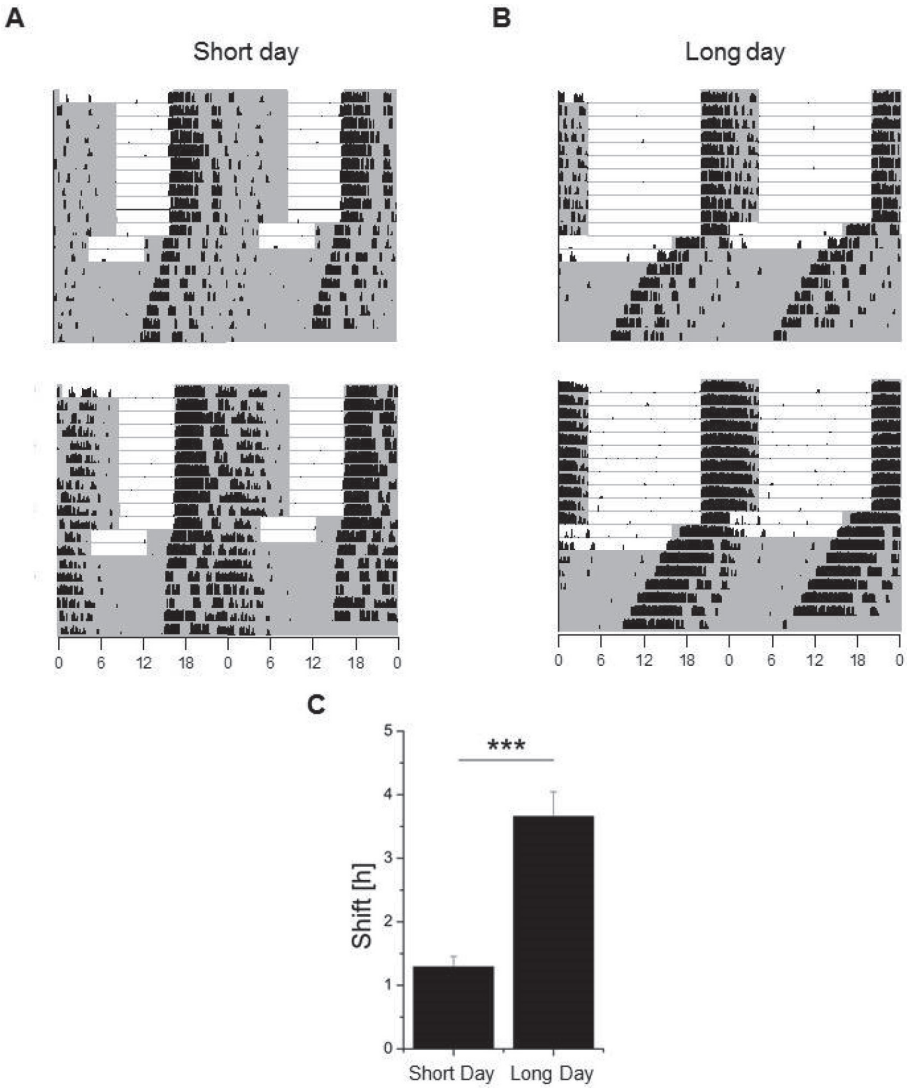

Figure 2. Phase advance in the wheel-running activity of mice following a 4-h phase advance in the LD cycle. (**A and B**) Examples of running-wheel activity in four representative mice entrained to either a short (**A**) or long (**B**) photoperiod. Each actogram is double-plotted and shows consecutive days on successive lines; the white areas are the light periods, and the shaded gray areas are the dark periods. The vertical black up-ticks indicate wheel-running activity. After 30 days of entrainment to a short or long photoperiod, the mice were exposed to a 4-h phase advance. The animals were then kept in the shifted LD regime for one additional complete cycle, then kept in continuous darkness. The phase-advance was measured as the difference between the time of wheel-running onset before the phase shift and the time of wheel-running onset in continuous darkness after the phase shift (transients were excluded). (**C**) Summary of the advances in the wheel-running behavior of mice in short days (n = 18) and long days (n = 18) mice. *, p<0.0001.

(Figure 3A and 3C); whereas the SCN from the mice from long days had a phase advance of only 0.4 ± 0.9 h (n = 6) (Figure 3B and 3C). Thus, the SCN phase-advancing capacity of mice from short photoperiods was significantly larger than from long photoperiods ($p<0.05$) (Figure 3C, Table 1).Importantly, these results did not differ significantly from the results obtained from SCN recordings from mice that had access to a running wheel ($p=0.06$ for the short day mice and $p=0.46$ for the long day mice). We pooled the data from the SCN recordings both with and without a running wheel and found a significantly higher normalized amplitude in the SCN from short days (0.58 ± 0.04) as compared to long days (0.45 ± 0.03; $p<0.05$).

In the absence of a running wheel, the behavioral phase advance is similar in mice from short and long days

We next compared the effect of a 4-h phase advance on the behavioral shift in mice that were entrained to short or long days in the absence of a running wheel. Following a 4-h advance in the LD regime, the resulting behavioral phase advance was determined in continuous darkness by recording the mouse's activity using passive infrared detectors (Figure 4A and 4B). The behavioral phase advance was similar between the mice from short days (1.0 ± 0.1 h, n = 9) and long days (1.0 ± 0.4 h, n = 6; $p=0.93$) (Figure 4C, Table 1). Interestingly, entraining the mice in the absence of a running wheel had no effect on the behavioral phase advance in the short day-entrained mice; in contrast, entraining the mice in the absence of a running wheel significantly reduced the phase-advancing capacity of the long day-entrained mice (1.0 ± 0.4 h vs. 3.7 ± 0.4 h in the presence and absence of a running wheel respectively; $p<0.005$) (compare Figure 4C with Figure 2C).

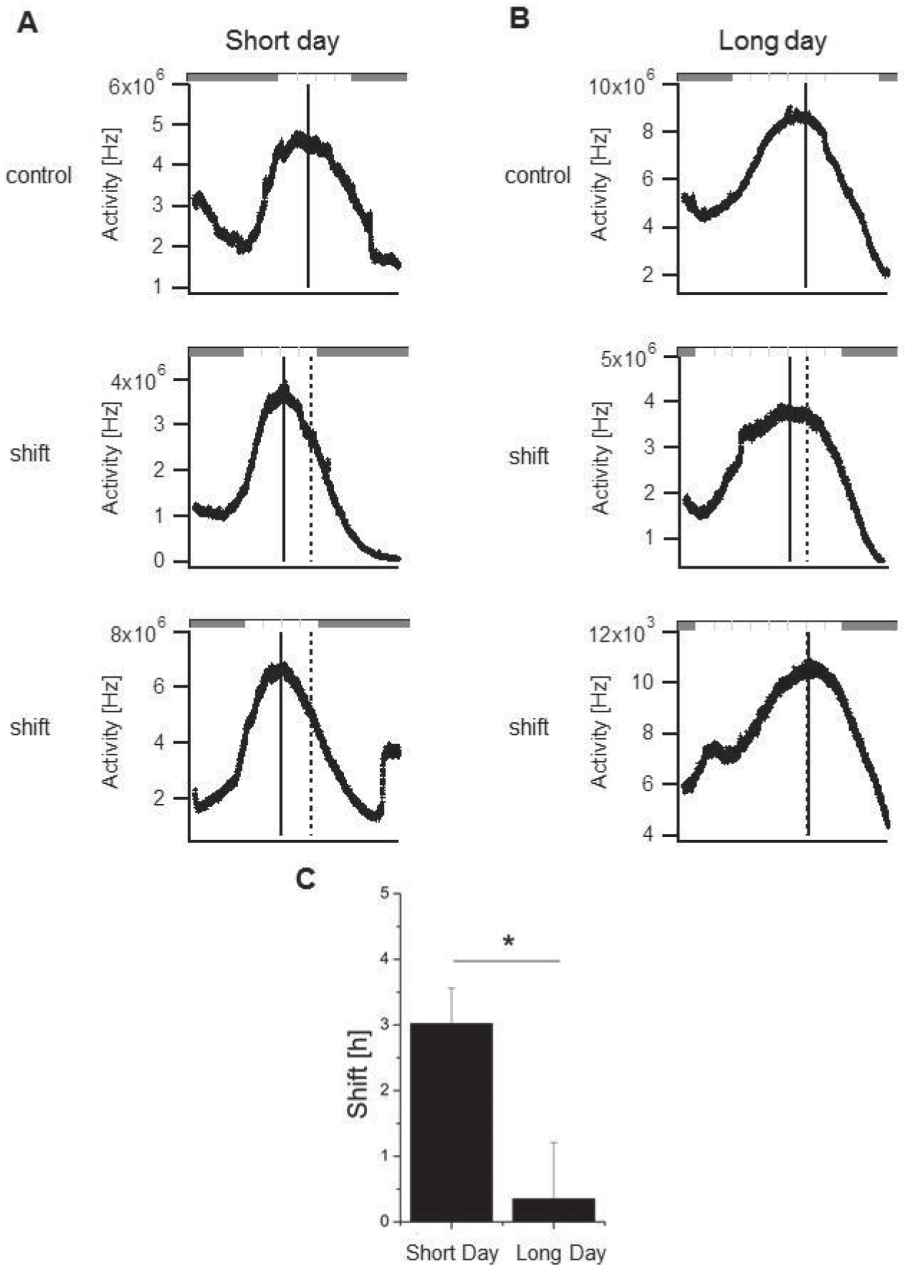

Figure 3. Phase advances in the electrical activity rhythm recorded in SCN from mice entrained to a short or long photoperiod in the absence of a running wheel. (**A and B**) Examples of electrical activity recorded from the SCN from mice entrained to either a short-day (**A**) or a long-day (**B**) protocol; see Figure 1 for details. (**C**) Summary of the advance in the activity rhythm of the SCN from mice entrained to short days (n = 6) and long days (n = 5) mice. *, p<0.05.

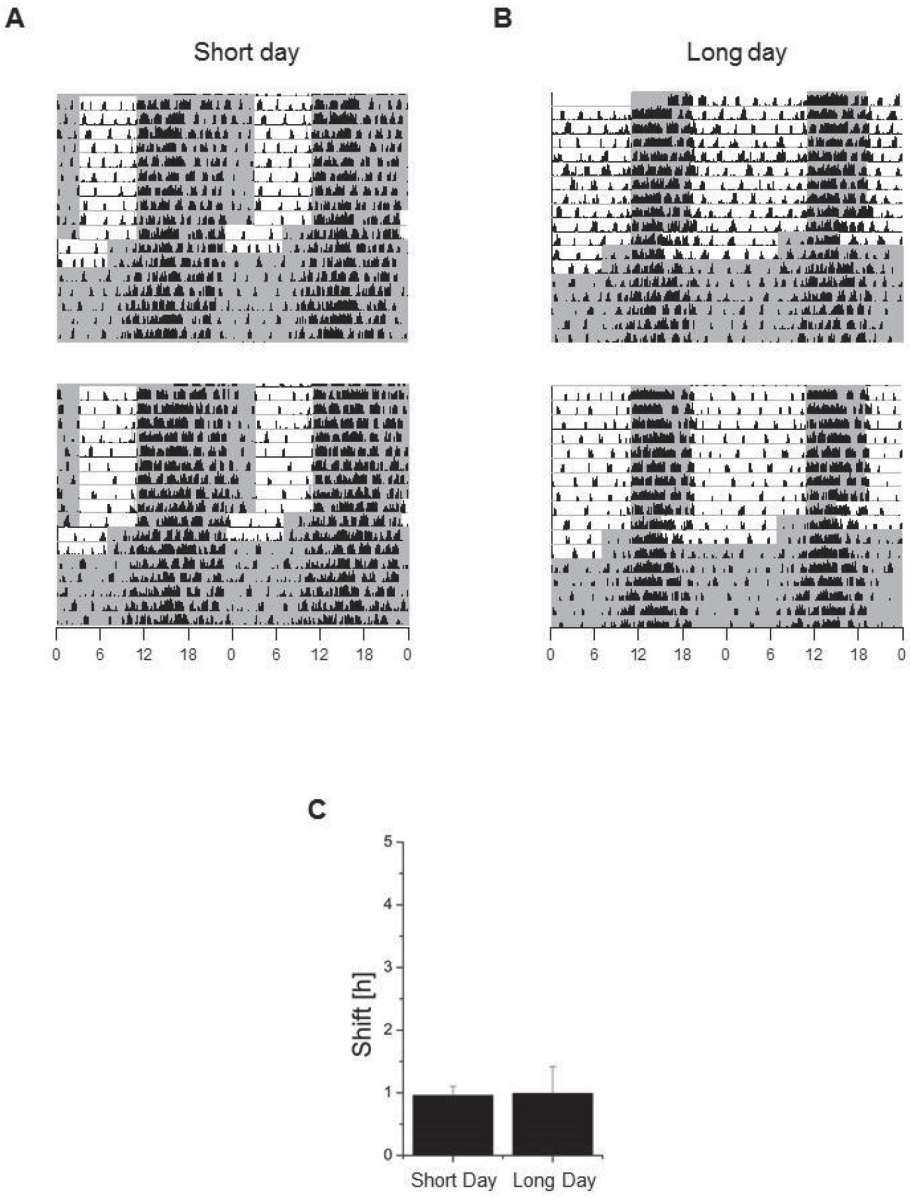

Figure 4. Phase advance in the activity behavior of mice following a 4-h phase advance in the LD cycle. (**A and B**) Behavioral activity was recorded using a passive infrared detector for mice entrained to either a short (**A**) or a long (**B**) photoperiod; see Figure 2 for details. (**C**) Summary of the advances in the behavioral activity of mice entrained to short days (n = 9) and long days (n = 9). The values did not differ significantly (p = 0.933).

Discussion

Previous studies have reported that SCN with high amplitude electrical activity rhythms phase delay significantly more than low amplitude SCN rhythms upon stimuli of equal sizes (vanderLeest et al. 2009). The aim of this study was to investigate the effect of a phase advancing perturbation on high- and low-amplitude SCN rhythms. We first confirmed that the activity in the SCN is more tightly synchronized in mice entrained to a short photoperiod than in mice entrained to a longer photoperiod; this increased synchronization was reflected by a narrower peak width and a higher-amplitude in the SCN activity rhythm of the short photoperiod-entrained mice. These results are consistent with previous reports of higher-amplitude rhythms in synchronized neuronal populations (Rohling et al. 2006a; Schaap et al. 2003; vanderLeest et al. 2009). We then induced a phase advance in the light-dark cycle and observed a large phase advance in the SCN of mice entrained to short photoperiod and a significantly smaller advance in the SCN of mice entrained to long photoperiod. These results confirm former studies on the phase delaying behavior of high- and low-amplitude SCN rhythms (vanderLeest et al. 2009). Our findings are however in contrast with the predictions of our own limit cycle oscillator simulations, in which we tested the validity of our protocol, and with predictions from limit cycle oscillator theory in general. We argue that neurons within a synchronized network population, with presumably consistent phase shifting responses, will result in a large amplitude PRC of the ensemble, whereas neurons within a desynchronized network will show diverse responses to the perturbing stimulus resulting in a small amplitude PRC (vanderLeest et al. 2009; Meijer et al. 2012). Therefore, our results are relevant as they illustrate that limit cycle oscillator behavior at the SCN network level is distinct from limit cycle behavior at the single cell level. We also observed striking differences between the phase-shifting capacity of the SCN and the behavioral phase-shifting capacity. Specifically, in mice from long photoperiod, the phase advance in the animal's behavioral activity rhythms was considerably larger than the advance in the SCN itself suggesting that other brain structures, in addition to the SCN, are in control of resetting behavioral activity rhythms.

In vitro results

In vitro recordings of control (i.e., non-phase-shifted) SCN revealed high levels of electrical activity during the subjective day and low levels of electrical activity during the subjective night; this pattern was observed in mice from short and long days. In both groups, the maximum SCN activity (SCN_{TMAX}) was close to midday. The waveform characteristics under short and long photoperiods are consistent with previous electrophysiological (VanderLeest et al., 2007; Brown and Piggins, 2009; vanderLeest et al., 2009) and molecular (Hazlerigg et al., 2005; Johnston et al., 2005; Inagaki

et al., 2007; Naito et al., 2008; Myung et al., 2012) studies. Specifically, the waveform was narrow in the SCN from short day mice (due to the tightly synchronized activity between the individual neurons in the network) and broad in the SCN from long day mice (due to asynchronous activity between the individual neurons) (vanderLeest et al., 2009). Thus, by entraining the mice to either a short or long photoperiod, we could drive the SCN's neuronal population into either high or low synchrony.

We found that SCN of mice taken from short days phase-advanced significantly more than SCN of long day mice. *In vitro* measurements revealed that the SCN rhythm of the short day group was significantly phase-advanced by 4.2 h and showed, accordingly, complete synchronization to the shifted light-dark cycle, while the SCN rhythm of the long day group remained in phase with the light-dark cycle prior to the shift. To examine the effect of behavioral activity on the phase-shifting capacity of the SCN, we repeated this experiment in the absence of a running wheel. This experiment was particularly relevant, as behavioral activity is more condensed in relative short nights (i.e., longer photoperiods) and therefore may have influenced our results. However, *in vitro* we again found a significantly larger phase advance in the SCN of mice entrained to short as compared to long photoperiod (3.0 hours vs. 0.4 hours, respectively), confirming that the phase-advancing response is larger in the synchronized network (i.e., in the SCN from short days) than in the non-synchronized network (i.e., in the SCN from long days). Thus, the shifts obtained *in vitro* were independent of the presence of behavioral activity.

Advances vs. delays

Testing the response to advances of the LD cycle was prompted by studies showing that the mechanisms underlying advances and delays differ. For example, light-induced glutamate release activates NMDA receptors, which triggers Ca^{2+} influx, activates nitric oxide synthase (NOS), and increases nitric oxide (NO) production. Blocking NMDA receptors or NOS activation inhibits glutamate-induced phase advances and phase delays (Ding et al., 1998). Several studies, investigating these intracellular signal transduction cascades, revealed that the divergence into the delaying and advancing signaling pathways occurs downstream of NO (Ding et al., 1998; Gillette and Mitchell, 2002; Antle et al., 2009; Schwartz et al., 2011). Important for light-induced resetting of the SCN are the clock genes *mPer1* and *mPer2* (Albrecht et al., 1997; Obrietan et al., 1998; Zylka et al., 1998; Field et al., 2000; Yan and Silver, 2002). Intracerebroventricular delivery of *mPer1* antisense oligonucleotides reduced the light-induced phase delay in locomotor activity (Akiyama et al., 1999). Moreover, applying *mPer1* antisense oligonucleotides suppressed glutamate-induced delays in neuronal firing rhythms *in vitro* (Akiyama et al., 1999). Finally, behavioral studies in hamsters revealed elevated *Per1* mRNA levels after entrainment to short T-cycles (advances), whereas entrainment to long T-cycles (delays) altered *Per2* expression

(Schwartz et al., 2011). Our results show that the SCN's response to an advancing shift is large for high-amplitude SCN rhythms and small for low-amplitude SCN rhythms, regardless of whether the mice were entrained in the presence or absence of a running wheel. Thus, despite the divergence between the advancing and delaying intracellular signaling pathways, the advances and delays appear to follow the same rule.

Behavioral results with a running wheel

In response to a 4h advance of the light cycle, followed by DD, we found a small advance in running wheel activity in mice from short days (1.3 h). The magnitude of the phase shift differs from the shift of the SCN *in vitro* (4.2 h). It has been previously shown that a shift in behavior is smaller than in the SCN (Vansteensel et al., 2003; van Oosterhout et al., 2012).The difference was attributed to interactions between non-SCN structures and the SCN, which attenuate the behavioral shift, and even the shift of the SCN when it is recorded *in vivo*. In contrast, when the SCN is measured in isolation, the endogenous phase is observable. Thus, in mice from short photoperiod, it is possible to explain the presence of both a small shift in behavior and a large shift in the SCN.

In contrast to this, mice that entrained to long days showed a large and immediate shift in wheel-running behavior after a 4-h phase advance of the LD cycle (3.7 h). The advance in wheel-running behavior of mice exposed to long days was found to be much larger than the advance measured in SCN isolated from mice exposed to long days in the presence of a running wheel (1.4 h). This result is rather surprising and raises the question of how to explain the large shift in the behavioral activity of mice in long photoperiod, despite the relatively small shift in the SCN itself.

To investigate this apparent difference in phase-advancing capacity between behavior and SCN activity, we examined the behavioral responses more closely. The phase advance in wheel-running activity in mice from long days was large in magnitude and did not show transients, unlike light pulse-induced advances. As a result of the shift in the light regime, the mice spent the latter part of the day in darkness, resulting in intensified behavioral activity. Dark pulses are considered to be non-photic stimuli and cause phase advances between midday and late day, where they induce behavioral activity (Janik and Mrosovsky, 1993; Biello and Mrosovsky, 1996). Although our protocol was designed to expose the mice to shifts in the light cycle, non-photic stimuli of unequal strength existed between the short and long day group (Reebs and Mrosovsky, 1989a; Reebs and Mrosovsky, 1989b; Yamanaka et al., 2008). Additional research would be required to identify brain structures that may function as secondary oscillators and shift to a larger extent than the SCN in response to behavioral activity.

Behavioral results without a running wheel

To test for possible effects of wheel-running activity on the behavioral phase advance, we repeated the behavioral experiments in the absence of a running wheel. Mice that were entrained to a short day without a running wheel had a small (1-hour) phase advance after a 4-hour advance in the LD regime. This relatively small advance is similar to the 1.3-hour advance in the mice that were entrained to a short day in the presence of a running wheel. These results suggest that wheel-running activity has little or no influence on the behavioral phase-advancing capacity of mice that are entrained to a short day. On the other hand, the behavioral phase advance in the long day-entrained mice was reduced significantly from 3.7 hours to 1.0 hour in mice that were entrained without a running wheel. From these results, we conclude that the phase-advancing response of the long day-entrained mice was induced predominately by wheel-running activity. Because the behavioral shift was much larger than the shift in the SCN, it is highly unlikely that the behavioral shift was driven by a shift in the SCN. Previous studies have reported accelerated re-entrainment of behavior to a shifted LD cycle when the animals had access to a running wheel (Deboer and Tobler, 2000; Yamanaka et al., 2008). Nevertheless, the most intriguing aspect of this finding was the absence of a large shift in the SCN, in the presence of a large behavioral shift. Previous studies revealed that peripheral tissues and/or non-SCN brain structures can have a higher phase-advancing capacity than the SCN (Yamanaka et al., 2008; Sellix et al., 2012; Cao et al., 2013; Mohawk et al., 2013). For example, the habenula plays a role in regulating several circadian behaviors, including locomotor activity, and this structure can regulate activity rhythms either on its own or as part of a larger network (Paul et al., 2011). Our results are consistent with these findings and suggest that additional (non-SCN) brain structures also control behavioral activity rhythms.

Conclusions

For many decades, the phase-shifting capacity of the biological clock was believed to reflect the behavior of a limit cycle oscillator. Based on limit cycle oscillator theory, one would expect that high-amplitude oscillations would have a smaller shift than low-amplitude oscillators in response to a given stimulus. This prediction has indeed been confirmed in unicellular organisms, including Chlamydomonas, Euglena, Gonyaulax, Paramecium, and Neurospora (Malinowski et al., 1985; Johnson et al., 1989; Hosokawa et al., 2013). The amplitude of the SCN network is determined by (a) the single cell amplitude and (b) synchrony among the single cells. In several previous studies the SCN rhythm amplitude was reduced as a result of a reduction in single cell amplitude, rather than as a reduction in neuronal synchrony (Guilding et al., 2013) (Vitaterna et al., 2006). According to expectations from limit cycle theory, the increase in phase shifting capacity in these mice is explainable by the reduction

in single cell amplitude. In our study we have modified the electrical activity amplitude of the SCN rhythm, not by varying the amplitude of the individual neurons, but by changing the synchrony among them (vanderLeest et al. 2007). We found that selective enhancement of neuronal synchrony leads to large phase shifting capacity. This enhanced phase-shifting capacity in SCN from short days is in agreement with previous studies that reported a larger phase-delaying capacity of high-amplitude SCN oscillations compared to low-amplitude SCN oscillations (vanderLeest et al., 2009). Taken together, these data reveal a positive relationship between neuronal synchronization and phase-shifting capacity at the level of the SCN neuronal network.

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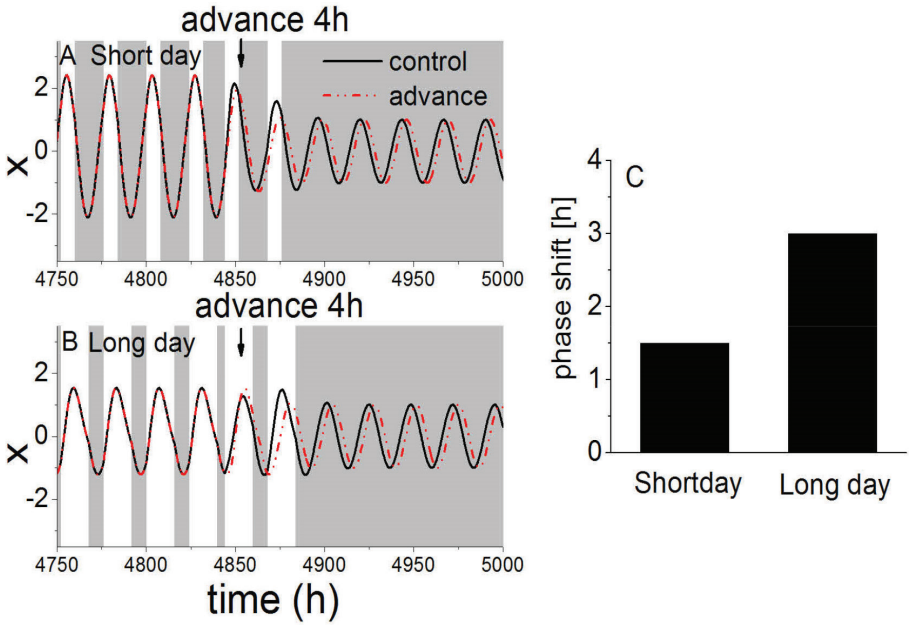
Supplemental figure



Supplemental Figure S1. Poincaré model simulation of short and long photoperiods with a 4-hour phase advance (indicated by the vertical arrow). The gray shading represents darkness, and the white bars represent light. (**A and B**) Time series of variable x under short-day (**A**) or long-day (**B**) conditions before and after the advance (the dotted red line) and for control conditions (no advance, indicated by the solid black line). The *y*-axis is in arbitrary units. (**C**) The phase shift that results from a 4-hour phase advance under short-day and long-day conditions, calculated from the data shown in panels (**A**) and (**B**), respectively.

3

THE PROPORTION OF LIGHT-RESPONSIVE NEURONS DETERMINES THE SCN'S LIMIT CYCLE PROPERTIES

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Abstract

In mammals, the central clock in the suprachiasmatic nuclei (SCN) controls physiological and behavioral circadian rhythms and is entrained to the external light-dark cycle. The ability of the SCN to entrain can be measured by exposing the animal to a light-dark cycle with a duration that deviates from 24 hours (T-cycles); a wider entrainment range reflects a higher ability to entrain. The neurons of the SCN are either light-responsive or light-unresponsive and are mutually synchronized. The coupling and synchronization between individual SCN neurons and between groups of neurons within the SCN influences the SCN's ability to entrain. Some studies suggest that enhanced coupling decreases the entrainment range, whereas others suggest that enhanced coupling increases the entrainment range. The latter results are surprising, as they are not consistent with the prevalent assumption that the SCN is a limit cycle oscillator that has larger phase shifts when the amplitude is smaller. Here, we used Poincaré and Goodwin models to test entrainment properties using various proportions of neurons that are responsive to an external stimulus. If all neurons receive external input, the SCN shows limit cycle behavior in all conditions. If not all neurons receive light input, we found that the entrainment range of the SCN was positively related to coupling strength when coupling was weak. When coupling strength was stronger and above a critical value, the entrainment range was negatively correlated with coupling strength. The results obtained from our simulations were confirmed by analytical studies. Thus, the limit cycle behavior of the SCN appears to be critically dependent on the coupling strength among the neurons, and the proportion of neurons that respond to the entraining stimulus.

Introduction

As a consequence of the Earth's rotation around its axis, virtually every organism has developed circadian rhythms in physiology and behavior. In mammals, these circadian rhythms are controlled by the suprachiasmatic nucleus (SCN), which serves as the central pacemaker and is located at the base of the brain. The SCN contains approximately 20,000 neurons and receives light input from the retina through the retino-hypothalamic tract (Welsh et al., 2010). SCN neurons are autonomous single-cell oscillators, generating an intrinsic 24-hour rhythm via an internal molecular feedback loop (Welsh et al., 1995; Reppert and Weaver, 2002; Herzog, 2007; Webb et al., 2009). These single-cell oscillators are mutually coupled and synchronized (Okamura, 2003; Quintero et al., 2003; Schaap et al., 2003; Honma et al., 2004; Aton et al., 2005; Welsh et al., 2010; Mohawk and Takahashi, 2011) .

The endogenous rhythm of the SCN is entrained to the external light-dark cycle. Light information is received through the retina and is transmitted to the ventrolateral aspect of the SCN. The ability of the SCN to entrain can be analyzed by varying the length of the external cycle (T-cycles). The entrainment range is defined as the difference between the shortest and longest T-cycles to which the SCN can entrain, and varies both between and within animal species—for example, the range is 22.5-25.5 hours for *Arvicanthis niloticus* and 20.5-29.0 hours for humans (Wever, 1983).

The entrainment range is believed to depend on the ratio between the amplitude of the oscillator and the strength of the external entraining stimulus (zeitgeber) (Pittendrigh et al., 1991; Abraham et al., 2010). The amplitude of the SCN rhythm depends both on the amplitude of the individual neuronal oscillators and on the coupling strength between SCN neurons (Abraham et al., 2010). Tighter coupling increases phase synchrony (Abraham et al., 2010). If the neurons are tightly coupled and are closely synchronized in phase, the amplitude of the SCN rhythm will be large. On the other hand, if the coupling is weak and the cells are poorly synchronized, the amplitude of the SCN rhythm will be small (Schaap et al., 2003; Rohling et al., 2006b; Abraham et al., 2010). Therefore, a high degree of coupling between SCN neurons is predicted to result in a narrow entrainment range (i.e., a low capacity to synchronize to external cycles that deviate from 24 hours). Indeed, the SCN has a narrower entrainment range than tissue with weakly coupled oscillators such as the lung, and this is consistent with the finding that the SCN network is more tightly coupled than the lung tissue (Abraham et al., 2010).

On the other hand, some results suggest that a decrease in coupling and in synchronization, corresponds with a smaller—not larger—phase-shifting capacity and with a smaller entrainment range (Colwell et al., 2003; Aton et al., 2004; vanderLeest et al., 2009; Farajnia et al., 2012). For example, mice that lack vasoactive intestinal polypeptide (VIP) show reduced coupling among neurons, and these mice

have a smaller-amplitude phase response curve whereas overexpression of VIP and enhanced synchrony leads to faster resetting (Colwell et al., 2003; Cao et al., 2013). Furthermore, the amplitude of the SCN rhythm is diminished in older animals due to desynchronization among the neurons (Farajnia et al., 2012; Chang and Guarente, 2013) and old animals have more difficulty entraining to a 22-hour T-cycle than young animals (Aton et al., 2004). In addition, animals that are housed under long photoperiod have a lower amplitude SCN rhythm than animals housed under short photoperiod, due to decreased synchronization within the SCN (vanderLeest et al., 2009). Nevertheless, the SCN of long-day exposed mice has a smaller phase-shifting capacity (vanderLeest et al., 2009; Ramkisoensing et al., unpublished data). These results are contradictory to the expectation that low-amplitude rhythms show larger phase shifts than high-amplitude rhythms in response to the same perturbation.

Several factors can determine the phase-response properties of the SCN. For example, it is easier to entrain the SCN to extreme T-cycles (i.e., 22 and 26 hours) if a higher proportion of neurons are responsive to the zeitgeber (Gu et al., 2012). Here, we mathematically explored the impact of changing the proportion of neurons that receive the external stimulus on limit cycle oscillator behavior in an attempt to resolve discrepancies between previous results. We used numerical simulations using both the Poincaré and Goodwin models, and we also explored this issue analytically.

We found that when 100% of the neurons are responsive to the stimulus, a monotonic relationship was revealed between coupling strength and entrainment range. On the other hand, when fewer than 100% of the neurons are responsive to the stimulus, the response characteristics became dependent upon the coupling strength between the neurons and showed an optimum curve as a function of coupling strength. Thus, the proportion of neurons that respond to an external stimulus determines whether the system behaves as a typical limit cycle oscillator. This finding can help resolve the differences in the results that have been observed in previous studies.

Methods

Two types of models that are often used to describe the SCN oscillatory network are the Poincaré model (Abraham et al., 2010; Bordyugov et al., 2011; Gu et al., 2013) and the Goodwin model, which describes oscillators in terms of a genetic feedback loop (Ruoff et al., 1999; Gonze et al., 2005; Locke et al., 2008; Gu et al., 2009; Xu et al., 2012). We use the Poincaré model in addition to the Goodwin model because it is better suited for mathematical analysis when the oscillators are part of a system of coupled oscillators. The analytical studies were performed as they lead to precise mathematical formulations that can be used for the entire domain of parameters, while in the simulations it is only one set of parameters that is explored. The approaches are complementary.

Poincaré model

The Poincaré model is a generic model containing two variables, x and y for each neuron. The coupled model is defined as follows:

$$\frac{dx_i}{dt} = yx_i(A_0 - r_i) - \frac{2\pi}{\tau} y_i + KF + L_i$$
$$\frac{dy_i}{dt} = yy_i(A_0 - r_i) - \frac{2\pi}{\tau} x_i \qquad (1)$$

where γ represents the relaxation parameter, A_0 and τ represent the amplitude and period, respectively, of one isolated individual oscillator, and i represents the i-th oscillator (i = 1,...,N). The individual oscillators are coupled via the coupling term KF, where K represents coupling strength and F is the mean field value of variable x, and r is the amplitude of a single oscillator. r and F are defined as follows:

$$r_i = \sqrt{x_i^2 + y_i^2}\ , F = \frac{1}{N}\sum_{i=1}^{N} x_i \qquad (2)$$

L_i represents the input of light (or any other external stimulus) as follows:

$$L_i = \begin{cases} K_f \sin(\Omega t) & (i \le pN) \\ 0 & (i > pN) \end{cases} \qquad (3)$$

where p represents the proportion of neurons that are responsive to the light signal ($0 < p \leqslant 1$), K_f represents light intensity, and Ω is the angular frequency of the light-dark cycle. When $K = 0$ and $L_i = 0$, then $r = A_0$. The following parameter settings were used:

$y = 0.2,\ A_0 = 1,\ \tau = 24,\ K_f = 0.1,\ N = 20.$

In experimental conditions the light-dark cycle has a square waveform rather than a sinusoidal function. We choose to use the sinusoidal function because it is easier to analyze mathematically. When we simulated light intensity changes by a square-form we obtained similar results (Fig. S1).

Goodwin model

The Goodwin model is widely used to describe mathematically the molecular negative feedback loop that generates circadian rhythms in mammals. In the model, the feedback loop is comprised of three variables: a clock gene mRNA (*x*), a clock protein (*y*), and a transcriptional inhibitor (*z*). Together, these three variables represent one single oscillator, and different oscillators are coupled through the mean field (*F*) of neurotransmitter concentration (*V*). A Goodwin model comprised of *N* oscillators is defined as follows:

$$\begin{aligned}
\frac{dx_i}{dt} &= \sigma\left(\alpha_1 \frac{k_1^n}{k_1^n + z_i^n} - \alpha_c \frac{KF}{k_c + KF}\right) + L_i \\
\frac{dy_i}{dt} &= \sigma\left(k_3 x_i - \alpha_4 \frac{y_i}{k_4 + y_i}\right) \\
\frac{dz_i}{dt} &= \sigma\left(k_5 y_i - \alpha_6 \frac{z_i}{k_6 + z_i}\right) \\
\frac{dV_i}{dt} &= \sigma\left(k_7 x_i - \alpha_8 \frac{V_i}{k_8 + V_i}\right) \\
F &= \frac{1}{N}\sum_{i=1}^{N} V_i
\end{aligned} \qquad (4)$$

$i = 1, 2, 3..., N$

where the state variables for clock gene mRNA x_i, clock protein y_i, and transcriptional inhibitor z_i form a negative feedback loop and constitute oscillator *i*. In this model the neurotransmitter concentration V_i is induced by mRNA x_i of neuron oscillator *i*. The oscillators are coupled through the mean field *F*, which is the mean of V_i, and coupling strength *K* represents the sensitivity of each individual oscillator to the mean field and is assumed to be identical for every oscillator.

The light term is defined as stated above for the Poincaré model with K_f = 0.01.

The following parameter set is the same set as described previously (Gonze et al., 2005):

$\alpha_1 = 0.7\mathrm{n}M/h,\ k_1 = 1.0nM,\ n = 4.0,\ \alpha_2 = 0.35nM/h,\ k_2 = 1.0nM,$
$k_3 = 0.7/h,\ \alpha_4 = 0.35nM/h,\ k_4 = 1.0nM, k_5 = 0.7/h,$
$\alpha_6 = 0.35nM/h,\ k_6 = 1.0nM,\ k_7 = 0.35/h,\ \alpha_8 = 1.0nM/h,$
$k_8 = 1.0nM,\ \alpha_c = 0.4nM/h,\ k_c = 1nM,\ N = 20.$

Definition of the entrainment range

The entrainment range of the SCN network is represented by its lower limit of entrainment (LLE) (Abraham et al., 2010). If the period difference between the endogenous cycle and the exogenous T-cycle is less than 0.000001 hour, the SCN network is considered to be entrained to the T-cycle.

In order to compare the effect of differing coupling strength on the entrainment range, it is necessary to make the free-running period uniform for various coupling strengths, as coupling strength *K* can affect the free-running period (Gonze et al., 2005; Gu et al., 2009; Abraham et al., 2010).

Here, we defined the normalized LLE in both the Poincaré and Goodwin models in accordance with (Abraham et al., 2010; Gu et al., 2012) as follows:

$$LLE_{normalized} = LLE \times \frac{24h}{\tau(K)} \qquad (5)$$

where $\tau(K)$ is the free-running period of the SCN with coupling strength *K*. When *K* = 0, the neurons are not coupled, and the intrinsic period of each individual neuron is 24 hours. For simplicity, we use LLE to represent $LLE_{normalized}$ throughout the paper.

We confirmed the results obtained for the LLE using the upper limit of entrainment (ULE) with the Poincaré and Goodwin model and with analytical studies. Furthermore, we added simulations where SCN neurons show enhanced light responses during the night, by introducing a circadian modulation of the light-sensitivity parameter. Also we investigated the impact of a heterogeneous -as opposed to homogeneous- population of neurons on our results by (1) varying the intrinsic periods of individual neurons from 23 h - 25 h and (2) varying the coupling strengths according to a normal distribution (mean value = 1; standard deviation = 0.02).

Simulation details

To numerically calculate the equations, we used the fourth-order Runga-Kutta method with time increments of 0.01 hour. The initial 1,000,000 time steps were neglected in order to avoid the influence of transients. The number of oscillators (*N*) was 20. The initial conditions for each variable were selected randomly from a

uniform distribution in the range (0-1) for *x* and *y* in the Poincaré model and for *x*, *y*, *z*, and *V* in the Goodwin model. We also calculated the results using $N = 100$ and time increments of 0.001 hours.

Results

We used the Poincaré model to evaluate how the proportion of SCN neurons that are responsive to a zeitgeber (e.g., light or temperature) determines the SCN's ability to entrain. We performed numerical simulations using five different proportions (10, 25, 50, 75, and 100%) of neurons that receive the external signal, where 25% (i.e., $p = 0.25$; see Methods) corresponds to normal light input of the SCN (Sawaki, 1979; Meijer et al., 1986; Cui and Dyball, 1996; Jiao et al., 1999). To estimate the entrainment range of the SCN, we determined the low limit of entrainment (LLE) as described previously (Abraham et al., 2010). A larger deviation of the LLE from 24 hours corresponds with a wider entrainment range.

We examined the response properties of the SCN with respect to varying both coupling strength (K) and the proportion of neurons that receive the external signal (Fig. 1, ULE: Fig. 2A). When 100% of the neurons receive the external signal (i.e., $p = 1$), the entrainment range decreases as coupling strength increases; thus, a tightly coupled system will have a narrower entrainment range than a weakly coupled system. However, a different result is obtained when a smaller proportion of neurons receive the external signal. With weak coupling strength, the entrainment range increases as a function of K and shows an optimum at the critical value (K_c), which is approximately 0.08. Above this critical value, the entrainment range then decreases as a function of K.

To verify the outcome of our Poincaré simulation using a secondary approach, we also applied the Goodwin model (Fig. 2B). The results obtained using the Goodwin model are consistent with the results obtained with the Poincaré model. When $p = 1$, the entrainment range decreased as a function of coupling strength; however, when $p < 1$ and with low coupling values, the entrainment range increased as a function of K, whereas the entrainment range decreased as a function of K with higher coupling values.

We performed two additional simulations, one in which the SCN neurons show enhanced light responses during the night (Meijer et al., 1998; Brown et al., 2011), and one in which the neurons are heterogeneous with respect to their period and coupling strength (Gu et al., 2012; Gu et al., 2013). The results were very similar to our main results (Fig. 2C-E).

Analytical studies

To obtain insight into our simulation results, we performed a series of analytical studies. The details of these analytical studies are provided in the Supplementary Material. We used Equation S7 when $K \leq K_c$ and Equation S10 when $K > K_c$ to show the analytical results, using $p = 0.25$ to mimic the light input of the SCN (Fig. 3 A) (Sawaki, 1979; Meijer et al., 1986; Cui and Dyball, 1996; Jiao et al., 1999). The results

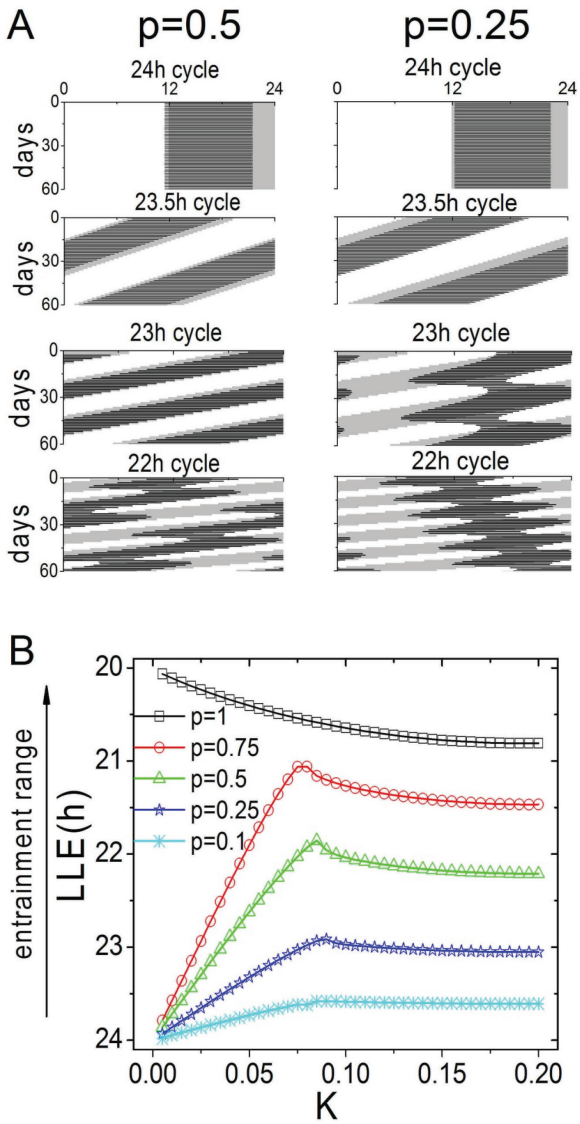

Figure 1. **Entrainment range depends on both the proportion (*p*) of neurons that receive the entrainment signal and the coupling strength (*K*) among the neurons.** (**A**) Simulated activity rhythms for $p = 0.5$ and $p = 0.25$ for different T-cycles, with coupling strength $K = 0.10$. The rhythm entrains to a T-cycle of 23 h when $p = 0.5$, but not when $p = 0.25$. The light-dark cycle is shown as white-gray background. (**B**) The lower limit of entrainment (LLE) is plotted against the coupling strength for different p. When $p = 1$, the entrainment range is a monotonic negative function of K. In contrast, when $p < 1$, the entrainment range follows a biphasic relationship with respect to K. When K is below a critical value (K_c), the entrainment range increases as coupling strength increases; when K is above this critical value, the entrainment decreases as coupling strength increases. Here, K_c is approximately 0.08. The data were simulated using the Poincaré model (see Methods).

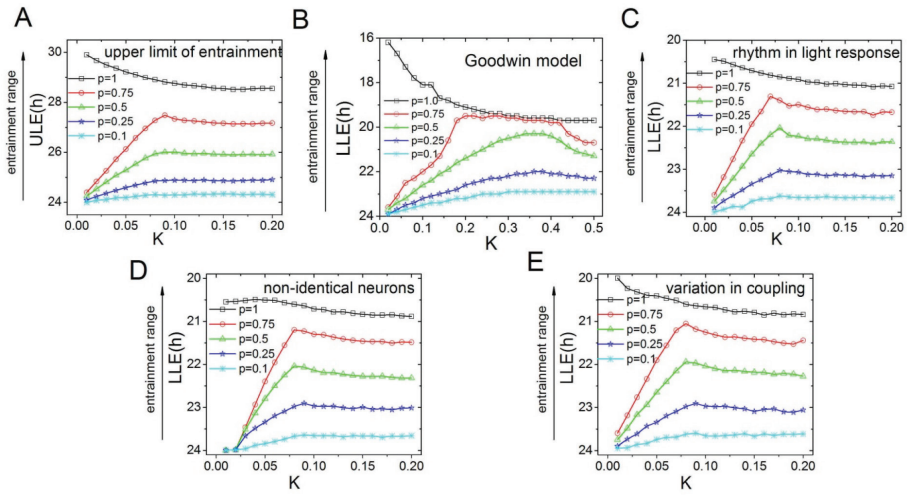

Figure 2. **Entrainment range is dependent on both the proportion (*p*) of light-receiving neurons and coupling strength for different conditions. (A)** Upper limit of entrainment for Poincaré model. The upper limit of entrainment (ULE) is plotted against the coupling strength for different values of *p*. The results are very similar to the results obtained for the lower limit of entrainment (LLE), and K_c = 0.08. **(B)** LLE for Goodwin model simulations, plotted as a function of coupling strength, for different *p* values. **(C)** LLE simulated with cells that are more sensitive to light during the night and less sensitive during the day, for different *p* values. **(D)** LLE simulated for non-identical cells with intrinsic periods ranging from 23-25 h, for different *p* values. **(E)** LLE simulated for non-identical coupling strengths between the neurons, for different *p* values. All results are qualitatively similar to the results obtained with the Poincaré model (Fig. 1).

of the theoretical analysis are similar to the simulation results and yielded a unimodal curve that contained a critical transition point K_c. To further investigate K_c, we plotted LLE as a function of the proportion of neurons that receive input (Fig. 3 B). When $K < K_c$ (i.e., below 0.08), we observed a sharp and steep jump in the entrainment range as *p* approaches 1, indicating a fundamental difference in response behavior between partial and full sensitivity to the external signal when coupling is weak. If coupling strength is higher than the critical value ($K > 0.08$), we observed a gradual and consistent increase in entrainment range as a function of *p*. In other words, an increase in *p* corresponded with a near-linear increase in entrainment range.

We analytically determined the parameter domain of *K* and confirmed the existence of K_c when $p < 1$. When $K < K_c$, Equation S7 shows that Ω_{LLE} is proportional to *K*. Because K_f and *K* are substantially smaller than A_0, it follows that $\frac{r_b}{r_a} \sim 1$. The rate of change between r_b and r_a is lower than the rate of change for *K*, which is ~10 (*K* ranges from 0.01 to K_c, which is around 0.08). Hence, when $p < 1$ and when $K \leqslant K_c$, the entrainment range increases as *K* increases. When $K > K_c$, Equation S11 reveals

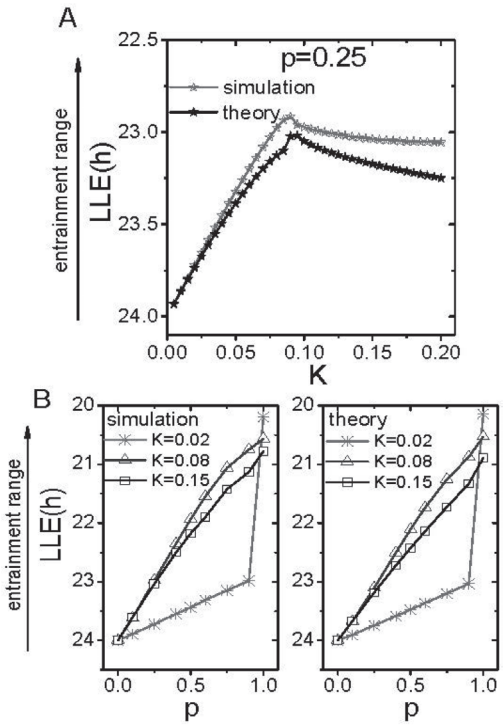

Figure 3. Comparison between the theoretical analysis and numerical simulations. (A) The results from Fig. 1 were theoretically analyzed for $p = 0.25$. The simulation data are repeated from Fig. 1 (note the change in the y--axis scale from Fig. 1). **(B)** The relationship between entrainment range and the proportion (p) of neurons that receive the entrainment signal for $K < K_c$, $K = K_c$, and $K > K_c$. The left figure shows the results of the numerical simulation, and the right figure shows the results of the theoretical analysis. In both simulation and theoretical analysis, the entrainment range is positively correlated with p. When $K = 0.02$ (i.e., $K < K_c$), the entrainment range changes abruptly from $p = 0.9$ to $p = 1.0$.

that Ω_{LLE} is negatively correlated to K. As p approaches 1 ($p \to 1$), the expression of Ω_{LLE} becomes $\omega_0 + \frac{K_f}{2(A_0 + \frac{K}{2\gamma})}$, which is the same equation that was used by Abraham and colleagues (Abraham et al., 2010). Hence, under these conditions, the entrainment range decreases as K increases.

The value of K_c is obtained using Equation S10 and is positively dependent on the value of p. We attributed a value to p in Equation S10 as follows:

$$K_c(p \to 0) = \frac{-[\gamma - \frac{K_f}{2A_0}] + \sqrt{[\gamma - \frac{K_f}{2A_0}]^2 + 4A_0\gamma K_f}}{2}, K_c(p \to 1) = \frac{-\gamma + \sqrt{\gamma^2 + 4\gamma K_f}}{2}$$

We substituted the values of other parameters and obtained $K_c(p \to 0) = 0.10$ and $K_c(p \to 1) = 0.07$. Consistent with the results shown in Fig. 1, K_c decreases as p increases. Note that when p is infinitely close to 1, K_c is 0.07; however, when $p = 1$, no critical value (K_c) emerges. All of our analytical results are fully consistent with our simulation results obtained using either the Poincaré model or the Goodwin model.

Discussion

In response to a given perturbation, low-amplitude rhythms are generally expected to cause a greater phase shift than high-amplitude rhythms. Although some studies support this expectation, other studies do not. Here, our aim was to investigate the conditions that determine the response properties of the SCN, to evaluate the influence of the proportion of neurons that receive the external stimulus, and to identify the role of coupling strength between neurons. Two models were used to examine the effects of external stimuli on SCN response properties. First, a modified Poincaré model was used, and these results were further tested and confirmed using a Goodwin model. Both models revealed that when all neurons receive the external signal, increasing coupling strength decreases the SCN's entrainment range. However, qualitatively different results were obtained when the proportion of neurons that receive the external signal drops below 1; specifically, the entrainment range is no longer a monotonic function of coupling strength, but has a maximum at a critical value of K (K_c, the transition point). When coupling strength increases from 0 to the critical value, the entrainment range of the SCN first increases; however, when coupling strength is higher than this critical value, the entrainment range decreases with increasing coupling strength. These simulation results were verified using analytical approaches.

Perturbing stimuli: temperature and light

Although the circadian clock can compensate for changes in temperature, external temperature can serve as a weak zeitgeber (Edery, 2010). However, in homeothermic animals such as mammals, the influence of external temperature on the pacemaker's phase is extremely small, and changes in external temperature have only a minimal influence on the phase of the pacemaker (Refinetti, 2010). The effect of temperature on the SCN can be investigated however, *in vitro* by changing the temperature of the bath medium in which the tissue explant is cultured. As expected from the Goldmann equation, temperature will affect the amplitude and frequency of action potentials of individual SCN neurons (Ruby and Heller, 1996; Ruby et al., 1999; Herzog and Huckfeldt, 2003). Such changes in temperature will affect all neurons, and may occur during exercise or sleep (Franken et al., 1991; Deboer and Tobler, 1997; Nybo, 2012). However, we regard this primarily as an experimental manipulation that enhances our insight in the response properties of the system. If 100% of the neurons in the SCN respond to a change in temperature, this would correspond to a situation in which $p = 1$. This result is consistent with the findings of Abraham and colleagues (Abraham et al., 2010), who observed that when peripheral oscillators (fibroblasts) are weakly coupled, they have a wider entrainment range to temperature cycles than the network of the more strongly coupled SCN. In addition, weakening

coupling within the SCN network by the application of *N*-(Cis-2-phenyl-cyclopentyl) azacyclotridecan-2-imine-hydrochloride (MDL) or tetrodotoxin (TTX) increases the entrainment range of the SCN to temperature cycles (Abraham et al., 2010).

Retinal stimulation causes acute changes in the discharge levels of light-responsive SCN neurons. Single-cell *in vivo* recordings in anesthetized animals have yielded the most reliable and consistent estimates of the proportion of light-responsive neurons within the SCN and all studies obtained values between 20–30% (Sawaki, 1979; Meijer et al., 1986; Cui and Dyball, 1996; Jiao et al., 1999). If only a fraction of SCN neurons is responsive to an external stimulus, changes in coupling strength will have an unpredictable effect on the entrainment range, as K_c is unknown. These results raise the question of whether we can estimate K_c. Although we cannot currently determine K_c with absolute precision, it might be possible to use existing empirical data to determine whether coupling strength within the SCN is weaker or stronger than K_c.

When days are relatively short (i.e., the light period is shorter than 12 hours), the neurons in the SCN are more closely synchronized in phase than during long days (Hazlerigg et al., 2005; Rohling et al., 2006a; Rohling et al., 2006b; Inagaki et al., 2007; VanderLeest et al., 2007; Naito et al., 2008; Brown and Piggins, 2009). Moreover, in short days, the phase shifts are larger than in long days (Pittendrigh et al., 1991; Refinetti, 2007; vanderLeest et al., 2009), and the SCN of short-day animals has enhanced synchrony and readjusts more rapidly to a shift of the light-dark cycle (Ramkisoensing et al., unpublished data). The increased phase synchrony by exposure to short days leads to an enhanced phase shift in response to light stimuli, while decreased phase synchrony leads to a decrease in phase shifting response.

Similar to the effect of exposing animals to long days, aged animals experience a reduction in phase synchrony, as revealed by electrophysiological techniques (Nygard et al., 2005; Duncan, 2006-07; Nakamura et al., 2011; Farajnia et al., 2012; Sellix et al., 2012; Chang and Guarente, 2013). This reduced phase synchrony in aged animals is associated with reduced intercellular communication, reduced GABAergic activity, and reduced electrical activity. Thus, in aged animals, a decrease in communication within the SCN network has been clearly established. Of note, aged animals have reduced phase-shifting capacity, even in response to saturating light intensities (Valentinuzzi et al., 1997; Biello, 2009; Farajnia et al., 2012; Sellix et al., 2012). Interestingly, when aged mice were exposed to a shift in the light-dark cycle, the shift in the expression of *Per1* was attenuated, whereas the shift in *Per2* expression was enhanced (Kolker et al., 2003; Sellix et al., 2012). The latter result is surprising as an enhanced amplitude in the *Per2* expression rhythm of single cells was observed, as well as decreased synchrony between the cells in old animals (Sellix et al., 2012). Not only in animals but also in elderly humans a reduced ability to adjust to new cycles has been observed, as evidenced by a decreased ability to work

in shifts (Duffy et al., 2007; Sletten et al., 2009). Conversely, transgenic mice with enhanced SCN coupling as a consequence of overexpressing the type 2 vasoactive intestinal peptide (VPAC2) receptor rapidly resynchronize in response to an advance in the light-dark cycle (Shen et al., 2000; Cao et al., 2013).

The finding that aging and entrainment to long days result in lower-magnitude phase shifts indicates that the circadian system operates on a trajectory in which coupling strength is positively correlated with the entrainment range. This suggests that under "normal" conditions (i.e., young-adult animals housed under a LD 12:12), the coupling factor K is lower than K_c, as a decrease in coupling will lead to a decrease in the entrainment range only when K is lower than K_c (Fig. 4).

Several studies have suggested that coupling within the SCN is weak (Liu et al., 1997; Honma et al., 2004; Indic et al., 2008; Myung et al., 2012). Weak coupling is defined as the condition in which coupling has little effect on the *amplitude* of single neuron oscillators but has a large effect on the *phase* of single neuron oscillators (Kuramoto, 1984; Strogatz, 2000; Winfree, 2001; Acebrón et al., 2005). In constant light conditions, the amplitude of a single neuron's oscillations changes little, despite considerable desynchronization among the neurons (Ohta et al., 2005).

Additional evidence supporting weak coupling within the SCN was obtained from phase-shifting studies. Following a phase-resetting light pulse or a shift in the external light-dark cycle, resetting of the rhythm is often accompanied by transient cycles, which are believed to reflect reorganization in the phase relationships between the underlying oscillators. Because many cycles are required to complete the reorganization (which can take 3-6 days), coupling among SCN cells is believed to be relatively weak (Yamazaki et al., 2000; Reddy et al., 2002; Nagano et al., 2003; Davidson et al., 2009 ; Sellix et al., 2012). Taken together, the experimental results suggest that under normal circumstances, the SCN neurons are weakly coupled, which is consistent with the coupling factor being lower than K_c.

Entrainment range

In our study, we distinguished mathematically between oscillators that are responsive to an external stimulus and oscillators that are not directly responsive to a zeitgeber. Entrainment is achieved if the zeitgeber-responsive neurons and indirectly, the zeitgeber-unresponsive neurons are able to entrain to the T-cycle (de la Iglesia et al., 2004; Schwartz et al., 2010; Gu et al., 2012). It is an inherent property of the models that, within the limits of entrainment, the zeitgeber-responsive neurons are entrained to the zeitgeber. For the SCN, this may not be the case, which would decrease the effective contribution of light to the SCN, resulting in a further decrease of the entrainment range (corresponding with a lower p value in our model). When 100% of the neurons in the SCN are zeitgeber-responsive, all oscillators in the model

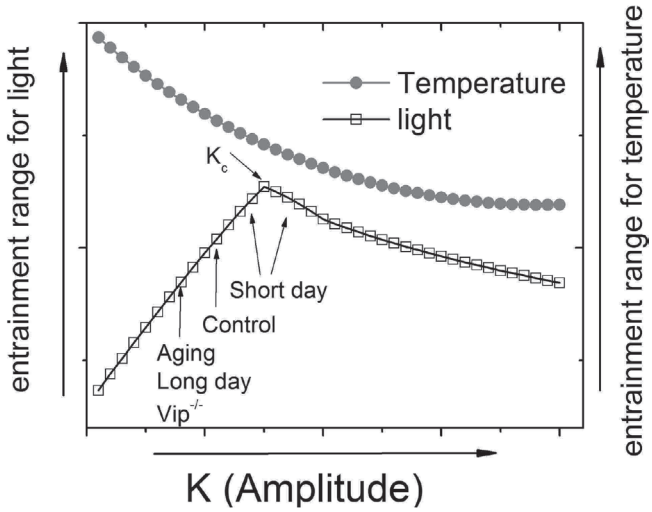

Figure 4. **Differences in entrainment range under various conditions can be explained by our model.** For temperature, $p = 1$ (as all of the neurons will respond to a change in temperature). For external light, $p = 0.25$. K_c is indicated, and the effects of the various conditions on K are indicated. "Control" refers to normal conditions (young mice housed under 12:12 light-dark conditions). Note that the two curves have different y-axis and can not be compared quantitatively.

are mathematically uniform. However, when fewer than 100% of the neurons are zeitgeber-responsive, the mathematical difference between zeitgeber-responsive and zeitgeber-unresponsive oscillators in our model accounts for the difference in entrainment below and above the critical coupling strength K_c.

When coupling strength is higher than K_c, the strength of the coupling will be strong enough to ensure that the zeitgeber-responsive and zeitgeber-unresponsive neurons will remain synchronized in phase, essentially forming one functional group of cells. Thus, the range of T-cycles to which the network can entrain will depend solely upon the coupling strength of the network, which is reflected in its amplitude. If the network amplitude is extremely high, the network will become too rigid to be entrained to an external T-cycle (Abraham et al., 2010). Thus, a higher amplitude leads to a narrower entrainment range, and a lower amplitude leads to a wider entrainment range. On the other hand, when coupling strength is lower than K_c, coupling between the zeitgeber-responsive oscillators and the zeitgeber-unresponsive oscillators is weak. Consequently, the zeitgeber-responsive neurons will be unable to synchronize the zeitgeber-unresponsive neurons to short T-cycles, resulting in dissociation between the two groups of neurons in the network (de la Iglesia et al., 2004). Thus, the entrainment range depends on whether or not the

zeitgeber-unresponsive neurons can be entrained by the zeitgeber-responsive neurons, from which follows that the entrainment range increases when coupling strength is increased.

Evidently, for all values of K, tighter coupling leads to more synchronization of the neuronal groups within the network and therefore to a larger amplitude of the network oscillation (see Fig. S2). However, we show here that the amplitude of the SCN and the entrainment range do not follow a monotonous relationship. Thus, for models that contain non-uniform oscillators (in which one group of neurons is zeitgeber-responsive and another group of neurons is zeitgeber-unresponsive), a critical coupling value (K_c) functions as a transition point between either entrainment determined by the synchrony within the network ($K < K_c$) or entrainment controlled by the strength (i.e., the amplitude) of the network oscillations ($K > K_c$).

Amplitude of SCN network

Importantly, SCN amplitude is the product of (1) single cell amplitude, and (2) neuronal synchrony. It is very likely that individual SCN cells behave as limit cycles and that cells with high amplitude rhythms shift less than cells with low amplitude rhythms. Intuitively, this would correspond with a pendulum with a long cord, which is more difficult to shift as compared to a pendulum with a short cord. Several studies have shown that a reduced amplitude of the SCN (at the population level), was associated with enhanced phase shifting capacity (Vitaterna et al., 2006; An et al., 2013; Guilding et al., 2013). In some studies, the population amplitude was reduced not as a result of desynchrony but as a result of a decrease in single cell amplitude (Vitaterna et al., 2006; Guilding et al., 2013). These studies therefore are not in disagreement with the present study. In another study, the population amplitude was decreased as a result of both a reduction in single cell amplitude and a desynchronization (An et al., 2013). In such a case it becomes unpredictable whether phase shift capacity will increase or decrease, as it depends on the relative changes in these two variables.

In short and long photoperiods, only the phase synchrony between the neurons is altered, while the single cell amplitude is not significantly different (VanderLeest et al., 2007). In this case, the phase shifting capacity decreases when rhythm amplitude of the population is lower. This is in accordance with the predictions of this study, and in contrast to the behavior of the individual components. Thus, when limit cycle oscillators are brought together, new properties arise in the behavior of the ensemble.

Can we still regard the SCN network as a limit cycle oscillator, or are these findings in conflict with the properties of a limit cycle oscillator? In the circadian rhythm field, amplitude has become closely associated with a limit cycle oscillator and it is generally believed that high-amplitude limit cycle oscillators have a smaller phase shifting capacity than low-amplitude limit cycle oscillators (Pittendrigh et al.,

1991). We show that high amplitude rhythms can shift more, if the high amplitude is the result of enhanced synchrony among the neurons. Our results suggest therefore that the SCN at the network level has more complex properties, that were not directly anticipated. These response properties of the SCN appear to follow logically from both the coupling strength of the SCN network and the proportion of neurons that respond to the perturbing external signal.

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PART II

CIRCADIAN PHOTORECEPTION

4

IRRADIANCE ENCODING IN THE SUPRACHIASMATIC NUCLEI BY ROD AND CONE PHOTORECEPTORS

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Abstract

Light information is transmitted to the central clock of the suprachiasmatic nuclei (SCN) for daily synchronisation to the external solar cycle. Essential for synchronisation is the capacity of SCN neurons to respond in a sustained and irradiance-dependent manner to light. Melanopsin has been considered to mediate this photosensory task of irradiance detection. By contrast, the contribution of the classical photoreceptors in irradiance encoding is less clear. Here we investigate the role of classical photoreceptors by *in vivo* electrophysiological responses in freely moving animals to specific wavelengths of light (UV; λ_{max} 365 nm, blue; λ_{max} 467 nm and green; λ_{max} 505 nm) in both melanopsin deficient mice (*Opn4*$^{-/-}$) and mice lacking rods and cones (*rd/rd cl*). Short and long wavelength light induced sustained irradiance-dependent responses in congenic wild-type mice (+19,6%). Unexpectedly, sustained responses to light persisted in *Opn4*$^{-/-}$ mice (+18,4%). These results provide unambiguous evidence that classical photoreceptors are able to transmit irradiance information to the SCN. In addition, at light intensities that would stimulate rod and cone photoreceptors, the SCN of *rd/rd cl* mice showed greatly reduced sustained responses to light (+7,8%). Collectively our data demonstrate a role for classical photoreceptors in illuminance detection by the SCN.

Introduction

The mammalian circadian pacemaker is located in the suprachiasmatic nuclei (SCN) and coordinates daily cycles in behaviour and physiology. Light is the primary cue for synchronisation (entrainment) of the SCN such that dawn and dusk adjusts the endogenous period of SCN neurons to the 24-h external cycle. Light information is detected in the retina by classical rod and cone photoreceptors and by a specialized subset of intrinsically photosensitive retinal ganglion cells (ipRGCs) which expresses the photopigment melanopsin. Photic information is then transmitted to the SCN via the retinohypothalamic tract (RHT) [1]. In addition to their direct photosensitivity, the ipRGCs receive an indirect light input from rod and cone photoreceptors [2-5], and by this route the classical photoreceptors also signal light information to the circadian system [6].

In response to retinal illumination, light responsive SCN neurons show an increase in electrical impulse frequency [7-12]. Typically, SCN responses to light consist of two components, a fast transient at the on- and off-set of the light pulse, and a sustained component that depends upon the irradiance level of the light [8, 13]. These two response components have been variously linked to differential melanopsin and rod/cone inputs, with sustained responses thought to arise from melanopsin and the transients from the rods/cones [9, 11, 12]. However, several studies have suggested a role for the rods and cones in circadian entrainment, melatonin suppression and SCN electrical activity [7, 9, 12, 14-21]. Whilst UV light via the stimulation UVS cones has been shown to drive sustained responses in SCN neurons [19], light in the visible range failed to drive this sustained response in *Opn4*$^{-/-}$ mice [11]. These findings are difficult to reconcile with behavioural data showing that light in the visible range can still drive illuminance detection in mice lacking melanopsin [15, 20, 21] [15]. For example, illuminance dependent period lengthening still occurs in *Opn4*$^{-/-}$ mice [20, 22].

In this study we explored the effect of both short and long wavelengths of light on SCN electrical activity in wild-type mice (*Opn4*$^{+/+}$), mice lacking melanopsin (*Opn4*$^{-/-}$) and mice lacking rods and cones (*rd/rd cl*). Electrical activity recordings from the SCN of freely moving mice show an acute irradiance-dependent firing of SCN neurons upon UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light exposure. These responses are sustained for the full duration of the stimulus. This sustained response was unaffected by the loss of melanopsin. Recordings in *rd/rd cl* mice showed intensity-dependent sustained responses in the SCN, but these were attenuated at lower irradiances across the sensitivity range of the rods and cones. Our findings provide strong evidence that classical photoreceptors, along with melanopsin, encode tonic irradiance information to the SCN.

Methods

Ethical approval

All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Center (the Netherlands).

Animals

Adults male *Opn4*$^{+/+}$ (n=4) and *Opn4*$^{-/-}$ (n=8) mice on a C57Bl/6 x 129 background (aged 3-9 months) and male *rd/rd cl* (n=4) and wild-type (n=4) mice on a C3H background (aged 3-9 months) were used for *in vivo* recordings of multiunit activity from the SCN. Mice were housed individually and food and water were available *ad libitum.* Ambient room temperature was maintained at 20 ± 2 ˚C.

In vivo electrophysiology

Surgical procedures were performed under deep anaesthesia (ketamine, 100 mg/kg; xylazine, 20 mg/kg; atropine 1 mg/kg). A tripolar stainless steel electrode (Plastics One, Roanoke, VA, USA) was implanted using a stereotactic instrument (Stoelting, Illinois, USA). Two twisted polyimide-insulated electrodes aimed at the SCN were used for differential recording and a third uncoated electrode was placed in the cortex as a reference. The electrode was implanted under a 5˚ angle using the following coordinates; 0.61 mm lateral from Bregma and 5.38 mm ventral to the dura. After a week of recovery animals were placed in custom-designed recording chamber and were connected to a counter balanced swivel recording system to where the animal was able to move freely. The electrical signal was amplified and bandwidth filtered (0.5-5 kHz), window discriminators were used to count digital pulses in 2 second epochs (CircaV1.9 custom made software). Data was stored for offline analysis.

Animals were exposed to monochromatic light using a custom-designed sphere. The diameter of the sphere was 30 cm and it was coated with high reflectance paint (Barium Sulphate; Labsphere WRC-680) to obtain uniform illumination levels. At the top of the sphere an opening was created for the connecting swivel system. Around the opening at the top of the sphere high power UV (λ_{max} 365 nm, NCSU033B, Nichia, Tokyo, Japan), blue (λ_{max} 467 nm, XML-PB01-0023, Luxeon Rebel, Philips, the Netherlands) and green (λ_{max} 505 nm, Luxeon Rebel, Philips, the Netherlands) LEDs were positioned and a baffle preventing the animal to look directly in the light source. The wavelength of light and its irradiance was measured using a calibrated spectrometer (AvaSpec2048, Avantes, the Netherlands). Using these parameters the actual amount of photons was calculated. Irradiance levels were manually regulated and the timing of the light pulses was computer controlled. To investigate the response characteristics (i.e. transient and sustained components), mice were exposed to light pulses of various durations (100 and 300 seconds) and intensities

(ranging from 9 to 13 log quanta/cm^2/s). These durations are similar or exceed those used in other studies to characterize SCN responses [7, 9, 11, 12]. All light pulses were applied between CT 14-18, which corresponds with the mid of the subjective night, the phase at which light pulses induce phase shifts. Animals were housed in constant darkness for a few days prior to the recording of the light responses. For the determination of the response characteristics mean changes in SCN electrical activity were compared to baseline discharge levels. The baseline level was defined as the average firing rate during the last 100 seconds before lights on and the sustained level of SCN electrical activity was defined as the average discharge rate during the entire duration of the light pulse excluding the first 25 seconds because of the transient response.

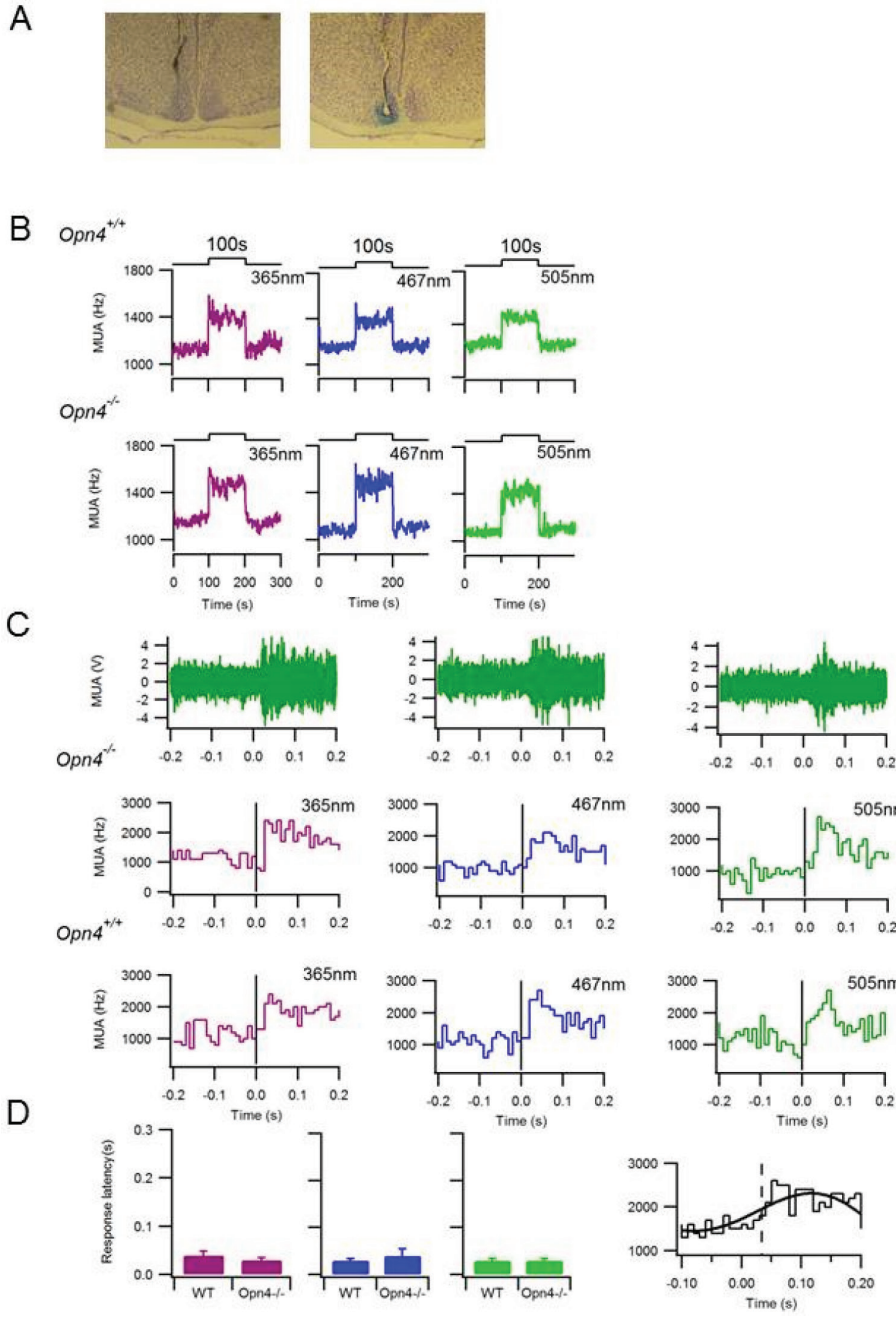
To determine the response latency of the SCN discharge rate in response to a light pulse at a high resolution the electrophysiological signal of the SCN neurons were digitized at 25 kHz using Spike2 hardware and software (Cambridge Electronic Design) and stored for off-line analysis. Analysis was performed as described previously [19]. The digitised recordings were imported into MATLAB as 'waveform data', including data from light and movement sensors, using parts of the sigTOOL SON Library. Imported waveform data was triggered at fixed voltage amplitude settings, and time and amplitude of these action potentials were used for analysis. To investigate the latency of the neuronal response to light onset, digitised action potentials were counted in 0.01 second bins. For a detailed analysis of population activity, a baseline recording (100 seconds before light pulse) was used to create a spike amplitude histogram. On the basis of this amplitude histogram, thresholds were set in such a way that the average number of counts within each threshold window was equal. Threshold windows were non-overlapping and started above noise level. Action potentials were counted within each step of set thresholds for the remained of the recording.

Histology

At the end of each recording, animals were sacrificed using CO_2 and a small electrolytic current was passed through the electrode to mark the recording site. Brain tissue was collected and immersed in a fixative solution containing potassium ferrocyanide to stain the recording site blue. The brain was sectioned coronally and stained with cresyl violet for microscopic reconstruction of the recording site (Figure 1 *A*).

Statistical analysis

Statistical analysis was performed using SPSS Statistics 17 (PASW). Two-tailed student's t-tests or one-way ANOVA, followed by a Bonferroni's *post-hoc* test were used to test significant differences between groups. Values were considered significantly when $p<0.05$.


A
B
Opn4+/+
100s
365nm
467nm
505nm
MUA (Hz)
Opn4-/-
Time (s)
C
MUA (V)
Opn4-/-
Opn4+/+
D
Response latency(s)
WT
Opn4-/-

◀ **Figure 1. A.** An example of a coronal slice of the mouse brain with the SCN right above the optic chiasm at the base of the hypothalamus (left figure). The location of the electrode can be verified by the blue spot which is marked using an electrolytic current (right figure). **B.** SCN electrical activity responses to various wavelengths of light in freely-moving melanopsin deficient mice *(Opn4$^{-/-}$)* and their wild-type littermates (*Opn4$^{+/+}$)*. Graphs show representative SCN multi-unit activity (MUA) traces in response to a 100 sec UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light pulse. Light pulses are indicated above the graphs. Bin size is 1 sec. SCN electrical activity show a sustained response during exposure to UV, blue and green light in both *Opn4$^{-/-}$* (+18,4%) and *Opn4$^{+/+}$* (+19,6%) mice. **C.** Response latencies to UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light exposure in *Opn4$^{-/-}$* and *Opn4$^{+/+}$*mice. Time of lights on are indicated by the vertical line in each graph. Upper graphs show a corresponding oscilloscope trace of SCN electrical activity and counts are shown in the lower graphs. Bin size is 0.01s. SCN firing rate is increased in both melanopsin deficient and wild-type mice with a response latency of 0.04s to the three different wavelengths of light. **D.** Histograms showing mean response latencies ± SEM of *Opn4$^{-/-}$* and *Opn4$^{+/+}$* mice in response to three wavelengths of light. The figure on the left is an example of a multiunit activity trace through which a polynomial fit is plotted. This fit was used to calculate the half maximum values. The dashed line indicates the half maximum value in the representative example. Half maximum values were used to determine the response latencies.

Results

We determined the effect of UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light on electrical activity of populations of SCN neurons in freely moving mice. Wild-type mice (n=8), possessing melanopsin ipRGCs, UVS cones (λ_{max}=360nm), rods (λ_{max} =498nm) and MWS cones (λ_{max} =508nm) showed a robust increase in SCN electrical discharge with a transient overshoot in firing at lights on and a sustained elevation in electrical activity throughout light exposure in response to 100 seconds of monochromatic light (Fig. 1). We found a sustained increase in SCN electrical activity in response to light exposure up to 30 minutes. At lights off a fast drop in electrical discharge rates was observed to levels that were transiently below baseline level. No differences in light-response characteristics of transient and sustained responses were found between responses to 365, 467 or 505 nm light (Fig. 1 *B*).

The possible contribution of rods/cones to the sustained response was investigated by determining whether the sustained component was affected by the absence of melanopsin in *Opn4$^{-/-}$* mice (n=8). In response to 100 seconds of monochromatic UV light of 365 nm, SCN electrical discharge showed a tonic firing rate that was consistent with our previous findings [19]. Surprisingly, exposure to longer wavelengths of light (467 and 505 nm stimuli) also elicited a sustained response for the full duration of the light presentation. The kinetics of the light response were similar to wild-type mice, and contained both phasic and tonic components (Fig. 1 *A*). Response latencies in *Opn4$^{-/-}$* mice to all wavelengths of light were in the range of 30-40ms (n=8, Fig. 1 *C, D*). The latencies were defined as the half maximum of a fitted polynomial function.

These data show that tonic activation of SCN cells to 365, 467 and 505 nm light can all occur independently of melanopsin, indicating that rods and/or cones can induce a sustained response at the level of the SCN.

To investigate the sensitivity to different wavelengths of light we undertook irradiance response measurements in *Opn4*$^{-/-}$ mice. Mice were exposed to 100 seconds of light of different irradiances ranging over 4 or 5 log units (9-13 log photons/cm^2/s). *Opn4*$^{-/-}$ mice and congenic wild-type mice showed a sustained response that was irradiance-dependent (Fig. 2 *A,B*).

In *Opn4*$^{-/-}$ mice both rods and MWS-cones would be strongly stimulated by 467 and 505 nm stimuli. Only a small increase in SCN electrical activity was observed in response to the lowest irradiances (9-11 log photons/cm^2/s). At this irradiance rod photoreceptors should be fully stimulated [23] and be able to induce a maximal change in SCN electrical activity. However, at high irradiances (12-13 log photons/cm^2/s) the increase in SCN electrical activity was significantly larger compared to the sustained response at low rod-mediated irradiances (p=0.01, one-way ANOVA).

To determine the possible contribution of classical photoreceptors to SCN light responses we undertook recordings in mice (*rd/rd cl*) lacking both rods, UVS cones and MWS cones. SCN firing frequencies were increased in *rd/rd cl* mice (n=4) in response to 300 second light pulses of 365, 467 and 505 nm (Fig. 3 *A*). Because of the slow response kinetics, pulses with a duration of 300 seconds were used to make sure we were able to observe the full response repertoire. Typically, the light-activated SCN discharge started with a transient overshoot at lights on followed by a relatively small sustained response which was significantly smaller than that observed in wild-type animals (p=0.02, independent samples t-test). No transient off inhibition was present at the end of light exposure, rather SCN electrical discharge rate slowly returned to baseline after termination of light exposure. Mice lacking rods and cones *(rd/rd cl*) showed a response latency of 200 ms (n=4 animals, Fig. 3 *B*) in reaction to light pulses of 365, 467 and 505 nm. The latencies were significant longer than the response latencies of 30-40ms seen in congenic wild-type mice (Fig. 3C, p<0.001, independent samples t-test).

To investigate the sensitivity of mice lacking rods and cones (*rd/rd cl,* n=4) to various wavelengths of light, these mice were exposed to 300 seconds of light at three different wavelengths (365, 467 and 505 nm) ranging from low to high irradiances. While a sustained response to 365 nm light was detected at the highest irradiance (~12 log photons/cm^2/s, p=0.22, one-way ANOVA) (Fig. 4 *A,B*), no light response was detected at lower irradiances. Only the highest irradiances of 467 and 505 nm stimuli induced a significant sustained response in SCN electrical activity levels (p=0.04, one-way ANOVA). Transient responses were also detected at the highest light intensities and showed a longer latency compared to wild-type congenics.

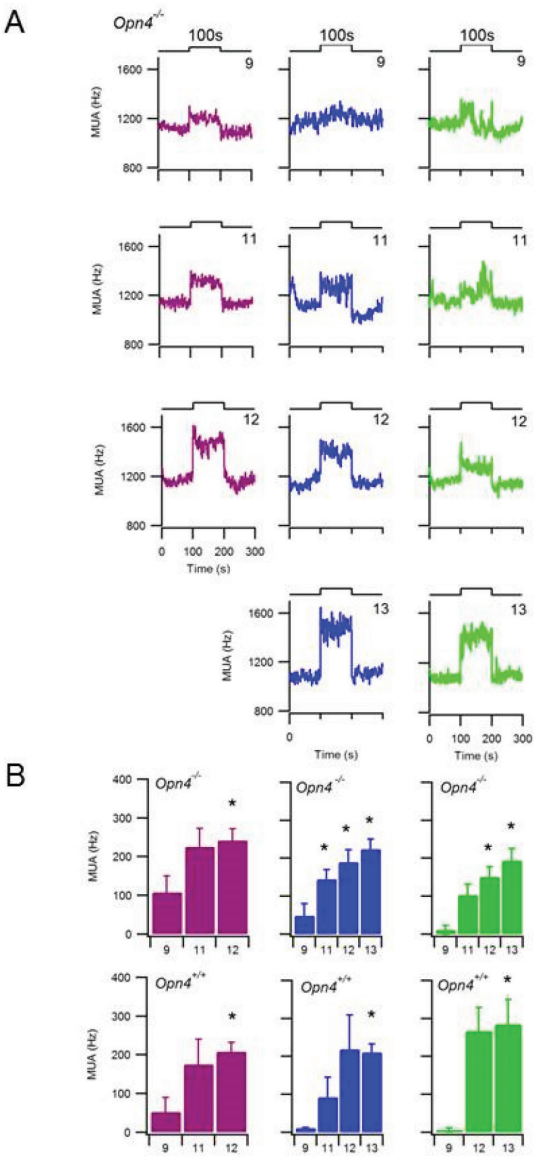


Figure 2. A. SCN electrical activity responses UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light exposure at three different irradiances of light in *Opn4*$^{-/-}$ mice. Mice were exposed to 100 sec of light which is indicated in a step diagram above each graph. One representative trace of SCN electrical activity is shown per irradiance, with log quanta/cm^2/s shown in each graph. Bin size is 1sec. **B.** Histograms showing mean sustained responses in SCN electrical activity as a function of irradiance of UV, blue and green light in *Opn4*$^{-/-}$ and *Opn4*$^{+/+}$ mice. Graphs show the difference in spike frequency between sustained and baseline levels for each irradiance level (n=4-7 per irradiance). Sustained responses to light in SCN electrical activity are increased in a irradiance-dependent manner in both *Opn4*$^{-/-}$ and *Opn4*$^{+/+}$ mice. No difference was detected between UV, blue and green light (asterisk: $p<0.05$).

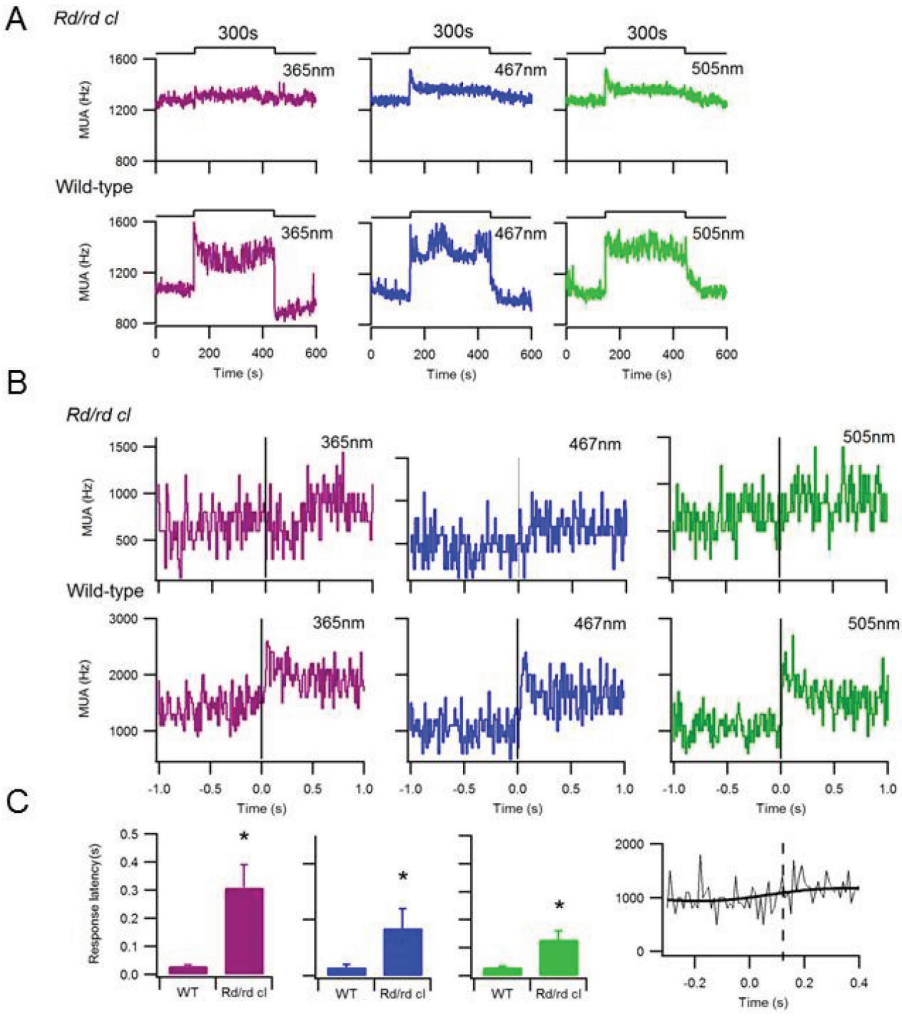


Figure 3. A. Representative traces of MUA in response to a 5-min light pulse in rodless-coneless mice (*rd/rd cl*) and their wild-type littermates. Mice were exposed to UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light. *Rd/rd cl* mice show a reduced sustained response to UV (+2,3% increase), blue (+12,5%) and green (+8,9%) light and do not show an off-response. SCN electrical activity slowly returns to baseline after light exposure. **B.** Response latencies to UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light exposure in *rd/rd cl* mice and wild-types. Time of lights on are indicated by the vertical line in each graph. Bin size is 0.01 s. SCN firing rate is increased in both *rd/rd cl* and wild-type mice, with a response latency of 0.04 s in wild-type mice and of 0.20 s in *rd/rd cl* mice. **C.** Histograms showing mean response latencies ± SEM *rd/rd cl* and wild-type mice in response to three wavelengths of light. The figure on the left is an example of a multiunit activity trace through which a polynomial fit is plotted. This fit was used to calculate the half maximum values. The dashed line indicates the half maximum value in the representative example. Half maximum values were used to determine the response latencies. Note the different scale on the x-axis compared to B (asterisk: $p<0.05$).

Discussion

Our findings show that the SCN exhibits sustained firing levels in the absence of melanopsin, and across a broad range of wavelengths. Sustained increases in electrical discharge were observed during exposure to UV, blue and green light, for the entire duration of the light pulse from the SCN of wild type and melanopsin-deficient *Opn4*$^{-/-}$ mice. These findings suggest strongly that classical photoreceptors are capable of inducing tonic responses to light at the level of the SCN. We undertook recordings from mice lacking all rods and cones (*rd/rd cl*) and found an attenuation of the sustained response across all wavelengths at irradiances that were sufficient to fully stimulate classical photoreceptors. Our findings are consistent with behavioral experiments that have shown preserved tonic responses to light on circadian period in rod-only mice [15]; attenuated phase shifting responses in mice lacking MWS-cones [14, 16]; and tonic responses to light recorded from retinal ganglion cells in the absence of melanopsin [24].

Origin of sustained responses in the SCN

In vivo electrophysiological recordings in the SCN showed that UV light (365 nm) can induce sustained light responses within the SCN, consistent with our previous studies [19]. The current results show that green (505 nm) and blue (467 nm) light pulses can also elicit transient and sustained responses to light within the SCN, fully mimicking the effect of white light on the SCN. Remarkably, in the absence of melanopsin, the full response characteristics were maintained. This finding was unexpected, as the ability to drive a tonic/sustained response is generally assumed to be the property of melanopsin-based phototransduction [9, 11, 12, 25]. In contrast to the present study, Mure et al., (2007) failed to detect sustained responses to light in mice lacking melanopsin. This difference might arise because Mure et al., (2007) recorded from the SCN of anaesthetized animals and at a different time of day, ZT4-12 *versus* CT14-CT18 in the present study. In agreement with our findings, direct recordings from illuminated ipRGCs of the macaque monkey have been shown to elicite sustained responses at irradiances considered too low to drive melanopsin phototransduction [2]. In addition, recordings from mice lacking melanopsin (*Opn4*$^{-/-}$) show that ipRGCs retain the ability to respond to light in a sustained manner [24]. These findings, with the data we present here, all strongly suggest that rods and cones are able to mediate sustained responses to light from SCN neurons. Furthermore, recent findings indicate that short wavelength light triggers sustained responses in electrical activity within the PON and SCN [19, 26]. The present findings show that illuminance detection by the SCN occurs across a broad spectral range (UV to visable) and can be mediated by both melanopsin and the classical photoreceptors.

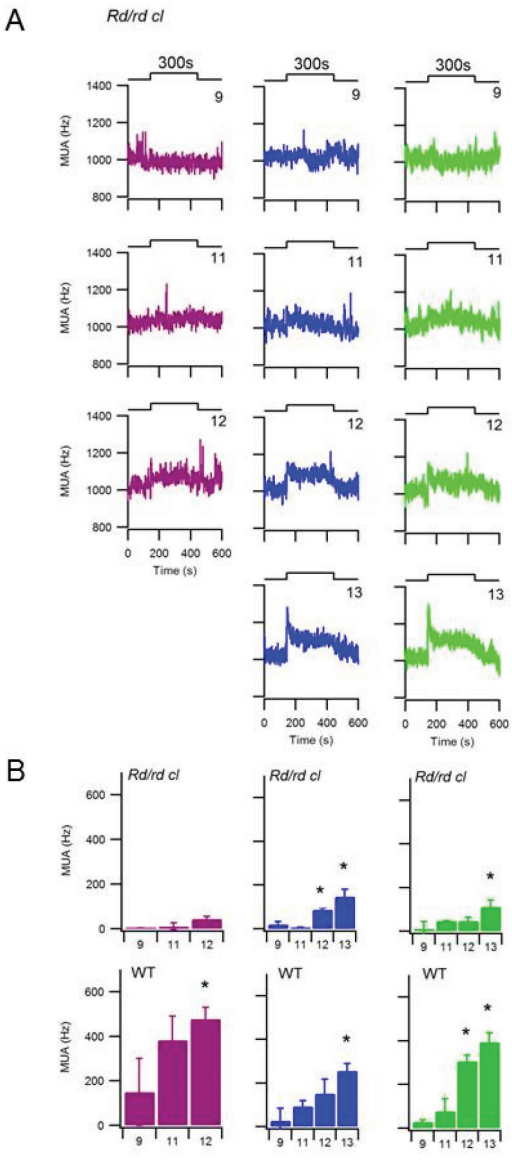


Figure 4. A. SCN electrical activity responses to UV (λ_{max} 365 nm), blue (λ_{max} 467 nm) and green (λ_{max} 505 nm) light exposure at three different irradiances of light in *rd/rd cl* and wild-type mice. Mice were exposed to 5 min of light which is indicated in a step diagram above each graph. One representative trace of SCN electrical activity is shown per irradiance, with log quanta/cm^2/s shown in each graph. Bin size is 1 sec. **B.** Histograms showing mean sustained responses in SCN electrical activity as a function of irradiance of UV, blue and green light in *rd/rd cl* and wild-type mice. Graphs show the difference in spike frequency between sustained and baseline levels for each irradiance level (n=4 per irradiance). Sustained responses to blue, green and UV light in SCN electrical activity are decreased in *rd/rd cl* mice compared to wild-type mice. *Rd/rd cl* mice only respond to high light intensities of UV light. Both *rd/rd cl* and wild-type mice show irradiance-dependent effects to green and blue light (asterisk: $p<0.05$).

We also examined light-induced electrical responses in the SCN of mice with melanopsin ipRGCs but lacking rods, UVS and MWS cones (*rd/rd cl*) and observed attenuated sustained responses to UV, blue and green light. The light response of *rd/rd cl* mice was characterized by an absence of the fast transient component, as well as by a reduction in the sustained discharge. In response to low light irradiances (9 log/quanta/cm^2) no light responses were observed, consistent with the relative insensitivity of melanopsin-based ipRGCs [3, 25, 27]. At higher irradiances (above 12 log/quanta/cm^2) an intensity-dependent increase in the sustained response was observed. In agreement with Wong et al, these responses were "sluggish", and slow, even for the moderate irradiances, and all transients were lost. Also in agreement with direct recordings from ipRGcs [3, 28] we failed to detect responses to low levels of light in the SCN in the absence of classical photoreceptors.

Origin of transient on and off responses

We observed transient "on-excitation" and "off-inhibition" in response to UV, blue and green light in both wild-type and melanopsin-deficient mice. The presence of these responses in the absence of melanopsin indicates a role for rods and/or cones in evoking the transient "on-excitation" and "off-inhibition". Interestingly, the "off-inhibition" following light exposure was not present in mice lacking rods and cones, which suggests a role for classical photoreceptors in mediating this response. The "on-excitation" was still present in *rd/rd cl* mice, but the reaction time of SCN electrical activity in response to light in these mice was 200 ms (Fig. 2 *B*). This is consistent with melanopsin-mediated response latencies measured from ipRGCs reported previously (>300 ms to minutes [2, 25, 29]and differs from both rod-mediated (150 ms) and cone-mediated (30-60 ms) response latencies [2, 3]. The response onset latency for all wavelengths of light in wild-type as well as in melanopsin-deficient mice has a very short duration of 30-40 ms (Fig. 2 *A*). This similarity to cone-mediated response latencies supports the conclusion that cones are capable of mediating broad spectrum light input to the SCN at high light intensities.

Key features of the response properties of SCN neurons correlate well with the effects of light on circadian behaviour. For example, mice possessing rods but lacking cones and melanopsin have been shown to entrain to both low and high light intensity light:dark cycles, suggesting that rods alone are capable of mediating photic entrainment [15]. These findings raise the question of whether rods can drive tonic responses within the SCN. In this study we observed small increments in SCN firing frequency in response to light intensities (9-11 log photons/cm^2/s) that should have been sufficient to maximally stimulate the rods. These responses were sustained and are, therefore, attributable to full stimulation of the rods and/or partial stimulation of the cones. Sustained responses siginificantly increased at higher light intensities compared to low light intensities that are expected to have saturated the

rods. These results suggest that cones can contribute to the sustained response of the SCN at higher light intensities. The observed short response latencies are also in accordance with cone activation as well as the reduction in phase shifting magnitude in the absence of MWS cones [9, 14]. By contrast, cone-only mice show poor entrainment to light-dark cycles [18] which is difficult to reconcile with a contribution of cones to the sustained response of the SCN. It is possible that cone-only mice have deficiencies that have occurred developmentally in retinal information processing, or alternatively, that the sustained cone driven discharge in the SCN is not sufficient for triggering functional entrainment pathways. This remains to be elucidated.

The discovery that the photic regulation of circadian behavior [30], melatonin suppression [31] and sleep induction [32] can all persist in the absence of rods and cones does not of course preclude a role for these photoreceptors in non-image forming responses to light in the intact retina. Behavioral studies in rodents have shown that the circadian system is very sensitive to small changes in light-intensity, such that small variations in light intensity can be sufficient for entrainment [15, 18, 33, 34]. Thus, activation of only a subset of retinal photoreceptors may be sufficient for photic entrainment. Collectively, the studies published to-date show a mixed and complex arrangement of photoreceptor inputs involved in irradiance detection. The relative contribution of melanopsin, rods and cones remains to be determined, and is most likely dependent on the intensity and wavelength composition of the light source.

Concluding remarks

An increasing body of evidence shows an important contribution of classical photoreceptors to circadian entrainment [14, 15, 18, 19], pupil constriction [26, 35] and SCN neuronal responses to light [7, 9, 11, 19, 20]. Both rods [15, 18] and cones [14, 16, 19] were suggested to be important for the circadian system, and our present findings offer a mechanism through which classical photoreceptors regulate the SCN (Fig. 5). We suggest that at lower to intermediate irradiances classical photoreceptors play a role in transmitting light information to the SCN. Whilst we cannot distinguish between the relative contribution of rods and cones, it seems that both rods and cones are involved. At low light intensities, only rods are stimulated, and we observed clear tonic responses in the SCN. An increment in light intensity to levels that should have saturated the rods, led to a further increment in light response levels, presumably mediated by cones. Our studies make clear that melanopsin is not required for sustained responses to light at the level of the SCN and suggest an advanced level of functional redundancy in the irradiance detection capacity of the SCN.

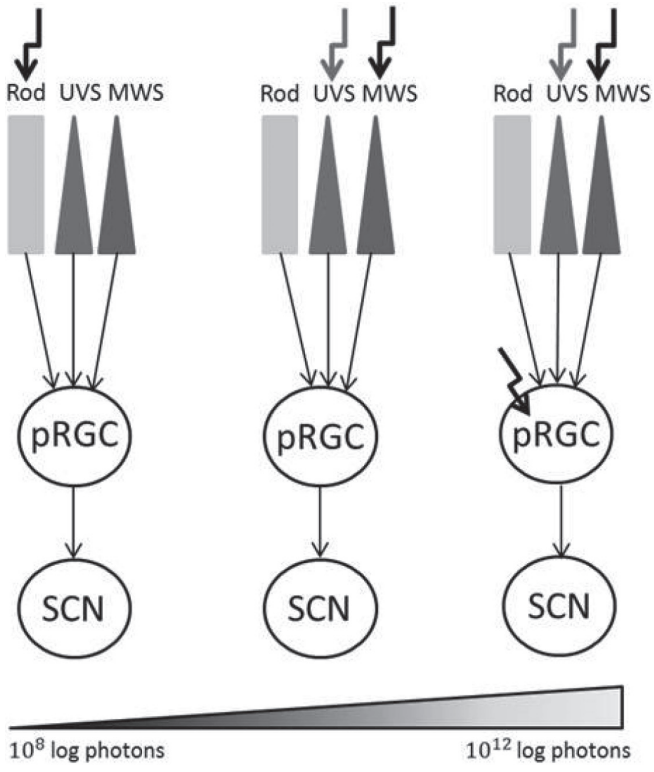


Figure 5. Schematic representation of the involvement of various photoreceptors in the photic input to the circadian system across a range of light intensities. Light intensities are depicted below the figure. Arrows depict light input; pink arrows indicate UV light and black arrows indicate long wavelength light. Our data suggest that at high irradiances cones can play a role in irradiance detection.

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5

CONE PHOTORECEPTORS CONTRIBUTE TO THE LIGHT RESPONSE OF THE MAMMALIAN BIOLOGICAL CLOCK

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In preparation

Abstract

The detection of ambient light is important for the circadian clock to synchronize to the external solar cycle. Light is detected by rods, cones and photosensitive retinal ganglion cells in the retina which send activating signals to the suprachiasmatic nuclei (SCN), the site of the major circadian pacemaker. Here we address the role of cone photoreceptors in the transmission of photic information to the SCN by evaluating SCN light responsiveness and photo-entrainment in mice having cones as the only functional photoreceptor. *In vivo* electrophysiological recordings in SCN from cone-only mice showed light responses in SCN neuronal activity in response to pulses of UV (λ max 365 nm) and green (λ max 505 nm) light. Higher irradiances of UV light led to irradiance-dependent enhancements in SCN neuronal activity, and the UV-light induced responses were not significantly different between cone-only and wild type mice. Interestingly, in cone-only mice higher irradiances of green light led to smaller sustained responses in SCN neuronal activity, and at the highest irradiance of green light, light-induced responses were significantly smaller in cone-only mice compared to wild type mice. In cone-only mice, light pulses of 15 minutes of UV light caused sustained responses in SCN neuronal activity that decayed with a half max-time of 525 seconds, while 15 minute pulses of green light only induced responses during the initial phase of the light exposure (half max-time of 52 seconds), indicating that the SCN is more sensitive to light of shorter wavelengths. Accordingly, recordings of wheel-running of cone-only mice exposed to light-dark (LD) cycles of UV or green light revealed that the majority of mice entrained normal to LD cycles of UV light, while the majority of mice exposed to green LD cycles entrained with a phase angle that was significantly earlier. Together, our data provide evidence for the ability of cone photoreceptors to transmit information about transitions of lighting conditions to the circadian clock.

Introduction

Light is sensed in the retina by classical rod- and cone photoreceptors and by a specialized subset of photosensitive retinal ganglion cells (pRGCs) containing the photopigment melanopsin. pRGCs project to non-image forming brain areas, such as the biological clock which is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The ablation of pRGCs results in the loss of photoentrainment (1). Although classical rod- and cone photoreceptors are not essential for entrainment of the biological clock to the external light-dark (LD) cycle, both rod- and cone photoreceptors can influence the SCN. This is evidenced by the experimental finding that mice can entrain to a LD cycle in the absence of melanopsin (2,3). In addition, recordings in the SCN of melanopsin-deficient mice show preservation of sustained light responses in the SCN, and the magnitude of which seems unaffected by the absence of melanopsin (4,5).

Whereas rods are capable of driving photoentrainment at a wide range of light intensities (6), mice with cones as the only photoreceptors in their retina show in general strongly reduced entrainment and surprisingly large inter-individual differences in their ability to entrain. The majority of cone-only (*Opn4*$^{-/-}$*Gnat1*$^{-/-}$) mice are not able to entrain to low intensity white light and some of them exhibit a positive phase angle of entrainment suggesting distortions in the ability to entrain (7,8). In addition, phase shifting responses in mice lacking mid-wavelength sensitive cones (M-cones) are attenuated (9,10), which is in agreement with the reduced ability of cone-only mice to entrain to a LD cycle. The question is therefore to what extent cones contribute to photic entrainment.

Photoentrainment is dependent on light-induced changes in SCN neuronal activity (11,12). Typically, SCN neurons respond to light with a transient increase in SCN electrical activity followed by a sustained component throughout light exposure. Rod- and cone photoreceptors together can mediate light responses at the level of the SCN including both the fast and the sustained component (4,5,13). These findings are consistent with responses of pRGCs (14-16). In this study we determined the specific contribution of the UV and green cone photoreceptors to circadian photoreception. We performed behavioral and *in vivo* electrophysiological recordings in cone-only mice to determine the effects of λ_{max} 365 nm (UV) and λ_{max} 505 nm (green) light on photoentrainment and on light-induced responses in electrical activity of SCN neurons.

Methods

Animals

Experiments were approved by the ethical committee of the Leiden University Medical Center and were carried out according to their guidelines. *Opn4-/-Gnat1-/-* mice originated from the lab of Prof. Samer Hattar (Johns Hopkins University, Baltimore) and were backcrossed on a C57/Bl6 background at the Leiden University Medical Center. Experiments were carried out with male mice with homozygous knockouts of the *Opn4* and *Gnat1* genes.

Behavioral activity recordings

Behavioral activity rhythms were assessed using running wheels. Mice (n=21) were housed individually in plastic cages which were equipped with running wheels to stimulate active behavior of the mice in a measurable way. The amount of rotations of the wheel was recorded using a ClockLab data acquisition system (Actimetrics, Wilmette, IL, US) and stored on a computer in 1-minute bins. Food and water were available *ad libitum* during the experiment. Mice were housed in a 12:12 LD schedule. During the light phase mice were exposed to either UV light (365 nm, NCSU033B, Nichia), blue light (470 nm, LXML-PB01-0023, RS company) or green light (505 nm, LXML-PE01-0070, RS company). After at least 7 days in a LD cycle, mice were released in continuous darkness.

In vivo electrophysiological experiments

Extracellular activity of SCN neurons was recorded in freely moving mice. Tripolar stainless steel electrodes (Plastics One, Roanoke, VA, USA) were implanted in an anaesthetized mouse. The mouse was fixed in a stereotactic frame (Stoelting, Wood Dale, IL, USA) and the electrodes were implanted in the brain targeting the SCN. Two polyimide insulated twisted electrodes were aimed at the SCN under a 5 degree angle, 0.61 mm lateral from bregma and 5.38 mm ventral to the dura and used for differential recording. The third uncoated electrode was placed in the cortex as a reference. The electrodes were fixed to the skull of the mouse with the use of additional screws and dental cement. After a week of recovery mice were connected to a custom designed recording chamber to measure extracellular activity of SCN neurons while the animal was able to move freely. The electrical signal was amplified and bandwidth filtered between 0.5 and 5 kHz. The electrical SCN signal was digitized at 25 kHz using Spike2 hardware and software (Cambridge Electronic Design Cambridge, UK) and stored for offline analysis.

A custom-designed sphere was introduced in the recording setup to expose the animal to specific monochromatic wavelengths of light. The diameter of the sphere was 30 cm and it was coated with high-reflectance paint (barium sulphate;

WRC-680; Labsphere Inc., North Sutton, NH, USA) to ensure uniform illumination levels. At the top of the sphere high power UV (365 nm, NCSU033B, Nichia), blue (470 nm, , LXML-PB01-0023, RS company) or green (505 nm, LXML-PE01-0070, RS company) LEDs were positioned at the top of the sphere with a baffle to prevent that the animal directly looks into the light source. The wavelength and intensity of light were measured with a calibrated spectrometer (AvaSpec2048, Avantes, Apeldoorn, the Netherlands). Animals were exposed to three wavelengths of light of various light intensities between CT12 and CT16, which corresponds to the beginning of the subjective night.

For detailed analysis of the response characteristics to light the digitized recordings were imported in MATLAB as waveform data using parts of the sigTOOL SON Library, including data from light sensors. Imported waveform data were triggered at fixed voltage amplitude settings. Time and amplitude of the action potentials were used for analysis. Digitized action potentials were counted in 1 sec bins. For a detailed analysis of population activity, a baseline recording was used to create spike amplitude histograms. On the basis of this amplitude histogram, thresholds were set in such a way that the average number of counts within each threshold window was equal. Threshold windows were non-overlapping and started above noise level. Actions potentials were counted within each step of a set threshold for the remainder of the recording.

Histology

At the end of each recording mice were sacrificed in a CO_2 chamber. A small electrolytic current was passed through the electrodes to mark the recording site. Brains were taken out and kept in 4% paraformaldehyde solution containing potassium ferrocyanide. The brains were sectioned coronally and checked with a microscope for reconstruction of the recording site.

Results

Behavioral photoentrainment

Photoentrainment was tested in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice under 12:12 LD cycles using either UV or green light during the light phase at relatively high irradiances (log13 photons/cm^2/s). Seven out of 21 mice (33%) showed normal entrainment under a 12:12 hour LD cycle. Twelve out of 21 mice (57%) were able to entrain to a 12:12 hour LD cycle, but with a positive phase angle of entrainment (Figure 1A). Two out of 21 mice (9.5%) were not able to entrain to the LD cycle. The phase angle of entrainment under a LD cycle with green light was significantly larger compared to UV light (205±56min and 49±17min respectively, t-test p=0.012) (Figure 1B).

In vivo electrophysiology

To determine the relative contribution of cone photoreceptors in light-induced effects on extracellular activity of SCN neurons *in vivo* electrophysiological recordings were performed in wildtype mice (n=4) and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice (n=4). We investigated the effects of light on the response properties of a subset of SCN neurons by exposing wildtype and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice to 60 seconds of UV (λ_{max} 365 nm) and green (λ_{max} 505 nm) light. In wildtype and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice, exposure to 60 seconds of monochromatic light of both wavelengths led to a robust increase in SCN neuronal activity with a typical transient overshoot (transient "on-excitation") in electrical discharge rate followed by a sustained component for the duration of lights on. When the light was switched off a fast decrease in SCN neuronal activity (transient "off-inhibition") was observed before the electrical discharge levels returned to baseline (Fig. 2A).

Sustained response

To examine the role of cone photoreceptors in the magnitude of light-induced responses to the different wavelengths of light, we determined the light-induced sustained increases in SCN neuronal activity in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice and wildtype mice in response to a range of light intensities (log12-15 photons/cm^2/s) (Figure 1A). In *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice, exposure to UV light pulses of irradiances ranging from 12.7 to 14.5 log photons/cm^2/s resulted in increases in SCN electrical discharge rate, and higher intensities caused larger enhancements in SCN neuronal activity (Pearson's r: 0.534 p=0.049, Fig2A,B). The sustained light response caused by high irradiance of green light (14.9 log photons/cm^2/s) was significantly larger in wildtype mice compared to *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice (p=0.011). Interestingly, in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice pulses of green light (log 13.3-14.9) induced enhancements of SCN firing rate, and exposure to higher irradiances of green light led to smaller responses in SCN neuronal firing rate (Pearson's r: -0.621, p= 0.024, Figure 2B).

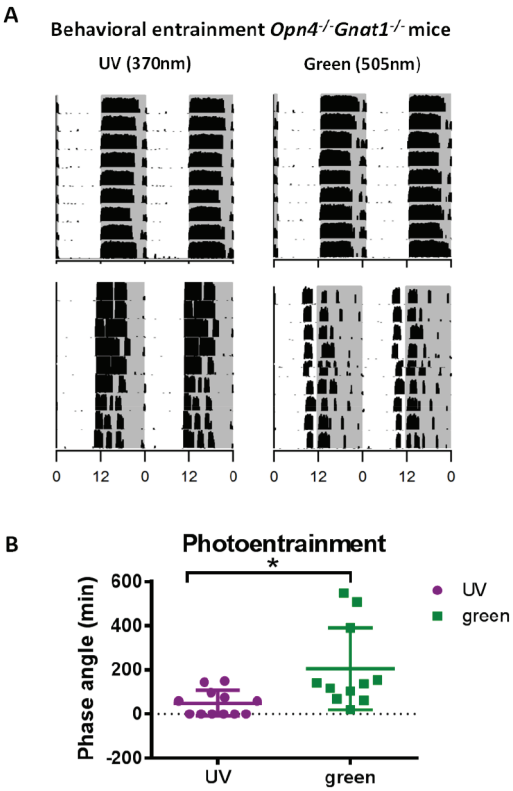

Figure 1. Double plotted behavioral activity recordings. A. Two examples of actograms of *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice mice kept on a 12:12 LD cycle. The gray shading indicates the dark phase, and the white background indicates the light phase. During the light phase the mice were exposed to UV (λ_{max} 365nm, left) or green (λ_{max} 505nm, right) light. Behavioral activity was measured using running wheel activity; the black bars indicate when the mouse runs in a wheel, and the bin size is 1 minute. **B.** Phase angle differences during 12:12 LD cycles compared to lights off in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice during UV (λ_{max} 365nm, left) or green (λ_{max} 505nm, right) light exposure (p =0.012).

Transient on-excitation and off-inhibition

To determine the contribution of cone photoreceptors to the magnitude of the light-induced transient on-excitation we analyzed the SCN firing frequency during the first minute of the light pulse. Wildtype mice and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice, displayed relatively large transient on-responses in response to UV light, and further increments in irradiance did not lead to larger magnitude transient on-responses (p=0.48 and 0.28 respectively, Figure 3A), indicating saturation at rather low light intensity levels. The transient on-excitations found in response to UV light were not significantly different between wildtype and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice. In *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice, higher

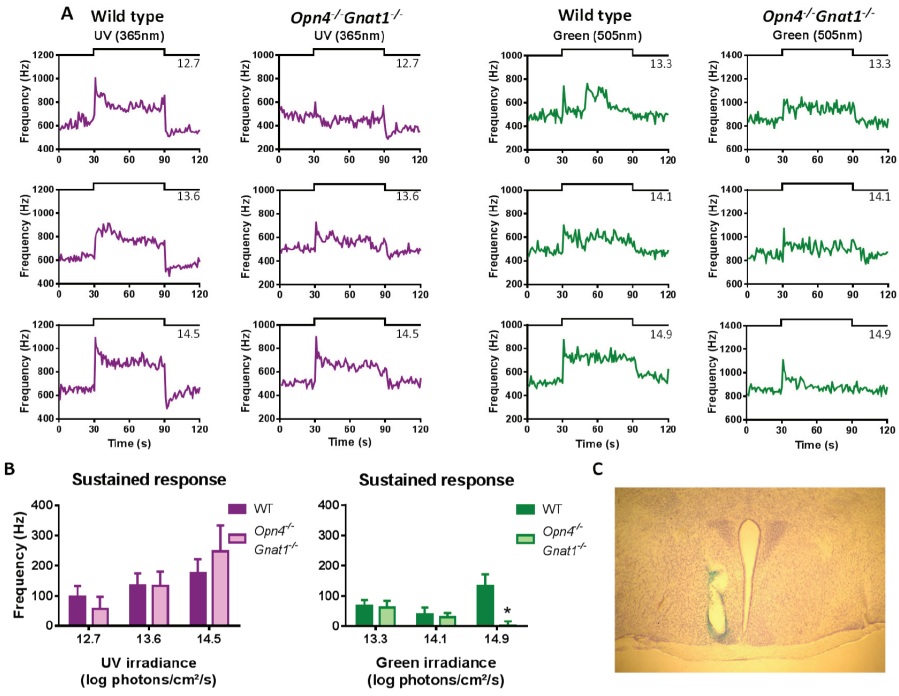

Figure 2. SCN *in vivo* electrophysiological responses to 1-minute UV (λmax 365nm, left) or green (λmax 505nm, right) light exposure at three different irradiance levels. A. Wild type and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice were exposed to 1-minute light pulses indicated in a step diagram above each graph. One representative trace of SCN electrical activity is depicted per irradiance, with the photon flux shown next to each graph as log photons/cm^2/s. Bin size is 1 second. **B.** Bar graphs showing mean (±SEM) sustained responses in SCN electrical activity as a function of irradiance of UV (left) and green (right) light in wildtype (filled bars, n=4) and in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ (n=4). *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ show irradiance-dependent light responses to green light (Pearson's r=-0.621, p=0.024). At 14.9 log photons/cm2/s of green light, the light response is significantly different between wild type and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice (independent t-test, p =0.11) **C.** An example of an coronal slice of the mouse brain with the SCN right above the optic chiasm. The location of the electrode was verified by the blue spot which was marked using an electrolytic current.

irradiances of green light led to an irradiance-dependent enhancement of the transient on-responses (Pearson's r: 0.711, p=0.006).

After exposure to UV light of various irradiances, *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ and wildtype mice showed large magnitude off-responses in SCN discharge rate. The magnitude of the off-inhibition after pulses of UV light were not irradiance-dependent, and not significantly different between wildtype mice and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice (Figure 2B). Light offset of green light caused relatively small off-inhibitions in SCN neuronal activity (Figure 3B). In wildtype mice, the magnitude of the off-inhibition in SCN firing rate were significantly smaller at higher green light intensities (Pearson's r=-0.679,

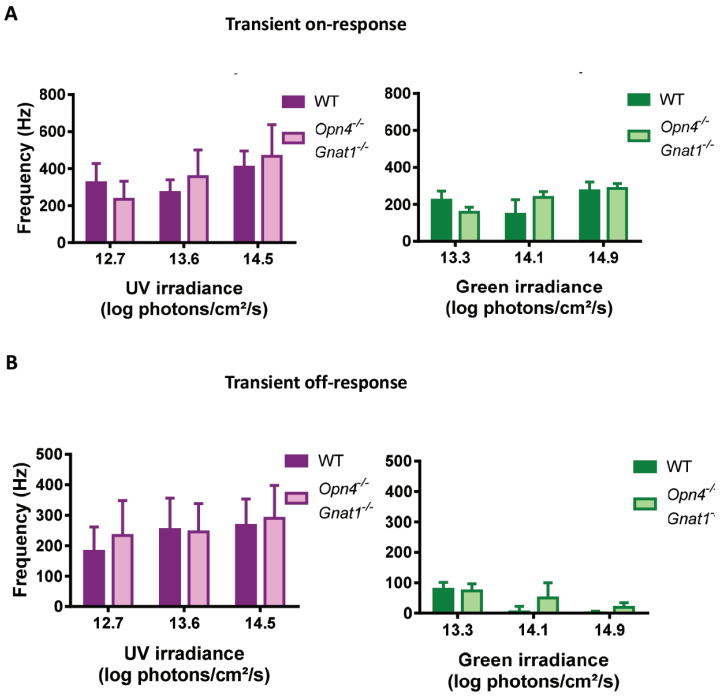

Figure 3. SCN *in vivo* electrophysiological responses to 1-minute UV (λmax 365nm, left) or green (λmax 505nm, right) light exposure at three different irradiance levels. Bar graphs showing mean (±SEM) transient on-responses (**A**) or off-responses (**B**) in SCN electrical activity as a function of irradiance of UV (left) and green (right) light in wildtype (filled bars, n=4) and in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ (n=4). *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ show irradiance-dependent transient on-responses to green light (Pearson's r=0.711, p=0.006). Wildtype mice show irradiance-dependent transient off-responses to green light (Pearson's r=-0.679, p=0.015).

p=0.015), and were at all light intensity test not significantly differ from off-inhibitions in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice.

15 min light pulses

15 minute pulses of UV light caused sustained light induced increases in SCN neuronal activity with half max-times of 524.6±200.4 seconds and 356.0±216.8 seconds respectively in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ and wildtype mice. In *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice, 15 minute pulses of green light led to light-induced responses that decayed to baseline levels after 24.6±13 seconds. In wild type mice, 15 minute green light pulses induced enhancements in SCN firing rate that showed half-times of 747.0±102.4 seconds. The magnitude of the light response and the duration of the sustained response were significantly larger in wild type mice compared to *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ mice (p =0.041 and p<0.001, respectively (Figure 4).

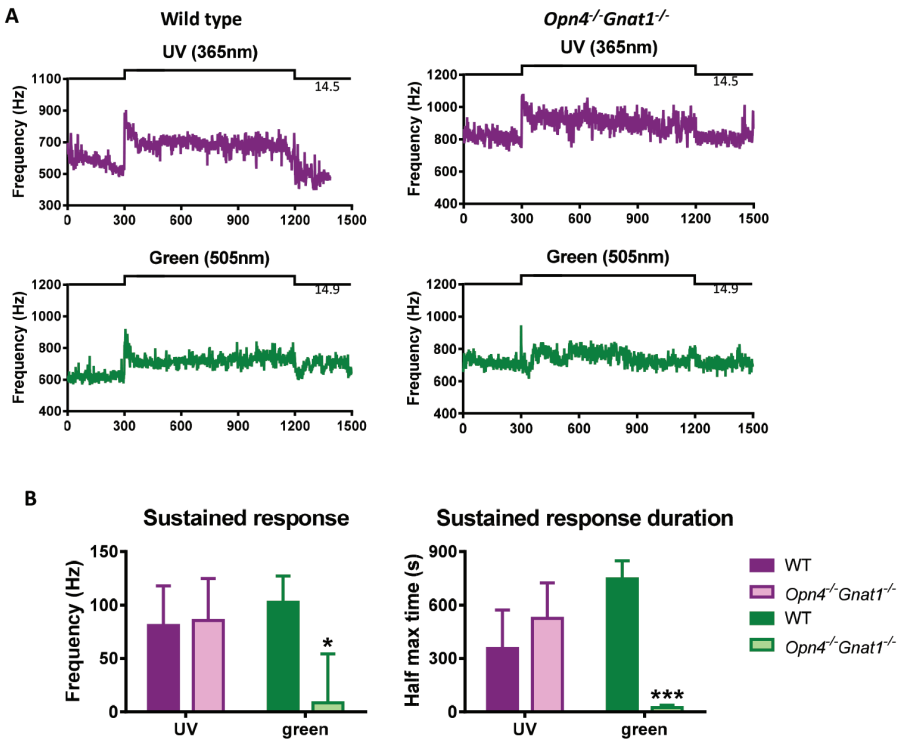

Figure 4. SCN *in vivo* electrophysiological responses to UV (λmax 365 nm, left) or green (λmax 505 nm, right) light exposure of 15 minutes. A. Wild type (upper traces) and *Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ (lower traces) mice were exposed to 15-minute light pulses which are indicated in a step diagram above each graph. One representative trace of SCN electrical activity is shown per group and per wavelength, with the photon flux shown next to each graph as log photons/cm^2/s. Bin size is 1 second. **B.** Bar graphs showing mean (±SEM) light response in SCN firing frequency caused by 15 min light pulses of UV or green light in wild type (n=4) and *Opn4*$^{-/-}$ *Gnat1*$^{-/-}$ (n=5) mice. Sustained light responses induced by 15 min of green light are significantly smaller in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ compared to wild type mice (independent t-test, p =0.041). **C.** Bar graphs showing mean (±SEM) half-max time of the light response in SCN firing frequency caused by 15 min light pulses of UV or green light in wild type (n=4) and *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ (n=5) mice. The duration of the sustained response is significantly shorter in *Opn4*$^{-/-}$*Gnat1*$^{-/-}$ than in wild type mice (independent t-test, p<0.0001).

Discussion

We undertook behavioral experiments and found that 'cone-only' mice are able to entrain to light-dark cycles of UV and green light, albeit with a large positive phase angle of entrainment. Our electrophysiological recordings show that the SCN exhibits sustained firing levels in the absence of melanopsin and rod photoreceptors in response to light over a broad range of wavelengths. Sustained increases in electrical discharge were observed during exposure to UV and green light, over various intensities. In wildtype and 'cone-only' mice, clear on-excitations in SCN firing rate were observed after onset of UV and green light. These findings strongly suggest that cone photoreceptors are capable of inducing transient responses to light at the level of the SCN. The results indicate that cone receptor-mediated light responses in the SCN are sufficient for proper circadian photoentrainment.

The majority (57%) of cone-only mice were able to entrain to light-dark (LD) cycles of UV light or green light. Under LD cycles with UV light, cone-only mice showed phase angles of entrainment that were in phase with the dark period. Under LD cycles with green light, cone-only mice displayed much behavioral activity during the light phase, and an earlier phase angle of entrainment (205±56 min, p=0.012) compared to LD cycles with UV (49±17 min) light. The results of the behavioral experiments indicate that short wavelength cones contributes more to photic entrainment than mid wavelength cones. To test this, we performed *in vivo* extracellular recordings of SCN neurons in "cone-only-mice" lacking melanopsin and rod photoreceptors in the retina. We observed light-induced sustained increase in SCN electrical activity in response to UV and green light. These results indicate that cone photoreceptors transmit photic information to the SCN during the initial phase of light exposure. After the sustained response the SCN discharge rate decayed to baseline levels. In cone-only mice, UV light of increasing irradiances led to an irradiance-dependent enhancement in SCN neuronal activity. Interestingly, increasing intensities of green light pulses did not cause an irradiance-dependent enhancement in SCN discharge rate. In contrast, increasing intensities of green light resulted in smaller sustained responses in SCN firing rate (Fig. 1B). Green light irradiance-dependent decrease of SCN neuronal firing rate in cone-only mice may be explained by bleaching of mid wavelength cones (7). In wildtype mice, light pulses of high irradiance of green light did lead to large magnitude sustained responses in SCN neuronal activity, which were probably driven by melanopsin (4,7).

We observed transient on-excitations in response to UV and green light in both wildtype and "cone-only" mice. The presence of this response in the absence of melanopsin and rod photoreceptors indicates a role for cone photoreceptors in evoking transient on-responses. Transient "off-inhibition" was observed in response to UV light and low intensities of green light in both wildtype and "cone-

only" mice, suggesting that cones mediate transient off-responses. Interestingly, off-response following higher irradiances of green light exposure was not present in wildtype and "cone-only" mice. In wildtype mice, high irradiances of green light activate malanopsin, which has a typical sluggish response (4,17-21) and could mask the relatively fast cone-mediated off-response. The absence of the off-response in "cone-only" mice in response to higher irradiance of green light is consistent with the small sustained responses found at higher irradiances of green light.

The data reveal that cone photoreceptors can drive light responses in the SCN over a range of wavelength and various light intensities. We subjected wildtype mice and cone-only mice to one minute monochromatic light pulses of UV (λ_{max} 365 nm) or green (λ_{max} 505 nm) light of irradiances from 12 to 15 log photons. Previous studies that used a similar range of irradiances of λ_{max} 360 nm, λ_{max} 480 nm or λ_{max} 530 nm light, showed that mice lacking the mid wavelength sensitive cones exhibit reduced phase shifting capacity in response to long wavelength light, while the response to short wavelength light was not altered (9,10). The differences in phase shifting responses were only present in response to short light pulses (1 and 5 min), which, in correspondence with our study, indicates a role for cones in the initial part of light detection. *In vivo* electrophysiological measurement in SCN of transgenic mice (*Opn1mw*^{R}) with human long-wavelength instead of mid-wavelength cones in the retina, revealed distinct responses in SCN neuronal activity induced by the activation of short- or long-wavelength cones. Moreover, the circadian entrainment of the *Opn1mw*^{R} mice improved in LD cycles with natural spectrum twilight as opposed LD cycles twilights which lacked changes in color or to rectangular LD cycles, indicating a biological relevance of "color-vision" by the SCN (22).

Several studies reported sustained responses to steady light stimuli during recordings directly from the membrane currents of retinal M- and S-cone photoreceptors (23-25). Furthermore, activation of classical photoreceptors can lead to sustained responses at the level of pRGCs in the absence of melanopsin (14-16). Both M- as well as S- cone activation in the outer retina can provide sustained synaptic input to the pRGCs in the inner retina (15,16). Other studies revealed an inhibitory role of S-cones on primate pRGCs (18) and the pupil response in humans (26). Together these studies indicate a significant contribution for cones in non-image forming functions. A role for cones in the regulation of light effects on the human circadian system was also elucidated. The level of melatonin suppression is similar in response to long and short wavelength light, which suggests the ability for cone photoreceptors to play a role in the regulation of melatonin suppression (27,28). The study shows that cones provide a substantial contribution to melatonin suppression during the first phase of light exposure (<90 min), whereas the cone contribution decayed during prolonged light exposure (27).

The melanopsin photo pigment plays a crucial role in regulation of the sustained response to light. More recently it became evident that classical photoreceptors also contribute to sustained light signaling to the SCN (4,5,13,29). Our findings show that cone photoreceptor can influence SCN electrical activity in the absence of both melanopsin and rod photoreceptor signaling. Our data reveal a contribution of cones during the first phase of light detection. There is evidence that cones contribute significantly to phase shifting responses during exposure to discontinuous light stimuli. This was tested in red cone knock in mice by presenting long wavelength red light as series of 1 minute pulses compared to a continuous light stimulus of 15 minutes. Discontinuous light stimuli lead to significant larger phase shifts in behavioral activity (7). In our study we made use of uniform illumination levels to ensure that the mice perceived a specific photon flux per wavelength of light. In nature animals are expected to be exposed to large fluctuations in illumination levels due to the movement of the animal in and out the shade or burrow. Therefore, cone photoreceptors may have a larger contribution to phototransduction in nature than in the lab, where illumination levels are artificially constant.

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PART III

SCN AND BEHAVIOR

6

ENHANCED ELECTRICAL OUTPUT OF THE SCN CLOCK BY BEHAVIORAL ACTIVITY FEEDBACK IN THE DAY-ACTIVE GRASS RAT *ARVICANTHIS ANSORGEI*

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In preparation

Abstract

Mammalian circadian rhythms are regulated by the suprachiasmatic nucleus (SCN). The SCN generates high levels of electrical activity during the day and low levels during the night, and transmits these to other brain areas. In nocturnal rodents, behavioral feedback to the SCN induces suppression of electrical firing rate. To examine the effects of behavioral activity on the SCN in a diurnal mammal we performed *in vivo* and *ex vivo* electrophysiological recordings in the SCN of diurnal grass rats (*Arvicanthis ansorgei*). To determine the characteristics of the system *in vivo* and *in vitro*, we performed a detrended fluctuation analysis (DFA). *In vivo* electrophysiological recordings revealed that episodes of behavioral activity of the animal coincided with epochs of enhanced SCN firing rates. These increments were acute and sustained for the full duration of behavioral activity. Consequently, the amplitude of the SCN activity rhythm is enhanced by behavioral activity of the grass rat when it occurs during the day. *Ex vivo* measurements of SCN firing rate from grass rats revealed circadian rhythms that were devoid from acute excitations, indicating that they are causally driven by extra-SCN areas. DFA analysis showed that the complexity of the signal in the SCN *in vivo* is scale invariant, whereas *in vitro* scale-invariance is not present. The results indicate that in diurnal and nocturnal species behavioral activity has opposite effects on the SCN discharge rate, leading to an enhancement of the amplitude of the SCN electrical activity rhythm, in both nocturnal and diurnal species through opposing mechanisms.

Introduction

Mammals have evolved an internal clock that enables anticipation and adaptation to 24-h changes in the environment on earth. The master clock of the body resides in the suprachiasmactic nucleus (SCN) of the ventral hypothalamus (1). Single neurons in the SCN generate a circadian rhythm based on the cyclic expression of clock genes and protein products (2,3). The rhythmic expression of clock proteins results in a rhythm in SCN electrical activity (i.e. high during the day and low during the night), and the rhythmic release of humoral factors, which results in circadian rhythms in physiology and behavior (4,5). Synchronization of the SCN clock to the ambient light-dark cycle is primarily accomplished by light information, which is perceived by the retina and projected to the SCN via the retinohypothalamic tract.

While light is the main synchronizer of the SCN, SCN's rhythmicity is also influenced by non-photic factors such as the animal's behavior. Behavioral activity (6) and substances that are involved in regulating behavioral activity, such as neuropeptide Y (NPY) (7) and serotonin agonists (8), affect the phase of the SCN (9). The application of NPY (10-13) or serotonin receptor agonist 8-OH-DPAT (14,15) to SCN neurons *in vitro* decreases the neuronal firing rate. *In vivo* measurements of the SCN's electrical activity in freely-moving night-active rodents such as hamsters (16), rats (17) and mice (18) revealed that behavioral activity induces acute suppression of the SCN's firing rate. In mice, suppressions of the SCN electrical activity induced by spontaneous behavior are superimposed on the SCN's circadian modulation, and the magnitude and duration of the suppressions are respectively dependent on the intensity and duration of the behavioral event (18). Because mice are night-active animals, they show elevated levels of activity during the night, which suppresses the SCN firing even more. Consequently, SCN rhythm amplitude increases, thereby improving rhythmicity at the periphery.

In humans, physical exercise accelerates the synchronization of sleep-wake rhythms to the external light-dark cycle (19-22) and improves health and well-being (23-29). We hypothesize that in day-actives species behavioral activity during the day will increase the amplitude of the SCN electrical output, as a result of behaviorally induced enhancements of SCN electrical firing. To test this, *in vivo* electrophysiological measurements were performed in the SCN of freely-moving day-active grass rats (*Arvicanthis ansorgei*). SCN discharge levels were overall higher during day time and lower during the night. Analysis of the *in vivo* recordings revealed that episodes of behavioral activity coincided with enhanced firing rates. Consequently, the amplitude of the SCN activity rhythm was increased by day-time activity. *Ex vivo* ("brain slice") recordings, in which the SCN is isolated from afferent and efferent connections, were performed to examine the causal direction of these effects and showed a complete lack of the short term increments in electrical

activity. The complexity of the signal was determined by a detrended fluctuation analysis (DFA). While *in vivo* the signal showed scale invariance, indicating a network of feedback interactions (30-32), *in vitro* this scale invariance was absent. The results show that the effect of behavioral activity on the SCN of the diurnal *Arvicanthis*, is opposite to the effect seen in nocturnal species, leading to an enhancement of the amplitude of the SCN electrical activity rhythm, in both diurnal and nocturnal species, via opposing mechanisms.

Methods

Animals and housing

Male grass rats (*Arvicanthis ansorgei*, breeding colony Chronobiotron, UMS 3415, CNRS University of Strasbourg, France) were subjected to12 hours light (200 lux) and 12 hours red dim light (<5 lux) cycles. All experiments were performed under the approval of the French Ministry of Higher Education and Research.

Micro-electrode implantations

Procedures for *in vivo* measurements of multiunit activity from SCN neurons have been previously described for rats (33) and mice (18). At a minimum of 8 weeks (150g-300g), tripolar stainless steel micro-electrodes (Plastic One MS333-3-BIU-SPC, Roanoke VA, United States) were implanted in anesthetized (0.13 ml Zoletil (Virbac, Carros, France) and 0.2 Rompun 0.5% (Bayer Pharma, Puteaux, France) per 100 g bodyweight) grass rats with the use of a stereotactic instrument. Two twisted electrodes (Polyamide-insulated; bare electrode diameter 125 µm) for differential recordings were aimed at the SCN (coordinates: 0.8 mm posterior to bregma, 0.9 mm lateral to the midline, 7.8 mm ventral to the dura mater, under a 5° angle in the coronal plane), and a third uncoated electrode (reference electrode) was placed in the cortex. The differential amplifier was based on the design of Yamazaki and coworkers (16).

In vivo SCN electrical recordings

After a recovery period of at least 5 days, the grass rat was individually housed in a temperature controlled recording chamber (22 °C), where water and standard laboratory food (SAFE, Augy, France) were available *ad libitum*. The grass rat was able to move freely during the time of recording. The electrical signal was amplified, bandwidth filtered (500 Hz – 5 kHz), and window discriminators were used to convert action potentials into digital pulses that were counted in 10 second bins by Circa V1.9 software (OriginLab)) and MATLAB (Mathworks Inc.).

Behavioral activity recordings

In vivo SCN electrical recording set-ups were equipped with a drinking sensor and a Passive Infra-Red (PIR) motion detector positioned 30 cm above the floor of the cage. Behavioral activity and SCN electrical activity were recorded simultaneously in 10 second time bins.

Data Analysis *in vivo* electrophysiology

Electrical activity date were smoothed in MATLAB (Mathworks Inc.). The magnitude of the behaviorally induced change in neuronal firing rate was defined as the difference between baseline of the electrical activity and the average electrical discharge during the episode of behavioral activity. The baseline level was defined as the mean neuronal firing rate during 2 minutes before the episode of behavioral activity.

Histological verification micro-electrode

After the *in vivo* measurements, the location of the micro-electrode was histologically checked. To sacrifice the grass rats, the animals received intraperitonally an overdose of sodium pentobarbital (CEVA, Libourna, France, 0.3 ml/100g bodyweight). An electrical current of 40µA was passed through the recording electrode to deposit iron at the electrode tip. The brains were collected and immersed in 4% paraformaldehyde with 0.5 g of C6FeK4N6·3H20 (Sigma Aldrich, Lyon, France) for 2 days. Brains were sectioned coronally in 40µm slices and stained with cresyl violet for microscopic verification of the recording site.

Ex vivo SCN electrical recordings

Grass rats were entrained to 12 h light : 12 h dark cycle for at least 30 days. The animals were scarified at ZT 21 (± 0.5 h) under dim red light., and the brains were removed within 1 minute of decapitation. Brain slices (~450 microns thick) were prepared using a tissue chopper, and the slice containing the SCN was transferred to a laminar flow chamber within six minutes after decapitation (31). The tissue was bathed in bicarbonate-buffered ACSF that was gassed by continuously blowing a warmed, humidified mixture of O2 (95%) and CO2 (5%) over the solution. The slice was submerged in the solution and stabilized using an insulated tungsten fork, and settled in the recording chamber for ~1 h before the electrodes were placed in the center of the SCN. Action potentials were recorded using 50-µm 90% platinum/10% iridium electrodes. The signals were amplified 10 k time and bandpass-filtered (0.3 Hz low-pass, 3 kHz high-pass). The action potentials that exceeded a predetermined threshold well above noise (~5 µV) were counted in 10-second bins using a custom-made automated computer program.

Data Analysis *in vitro* electrophysiology

The electrophysiological data were analyzed using a custom-made program in MATLAB as described previously (32). The time of maximum activity was used as a marker of the phase of the SCN and was determined as the first peak in multiunit activity. Multiunit recordings of at least 24 hours in duration that expressed a clear peak in multiunit activity were moderately smoothed using a least-squares algorithm (34). Subsequently, the SCN peak time, the peak width, and the relative

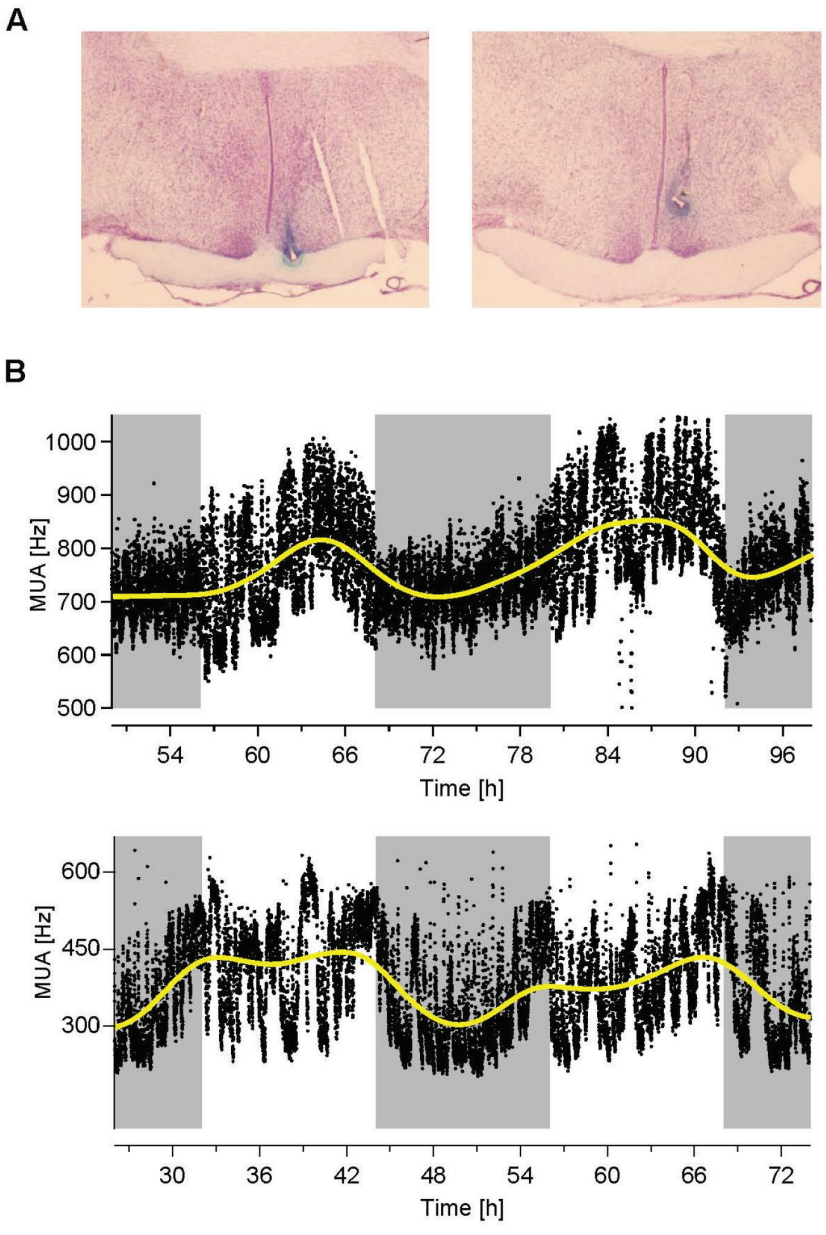

Figure 1. SCN multiunit electrical activity (MUA) *in vivo*. A. Two examples of a coronal slice of the *A. ansorgei* brain with the SCN right above the optic chiasm at the base of the hypothalamus. The location of the electrode was verified by the blue spout which is marked using an electrolytic current. **B**. Two examples of a raw trace of SCN electrical activity *A. ansorgei* kept in LD 12:12. The x-axis shows actual clock time in hours and the y-axis shows SCN MUA in Hz. The grey background represents periods of darkness. The smoothed fitted line of the MUA is plotted in yellow.

peak amplitude (peak-to-trough ratio) of the first cycle in vitro were determined. Statistical analyses were performed using SPSS. All summary data are reported as the mean ± the standard error of the mean (SD). P-values were calculated using the two-tailed Student's t-test, and differences with $p<0.05$ were considered to be statistically significant.

Assessment of scale invariance using detrended fluctuation analysis

In order to measure the scale invariance behavior in the behavior activity fluctuation, the detrended fluctuation analysis (DFA) was performed (32,35,36). The DFA was implemented as follows: (i) The integrated time series was divided into m non-overlapping "boxes". The number of data points in each box was n = N/m, where n represents the timescale. (ii) In each box, the n data points were fitted by a second-order polynomial function which represented the "local trends". (iii) In each box, we defined the "residuals" as the n data points subtracting the local trends. (iv) The root-mean-square of the residuals for each box was calculated. For the integrated time series, the mean of the root-mean-square from each of the m boxes stands for the fluctuation amplitude F(n). (v) We changed the timescale n satisfying $4 \leqslant n \leqslant 8640$, and repeated (i-iv). (vi) The fluctuation amplitude F(n) as a function of the timescale n was plotted in double-logarithmic coordination.

The activity fluctuation function can be presented by a power-law form $F(n) \sim n^{\alpha}$, if the fluctuations maintain the scale-invariant behavior. The scaling exponent α describes the scale-invariant correlation of the activity fluctuations. If α is equal to 0.5, the correlation is absent in activity fluctuation, which corresponds to white noise. If α is smaller than 0.5, there are negative correlations in the activity fluctuations, i.e. larger recording data values have more probability of being followed by smaller recording data values and vice versa; If α is larger than 0.5, there are positive correlations in the activity fluctuations, i.e. larger recording data values are more likely to be followed by larger recording data values and vice versa.

Results

In vivo SCN firing pattern in diurnal grass rats

In vivo SCN electrical activity measurements were performed successfully in 4 day-active grass rats (*Arvicanthis ansorgei*) and showed high levels during the day and low levels during the night (585 ± 220 Hz vs 562 ± 228 Hz, respectively). These rhythms were in phase with the animal's behavioral activity rhythm.

Acute excitations of the SCN firing rate by behavioral activity

Examination of the recordings of SCN electrical activity revealed in 3 out of 4 animals enhancements of the SCN firing frequency that were superimposed on the circadian rhythm in SCN discharge rate. Detailed analysis of the locomotor activity data showed that episodes of spontaneous behavioral activity were consistently present at times of enhanced SCN discharge rates (Figure 2a). Typically, at the start of the behavioral activity, an acute increase in firing rate was displayed, and the SCN firing frequency remained elevated for the complete duration of behavioral activity (Figure 2b).

SCN firing pattern *ex vivo*

To test whether increments in SCN electrical activity found *in vivo* are caused by behavioral activity or vice versa, we performed *ex vivo* electrophysiological recordings on the isolated SCN. *In vitro* electrophysiological recordings in SCN from grass rats revealed unimodal sinusoidal waveform patterns in the isolated SCN. SCN electrical activity was maximally high around midday (ExT 12.4 ± 1.6 h), and the elevated electrical activity interval was 10.6 ± 2.2 h. Increments in SCN electrical activity superimposed on the circadian modulation, as found *in vivo*, were absent *ex vivo* (Figure 3).

Fractal patterns of SCN discharge rate *in vivo* and *ex vivo*

A detrended fluctuation analysis (DFA) was performed over a recording period of 3 days (LD 12h: 12h) *in vivo*. The analysis showed that the fluctuation function *F(n)* of the electrical activity possessed a power-law form (a straight line in the log-log plot: $F(n) \sim n^{\alpha}$) at time scales from ~10 seconds up to 10 hours (Figure 4a). The power-law form reflects a fractal temporal structure and the scaling exponent α of 0.93 ± 0.09 indicates strong fractal correlations in SCN electrical activity fluctuations. DFA showed that the fluctuation function *F(n)* of *in vitro* SCN electrical activity possessed a power-law form with a breakpoint around 10 minutes (Figure 4b). At time scales from ~ 10 seconds up to 10 minutes the scaling exponent α was similar to that found *in vivo* (0.93 ± 0.07), and at time scales from 10 minutes to 10 hours the scaling exponent was 1.5 ± 0.09 (Figure 4c).

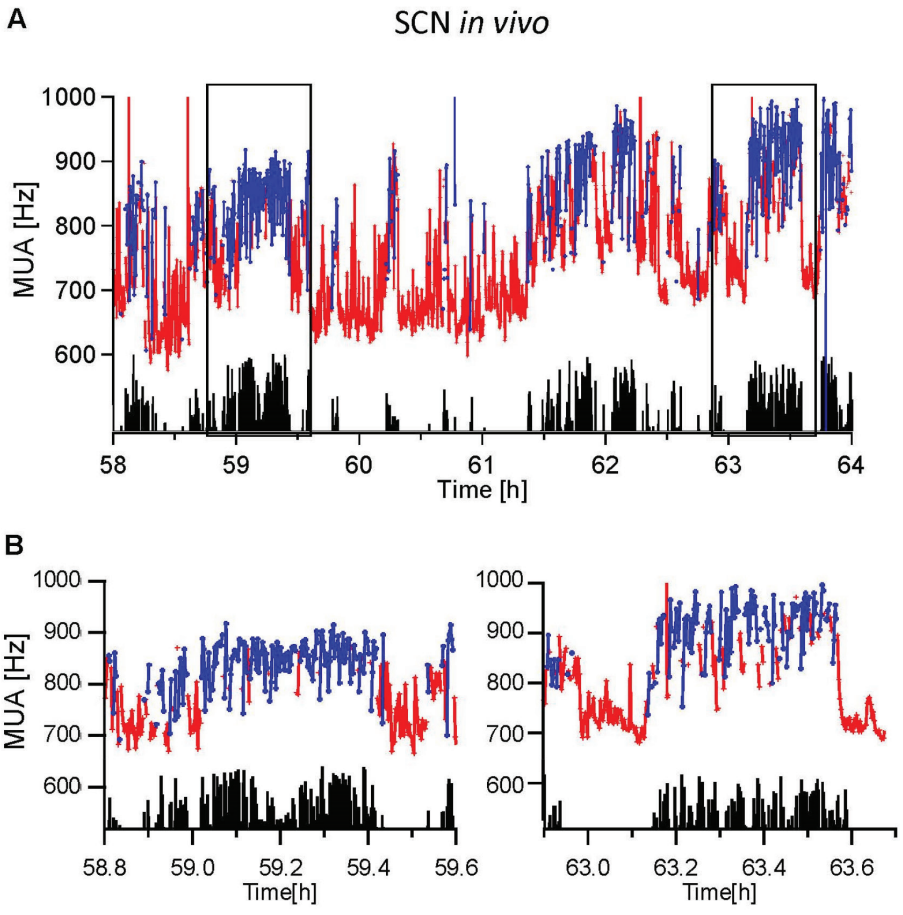

Figure 2. SCN multiunit electrical activity (MUA) *in vivo* during active (blue) and inactive (red) episodes. A. To visualize the effect of behavioral activity on SCN firing rate, SCN MUA recorded while the animal displayed behavioral activity (measured by passive infrared detectors) are plotted in blue, and MUA traces recorded while the animal was inactive are shown in red. Behavioral activity is plotted below the SCN electrical activity traces. The x-axis shows actual clock time in hours and the y-axis shows SCN MUA in Hz. Behaviorally-induced enhancements of SCN MUA are present during the day (upper graph) and during the night (lower graph). **B.** Expanded plots of SCN MUA during episodes of behavioral activity during the day as indicated by the boxes in A.

Enhancement of the amplitude of the SCN electrical output rhythm by behavioral activity

The effect of behavioral activity on the SCN firing rate was investigated by determining behavioral-induced enhancement of the normalized SCN discharge rates. During the dark period (night), epochs of behavioral activity caused a 17% increase of SCN

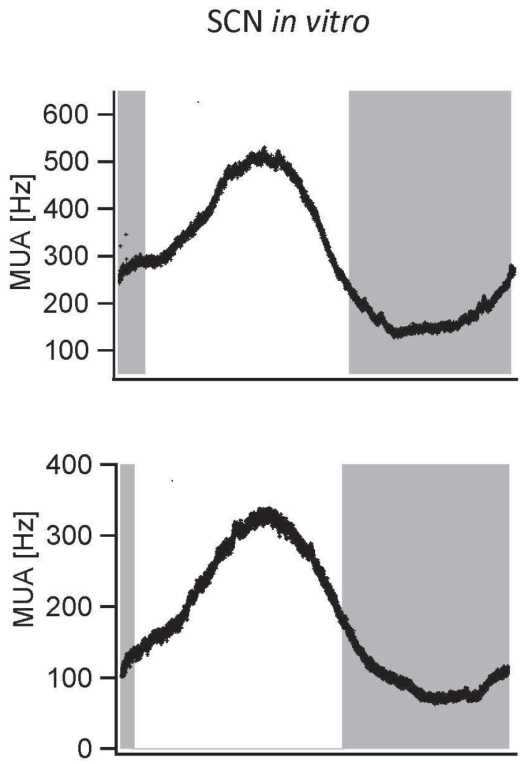

Figure 3. SCN multiunit electrical activity (MUA) *ex vivo*. Two raw traces of SCN electrical activity measured *ex vivo*. The white-grey background represent the light-dark regime to which the grass rats were entrained and the y-axis shows MUA in Hz. SCN discharge rate of *A. ansorgei* follow a unimodal circadian pattern, with maximal electrical activity at midday (ExT 12.4 ± 1.6 h, n=8). MUA in diurnal grass rats were elevated (measured by the peak width of the waveform) for a duration of 10.6 ± 2.2 h.

firing rates, and during the light period, behavioral-induced enhancements led to a 20% increase of the SCN discharge rate. We plotted the number of action potentials as a function of the time, and distinguished between behaviorally active and inactive episodes of the grass rats. Clear circadian rhythms exist both in absence and in the presence of behavioral activity (Figure 5a). The smoothed curves through the SCN neuronal activity rhythms revealed that the level of the rhythm was enhanced in the presence of activity. When the day-active grass rat only displays behavioral activity during day-time and behavioral in-activity during night time, the amplitude of the SCN electrical activity rhythm will be maximally high (Figure 5b). On the other hand, if the animal is active during night-time and rests during day-time, the rhythm of the SCN will be dampened or even reversed (Figure 5c).

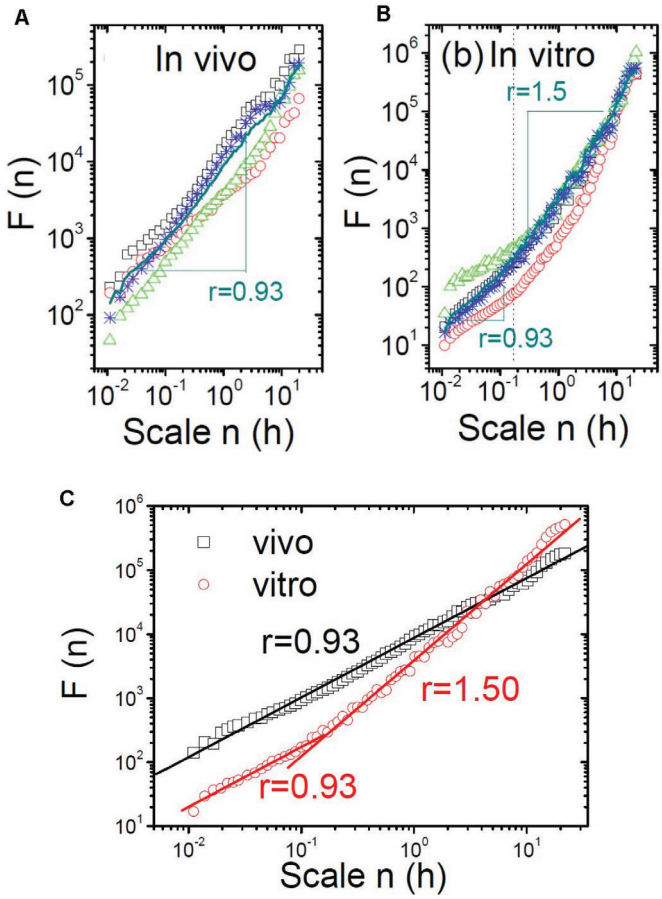

Figure 4. Fractal patterns of MUA fluctuations *in vivo* and *ex vivo*. A,B Results of individual A. ansorgei: four for *in vivo* recordings during 3 days of LD 12:12 and four for *ex vivo* recordings during at least 24 h. Examples of raw data are shown in Figure 1 and 3. **C.** Group averages of *in vivo* and *ex vivo* recordings. Data are shown in log-log plots. At time scales from ~10 seconds up to ~10 hours, the function *in vivo* shows a power-law form (straight line in the log-log plot) with the scaling exponent α=0.93, indicating fractal correlations in raw data. At time scales < ~6 minutes, the function *ex vivo* shows a power-law form with a virtually identical scaling exponent as *in vivo*. At time scale > ~6 minutes, the function *ex vivo* shows a power-law with the scaling exponent α=1.5.

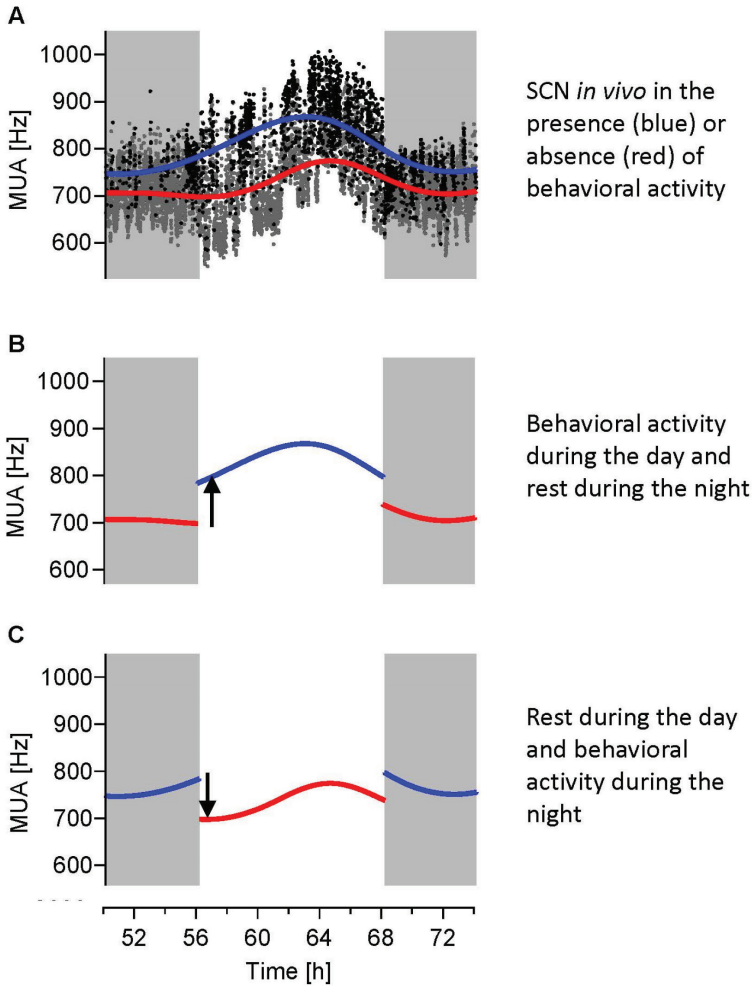

Figure 5. Circadian profile of MUA in the presence or absence of PIR-recorded behavioral activity. A. To visualize the effect of behavioral activity on SCN rhythm amplitude, the passive infrared data are integrated in the MUA data: whenever passive infrared movement was detected within a 10 s recording bin, the MUA measured in that bin is displayed with a black dot. Grey dots show MUA points from bins when no movement was detected. Smoothed lines were drawn through the black dot (blue line) and grey dots (red line) to illustrate the presence of a circadian rhythm in either profile. **B.** To visualize the effect of a proper behavioral activity rhythm on SCN rhythm amplitude, the fitted line of MUA measured during behavioral activity is plotted for the day and the fitted line of the MUA measured during inactivity is plotted for the night. **C.** To visualize the effect of a completely reversed behavioral activity rhythm on SCN rhythm amplitude, the fitted line of the MUA measured during inactivity is plotted for the day and the fitted line of the MUA measured during activity is plotted for the night. The x-axis shows actual clock time in hours and the y-axis shows SCN MUA in Hz. The grey background represents periods of darkness.

Discussion

In this present study we performed *in vivo* measurements of the SCN's electrical firing pattern in day-active grass rats (*Arvicanthis ansorgei*). The results show that in grass rats SCN discharge is overall higher during the day and lower during the night. Analysis of the *in vivo* electrical activity recordings revealed that electrical discharges were acutely enhanced during the full duration of behavioral activity. A DFA analysis showed the presence of long-range fractal regulation in the SCN, indicating a healthy physiological state. *In vitro*, the SCN's electrical activity followed a unimodal waveform and was free from acute enhancements of SCN discharge rate. Moreover, the patterns of long range fractal regulation completely broke down, reflecting a lack of feedback network interactions. Together the results indicate that other than in nocturnal rodents, behavioral activity leads to increments in SCN electrical activity, consequently leading to an enhancement of the amplitude of the circadian pacemaker in day-active rodents.

SCN electrical activity pattern *in vivo*

By the use of implanted microelectrodes we were able to measure the electrical activity of the SCN in freely-moving *A. ansorgei*, while monitoring their behavioral activity with passive infrared detectors. The recordings show that the SCN firing pattern from diurnal grass rats follow a circadian pattern that is higher during daytime and low during night time. This finding is similar to previous *in vivo* measurements of the SCN's discharge rate in the diurnal chipmunk (Eutamias sibiricus) (45) and nocturnal rodents such as hamsters (16), rats (17,37) and mice (18,46). Furthermore, our data is in agreement with *in vitro* recordings performed on the SCN of several nocturnal rodents (37,37-41) and indicates that the SCN's electrical activity waveform *in vitro* is similar in diurnal and nocturnal rodents. This finding is supported by previous studies showing similarities in daily variation of clock gene expression (42-44) and in the circadian expression of Fos in the SCN of nocturnal and diurnal rodents (45,46).

Behavioral induced-enhancements of SCN discharge rate

We observed epochs of increased SCN discharge rate superimposed on the circadian electrical activity rhythm of the SCN in day-active grass rats. Detailed investigation of the SCN electrical activity recordings revealed that in 3 out of 4 animals acute enhancements of the SCN firing rate correlated to bouts of behavioral activity. Accordingly, the mean firing rate of the SCN measured during behavioral activity was increased relative to SCN firing rates measured during inactive behavioral states. As behavioral activity of day-active animals is concentrated during the day, enhancements of SCN electrical activity is especially present during the day. As a result, the peak in SCN electrical activity is elevated and thus the SCN rhythm amplitude can be

boosted by behavioral activity during the animal's active phase, i.e. the phase of the cycle where SCN electrical activity is high. Previous studies performed in nocturnal hamster (16), rat (17) and mice (18) revealed behaviorally-induced suppressions of SCN firing rate. As nocturnal animals show high levels of behavioral activity during the night, the suppression of SCN firing rate is predominantly present during the night. Because of this, the SCN electrical activity levels are lowered even more (18), and the amplitude of the SCN rhythm is boosted. Our results indicate that the amplitude of the SCN rhythm of both diurnal and nocturnal animals can be enhanced by behavioral activity, but via opposite behaviorally-induced alterations of the SCN firing rate.

Unimodal SCN electrical activity pattern *ex vivo*

To investigate the direction of the relationship between enhancements SCN electrical and behavioral activity, we performed *ex vivo* measurements of SCN electrical activity from diurnal grass rats, to characterize the endogenous firing pattern of the SCN without feedback from other brain areas. Our data reveal that the firing rate of the SCN displays a circadian pattern, showing highest electrical activity during day time and lowest electrical activity during night time. Importantly, the circadian firing pattern of the isolated SCN of day-active grass rats are unimodal, and absent from acute enhancements of SCN discharge rate. The absence of variability *ex vivo* indicates that the variability arises from communication between the SCN and extra-SCN areas.

Fractal patterns of SCN discharge rate *in vivo* and *ex vivo*

To test whether fractal patterns of SCN electrical activity in grass rats are determined by network interactions between the SCN and extra-SCN areas, we performed detrended fluctuation analysis (DFA). The analysis showed that the fluctuation function *F(n)* of the *in vivo* SCN electrical activity recorded during 3 days (LD 12h :12h) possessed a power-law form (a straight line in the log-log plot: $F(n) \sim n^{\alpha}$) at time scales from ~ 10 seconds up to 10 hours (Fig 4). The power-law form indicates a fractal temporal structure and the scaling exponent α of 0.93 ± 0.09 indicates strong fractal correlations in SCN electrical activity fluctuations. DFA showed that the fluctuation function *F(n)* of *in vitro* SCN electrical activity possessed a power-law form with a breakpoint around 10 minutes. At time scales from ~ 10 seconds up to 10 minutes the scaling exponent α was practically the same as found *in vivo* (0.93 ± 0.07), and at time scales from ~ 10 minutes to 10 hours the scaling exponent was 1.5 ± 0.09. Thus, it seems that fractal patterns at time scales > 10 minutes emerge from the interaction between the SCN and extra-SCN areas.

Neuronal pathways involved in behavioral feedback to the SCN

In vivo, the SCN receives non-photic information via major inputs from the raphe nuclei, which contains serotonin (5-HT) (47), and from the intergeniculate leaflet (IGL), which contains among others neuropeptide Y (NPY) and GABA (48). In nocturnal rodents, increased levels of behavioral activity increase the levels of 5-HT (49) and NPY in the SCN (50). Interestingly, 5-HT receptor agonist 5-HT1A (8) and NPY (7) are able to induce phase shifts in the SCN's rhythm. Accordingly, the ablation of serotonergic afferent SCN pathways (9,51,52) or the administration of NPY antibodies (53) to the SCN attenuates the phase shifting effects of non-photic stimuli on the SCN. The application of NPY (10-13) or 5-HT receptor agonist 8-OH-DPAT (14,15) to SCN neurons *in vitro* decreases the neurons' firing rate, which suggest the potential involvement of these factors in behaviorally-induced suppression of SCN firing rate found in nocturnal rodents (16-18).

Our *in vivo* recordings of SCN electrical activity in the day-active *A. ansorgei* revealed behaviorally-induced enhancements of the SCN discharge rate, opposed to behaviorally-induced suppression found in nocturnal rodents (16-18). We hypothesize that neurotransmitters involved in behavioral-feedback to the SCN clock effect the SCN firing rate adversely in diurnal and nocturnal species. This hypothesis is supported by research on the effect of serotonergic activation on light resetting of the SCN in the day-active *A. ansorgei* (54). The authors showed that injections of 5-HT receptor agonists induce small phase advances during the subjective night (54), while in nocturnal species 5-HT receptor agonists cause large phase advances only during the subjective midday (55-57). Also GABA is able to induce different responses in the SCN of nocturnal and diurnal rodents; activating GABAA receptors mice during the subjective day induces phase advances in nocturnal rodents, whereas the SCN of diurnal grass rats display a phase delay (58). Another potent factor projecting behavioral information to the SCN is neuropeptide Y (NPY). In the *Arvicanthis nicloticus*, behavioral activity increase the levels of NPY in the IGL of nocturnal rodents (59), and behavioral activity is associated with Fos expression in NPY-containing neurons in the IGL of the *A. niloticus* (60). Moreover, in the *A. nicoloticus*, the elimination of the IGL leads to an absence of NPY fibers within the SCN, suggesting the presence of functional NPY projections from the IGL to the SCN (60). Our study together with existing literature indicate differences in SCN's responsiveness to neurotransmitters between nocturnal and diurnal animals. These differences should be investigated more elaborate in order to make recommendations to improve the well-being of humans based largely on results from studying nocturnal species.

Conclusions

We show that behaviorally-induced enhancements of SCN firing rate in the day-active grass rat significantly increases the amplitude of the SCN rhythm. In diurnal animals, when maximal behavioral activity occurs in phase with maximal SCN electrical activity (i.e. during day time), the electrical activity increases as a result of behaviorally-induced enhancements. Previous studies have revealed that in humans behavioral activity during the popper time of day can synchronize the circadian system (24,25,61-63). For example, in elderly and people suffering from Alzheimer's disease increased daytime exercise leads to improvements of sleep-wake cycles, mood and performance (23,26-29,64,65). Our results indicate that behavioral activity can boost the amplitude of the circadian clock, and contribute to the understanding of the interplay between the SCN and the periphery in humans.

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7

TEMPORAL ORGANIZATION OF BEHAVIOURAL ACTIVITY BY THE SCN IN THE DIURNAL GRASS RAT *ARVICANTHIS ANSORGEI*

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Abstract

The suprachiasmatic nuclei's (SCN) electrical activity regulates behavioral temporal patterns. In nocturnal animals the SCN electrical activity rhythm and behavioral activity rhythm are anti-phasic, while in diurnal animals these rhythms are in phase. The aim of this study was to investigate the regulation of behavioral patterns by the SCN's electrical output in a diurnal animal. To this purpose, we performed *in vivo* and *in vitro* recordings of SCN neuronal activity in grass rats with unimodal (full diurnal) or crepuscular behavioral phenotypes. Additionally, we assessed SCN electrical activity patterns *in vitro* after manipulation of behavioral patterns, by subjecting grass rats to long or short photoperiods. Our *in vivo* measurements revealed unimodal or bimodal in SCN of full diurnal or crepuscular grass rats respectively. *In vitro* recordings in isolated SCN from full diurnal or crepuscular grass rats revealed unimodal patterns of SCN electrical activity in both groups. The electrical activity patterns exhibited similar peak time and peak width. Exposure of grass rats to short or long days induced compressed or decompressed behavioral activity phases respectively, but recordings of the SCN of these animals *in vitro* revealed no differences in SCN electrical activity rhythms. The results indicate that in day-active grass rats, behavioral phenotypes (i.e. unimodal or crepuscular) nor the daily duration of the behavioral activity (as seen under long and short photoperiod) are predictable from the SCN electrical activity *in vitro*. This is in contrast to nocturnal animals, which show a close correspondence between SCN electrical activity and behavioral activity.

Introduction

The daily revolution of the earth around it axis causes a 24h cycle in environmental conditions, and the annual rotation of the around the sun brings about seasonal changes. To anticipate and adapt to these environmental changes, mammals are equipped with an internal clock which resides in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The SCN display a coherent circadian rhythm in electrical activity, which is based on the cyclic expression of clock genes and protein products within the single SCN neurons (1,2) as well as on synchronization among single cell neuronal oscillations (3). The electrical output of the SCN synchronizes peripheral rhythms in endocrine and physiological functioning (4) and drives rhythms in behavioral activity (5,6).

The ensemble of the SCN electrical activity has a sinusoidal-like waveform that peaks during the day and is low during the night (7,8), and thus is in anti-phase with the behavioral activity rhythm. *In vivo* recordings of SCN electrical activity in freely moving mice revealed close inverse correspondence between the SCN's pattern of electrical activity and the behavioral state of the animal. Specifically, the behavioral transitions from rest to activity – and vice versa – occur at the mid-point in the declining and increasing slopes in SCN activity, respectively (5).

Relatively little is known about the contribution of the SCN to behavioral activity patterns in diurnal animals other than that in diurnal animals the output of the SCN is in phase with behavioral activity (9,10). The aim of this study was to investigate the role of the SCN's electrical activity in the regulation of behavioral patterns in a diurnal animal. To this purpose, we examined electrical activity rhythms of the SCN from diurnal grass rats that displayed distinct behavioral phenotypes; unimodal (i.e. "full diurnal") or crepuscular (i.e. active around dawn and dusk) by performing *in vivo* electrophysiological measurements. In addition, we performed *in vitro* measurements in SCN from full diurnal or crepuscular grass rats, and from grass rats after manipulations of their behavioral patterns by exposure to long or short days. Our *in vivo* recordings in crepuscular grass rats revealed bimodal rhythms in SCN discharge rate with peaks occurring during dawn and dusk (i.e. times of maximal behavioral activity). *In vitro* recordings in SCN isolated from full diurnal or crepuscular grass rats revealed unimodal electrical activity patterns with similar peak-times and peak-widths (i.e. duration of elevated electrical activity). Moreover, photoperiod-induced compression or decompression of the behavioral activity phase did not correlate with SCN electrical activity waveform *in vitro*. The results indicate that in day-active grass rats behavioral activity patterns are not directly driven by the SCN, but presumably result from the interplay between SCN and extra-SCN areas.

Methods

Animals and housing

Male grass rats (*Arvicanthis ansorgei*, breeding colony Chronobiotron, UMS 3415, CNRS University of Strasbourg, France) were subjected to12 hours light (200 lux) and 12 hours red dim light (<5 lux) cycles. All experiments were performed under the approval of the French Ministry of Higher Education and Research.

Micro-electrode implantations

Procedures for *in vivo* measurements of multiunit activity from SCN neurons have been previously described for rats (13) and mice (14). At a minimum of 8 weeks (150g-300g), tripolar stainless steel micro-electrodes (Plastic One MS333-3-BIU-SPC, Roanoke VA, United States) were implanted in anesthetized (0.13 ml Zoletil (Virbac, Carros, France) and 0.2 Rompun 0.5% (Bayer Pharma, Puteaux, France) per 100 g bodyweight) grass rats with the use of a stereotactic instrument. Two twisted electrodes (Polyamide-insulated; bare electrode diameter 125 µm) for differential recordings were aimed at the SCN (coordinates: 0.8 mm posterior to bregma, 0.9 mm lateral to the midline, 7.8 mm ventral to the dura mater, under a 5° angle in the coronal plane), and a third uncoated electrode (reference electrode) was placed in the cortex. The differential amplifier was based on the design of Yamazaki and coworkers (15).

In vivo SCN electrical recordings

After a recovery period of at least 5 days, the grass rat was individually housed in a temperature controlled recording chamber (22 °C), where water and standard laboratory food (SAFE, Augy, France) were available *ad libitum*. The grass rat was able to move freely during the time of recording. The electrical signal was amplified, bandwidth filtered (500 Hz – 5 kHz), and window discriminators were used to convert action potentials into digital pulses that were counted in 10 second bins by Circa V1.9 software (OriginLab)) and MATLAB (Mathworks Inc.). *In vivo* SCN electrical recording set-ups were equipped with a drinking sensor and a Passive Infra-Red (PIR) motion detector positioned 30 cm above the floor of the cage. Behavioral activity and SCN electrical activity were recorded simultaneously in 10 second time bins.

Data Analysis *in vivo* electrophysiology

Electrical activity date were smoothed in MATLAB (Mathworks Inc.). The magnitude of the behaviorally induced change in neuronal firing rate was defined as the difference between baseline of the electrical activity and the average electrical discharge during the episode of behavioral activity. The baseline level was defined as the mean neuronal firing rate during 2 minutes before the episode of behavioral activity.

Histological verification micro-electrode

After the *in vivo* measurements, the location of the micro-electrode was histologically checked. To sacrifice the grass rats, the animals received intraperitonally an overdose of sodium pentobarbital (CEVA, Libourna, France, 0.3 ml/100g bodyweight). An electrical current of 40µA was passed through the recording electrode to deposit iron at the electrode tip. The brains were collected and immersed in 4% araformaldehyde with 0.5 g of C6FeK4N6·3H20 (Sigma Aldrich, Lyon, France) for 2 days. Brains were sectioned coronally in 40µm slices and stained with cresyl violet for microscopic verification of the recording site.

Behavioral Experiments

Grass rats were entrained to either a long (LD 16:8) or short (LD 8:16) photoperiod for 4 weeks. After 4 weeks the animals were subjected to at least 3 days of constant darkness. The grass rats were housed in clear plastic cages, and locomotor activity was monitored by either passive infrared detectors.

In vitro SCN electrical recordings

Grass rats were entrained to LD12:12, LD8:16 or LD16:8 for at least 30 days. The animals were scarified at ZT 21 (± 0.5 h) under dim red light., and the brains were removed within 1 minute of decapitation. Brain slices (~450 microns thick) were prepared using a tissue chopper, and the slice containing the SCN was transferred to a laminar flow chamber within six minutes after decapitation. The tissue was bathed in bicarbonate-buffered ACSF that was gassed by continuously blowing a warmed, humidified mixture of O2 (95%) and CO2 (5%) over the solution. The slice was submerged in the solution and stabilized using an insulated tungsten fork, and settled in the recording chamber for ~1 h before the electrodes were placed in the center of the SCN. Action potentials were recorded using 50-µm 90% platinum/10% iridium electrodes. The signals were amplified 10 k time and bandpass-filtered (0.3 Hz low-pass, 3 kHz high-pass). The action potentials that exceeded a predetermined threshold well above noise (~5 µV) were counted in 10-second bins using a custom-made automated computer program.

Data Analysis *in vitro* electrophysiology

The electrophysiological data were analyzed using a custom-made program in MATLAB as described previously (11). The time of maximum activity was used as a marker of the phase of the SCN and was determined as the first peak in multiunit activity. Multiunit recordings of at least 24 hours in duration that expressed a clear peak in multiunit activity were moderately smoothed using a least-squares algorithm (12). Subsequently, the SCN peak time, the peak width, and the relative peak amplitude (peak-to-trough ratio) of the first cycle in vitro were determined.

Statistical analyses were performed using SPSS. All summary data are reported as the mean ± the standard deviation (SD). P-values were calculated using the two-tailed Student's t-test, and differences with $p<0.05$ were considered to be statistically significant.

Results

In vivo SCN firing pattern in diurnal grass rats

In vivo SCN electrical activity measurements were performed successfully in 4 day-active grass rats (*Arvicanthis ansorgei*). Three grass rats displayed a crepuscular behavioral phenotype with high levels of behavioral activity around transitions from lights offset to lights onset (dawn) and around transitions from light onset to light offset (dusk), and lower levels of behavioral activity around the middle of the day and the middle of the night (Fig 1A). In these grass rats, SCN electrical activity followed a bimodal waveform that showed pronounced peaks around light onset and around light offset, which coincide with episodes of behavioral activity. SCN discharge levels were overall higher during the day and lower during the night (508±110 Hz vs 490±125 Hz, respectively).

One grass rat displayed a full diurnal behavioral phenotype with high levels of behavioral activity during daytime and low levels of behavioral activity during night time (Fig 1B). SCN discharge followed a unimodal waveform that showed one pronounced peak around midday and a trough around the middle of the night, and were overall higher during the day and lower during the night (815 Hz vs 777 Hz, respectively). After release into DD, rhythm in SCN electrical activity continued in all recordings.

In vitro SCN electrophysiological measurements.

To test whether the SCN regulate behavioral patterns or vice versa, endogenous firing rate rhythms were measured in SCN from grass rats with full diurnal or crepuscular behavioral phenotypes (Fig.2A). *In vitro* electrophysiological recordings in SCN from grass rats with unimodal or bimodal behavioral phenotypes revealed unimodal sinusoidal waveform patterns in the isolated SCN in both groups (Fig.2B). The circadian phase of the SCN was defined by the time of maximum discharge rate. In SCN from grass rats with unimodal behavioral phenotype, the electrical activity was maximally high around midday (ExT 12.3±0.9 h). In SCN from grass rats with bimodal activity patterns, the time of maximal SCN firing rate occurred also close to midday (ExT 12.4±0.8). SCN from unimodal and bimodal grass rats were not significantly different in phase (time of maximal firing rate) ($p=0.96$, Fig.2C). Evaluation of the peak width (i.e. duration of elevated electrical activity) of the multiunit recordings provides an indication of the phase-synchrony among activity patterns of individual neurons. SCN from unimodal grass rats showed a peak-width of 9.9±1.2 h, and SCN from bimodal grass rats displayed a peak-width of 11.23±1.1 h. These values were not significantly different ($p=0.43$, Fig.2D).

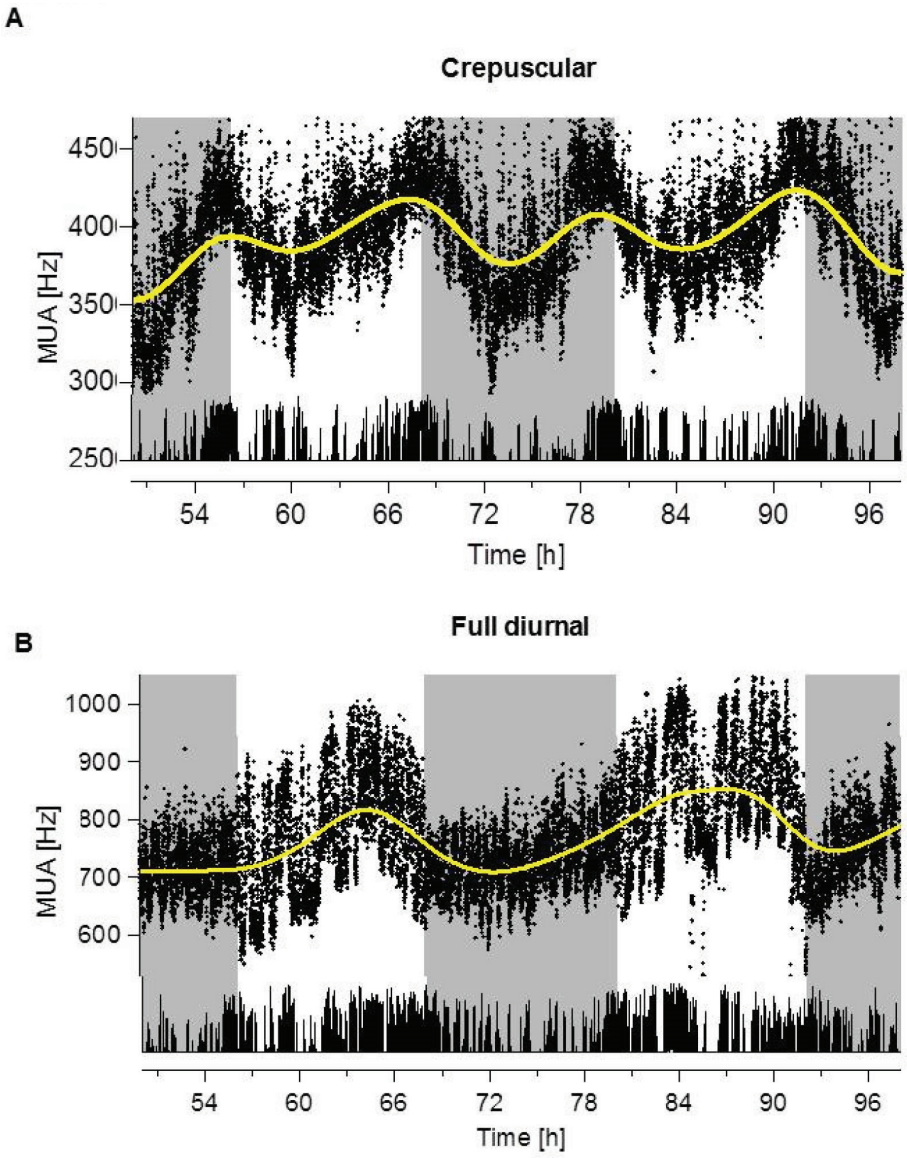

Figure 1. SCN multiunit electrical activity (MUA) *in vivo*. One raw trace of SCN electrical activity measured in a crepuscular *A. ansorgei* **(A)** and in a full diurnal *A. ansorgei* **(B)** kept in LD 12:12. The x-axis shows actual clock time in hours and the y-axis shows SCN MUA in Hz. The grey background represents periods of darkness. Behavioral activity was measured with passive infrared detectors, and is plotted below the SCN electrical activity traces. SCN discharge rate of the crepuscular *A. ansorgei* shows a bimodal circadian pattern, with peaks around dawn and dusk. SCN discharge rate of the full diurnal *A. ansorgei* follows a unimodal circadian pattern, with maximal electrical activity at midday. The smoothed fitted line of the MUA is plotted in yellow.

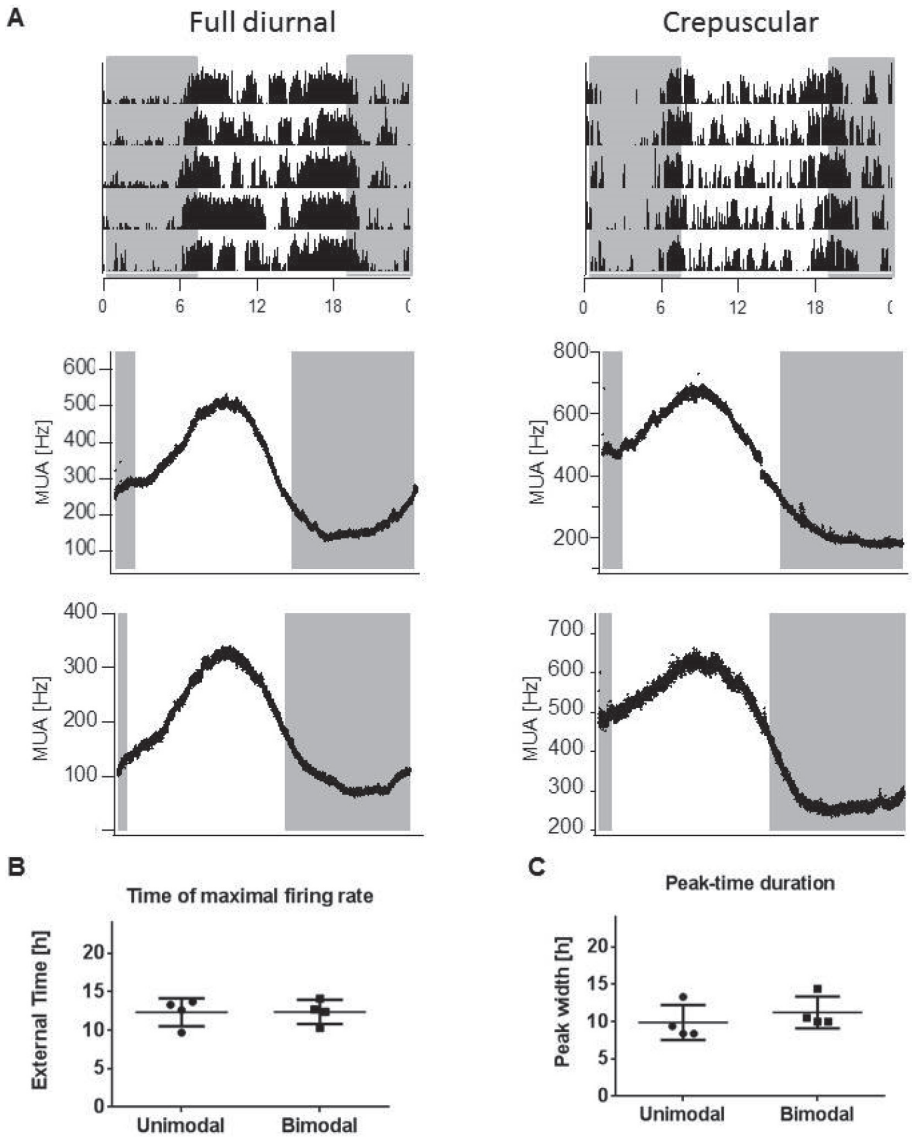

Figure 2. SCN multiunit electrical activity (MUA) *in vitro*. A. Example of actograms from full diurnal (left) and crepuscular (right) **A.** ansorgei kept in LD 12:12. Behavioral activity was measured with passive infrared (PIR) detectors. Consecutive days are plotted on successive lines. Raw traces of SCN electrical activity measured *in vitro* are plotted below. The white-grey background represent the light-dark regime to which the grass rats were entrained and the y-axis shows MUA in Hz. SCN discharge rate of full diurnal (left graphs) and crepuscular (right graphs) **A.** *ansorgei* follow a unimodal circadian pattern, with maximal electrical activity at midday. In full diurnal grass rats MUA was maximally high at ExT 12.3±0.9 h (n=4) and in crepuscular grass rats at ExT 12.4±0.8 h (n=4, p=0.96). MUA in full diurnal grass rats were elevated (measured by the peak width of the waveform) for a duration of 9.9±1.2 h (n=4), and in crepuscular grass rats 11.23±1.1 h (n=4, p=0.43).

Behavioral adaptations to long and short photoperiods

To investigate whether the SCN encodes the duration of the behavioral activity phase, we exposed grass rats to a short photoperiod (8 h light and 16 h darkness, LD8:16) or a long photoperiod (16 h light and 8 h darkness, LD16:8) (Fig.3A). Behavioral adaptations were assessed by comparing the activity phase (α; activity onset to activity offset) in LD 12:12 photoperiod to the activity phase found under the short or long photoperiod. Within 30 days of entrainment to the short photoperiod, the grass rats displayed an activity phase of 12.5±1.8 h. The activity phase found under a short day protocol was significantly decreased (i.e. compressed) compared to the activity phase during LD 12:12 (13.9±0.8 h; $p<0.001$). Grass rats exposed to the long photoperiod, showed an activity phase of 16.2 ± 0.4 h. The behavioral activity phase found under a long photoperiod protocol was significantly longer (i.e. decompressed) compared to the activity phase under LD 12:12 (13.4±0.6 h, $p<0.001$). To investigate memory of the short and long photoperiod, grass rat were subjected to constant darkness (DD) for 3 days. In short photoperiod, the activity phase was significantly compressed after release in DD during all three recordings days (day 1 12.2±1.8 h, $p<0.001$; day 2 12.2±1.5 h, $p<0.001$; day 3 12.8±1.5 h, $p<0.05$)(Fig. 3B,C). After long photoperiod, the behavioral activity phase was significantly decompressed after release in DD (day 1 15.4±1.0 h, 14.2±0.7 h, 14±0.9 h, $p<0.001$) (Fig. 3B,C).

Aftereffects on SCN electrical activity profiles after long and short photoperiod

To examine whether photoperiod-induced changes in behavioral patterns arise at the level of the SCN, we performed *in vitro* electrophysiological measurements in the SCN of grass rats entrained to a long photoperiod or a short photoperiod for at least 30 days. *In vitro* SCN electrophysiological measurements were conducted under constant conditions continuously for a period of at least 24 hours and revealed a sinusoidal unimodal electrical activity pattern. Electrophysiological recordings of SCN from grass rats exposed to a long photoperiod revealed a peak-width (i.e. duration of elevated levels of electrical activity) of 9.5±2.3 h and SCN recordings of diurnal grass rats from a short photoperiod showed a peak width of 10.3±1.2 h. The peak-width of the SCNs multiunit electrical activity of grass rats measured after exposure to long days and short days were not significantly different (Fig. 4B). The *in vitro* traces reflect the endogenous rhythm of the SCN on the first day of DD after exposure to short and long days. The behavioral activity phase (alpha) on the first day of DD after exposure to short or long days was significantly different between the groups (t-test, $p<0.0001$ Fig 4C).

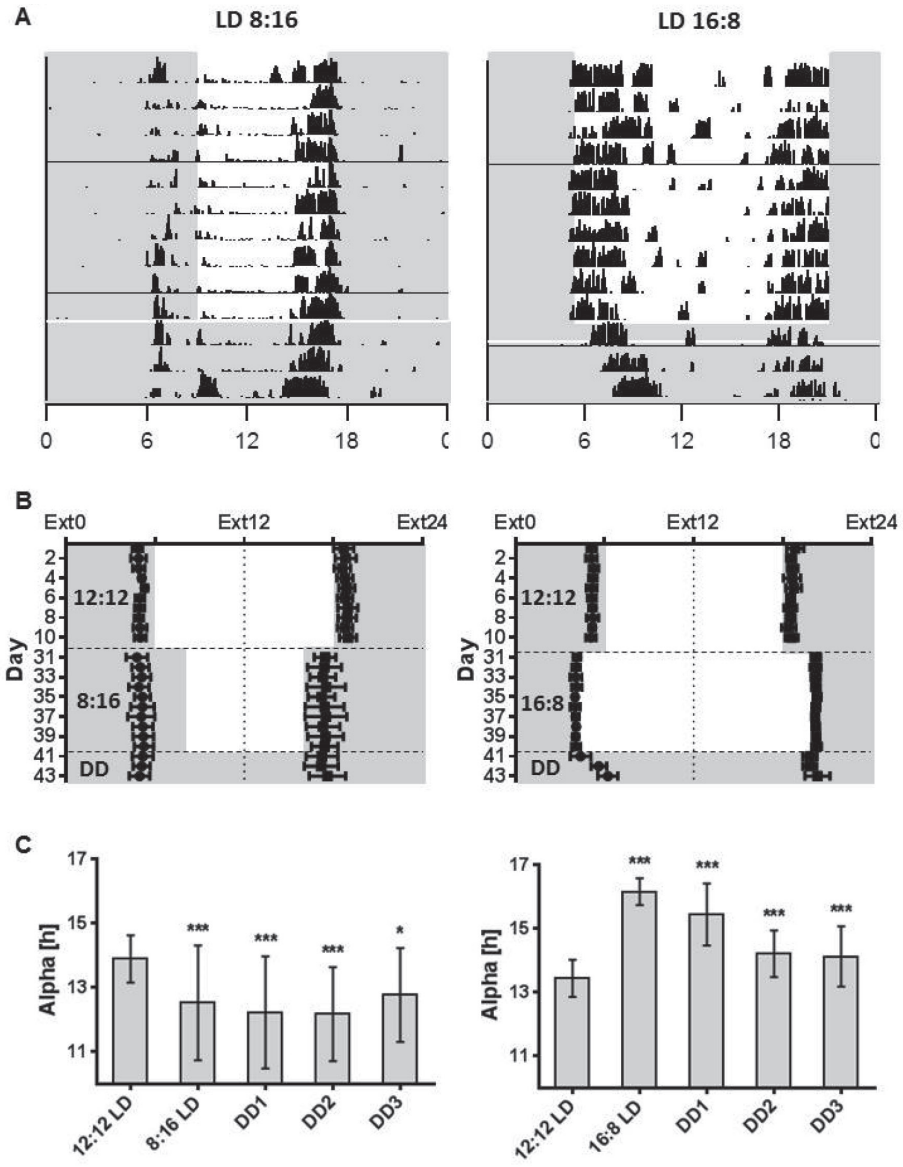

Figure 3. Photoperiod-induced adaptations in behavioral activity. A. Example of actograms from animals kept in short (left) and long (right) photoperiods. The actograms show the last 10 days of exposure to the short or long photoperiod and 3 days of constant darkness (DD). Consecutive days are plotted on successive lines. Behavioral activity was measured with passive infrared (PIR) detectors. **B.** Activity onset and offset relative to light onset and offset (mean ± SD), under LD 12:12 and LD 8:16 (left, n=14) and LD 12:12 and LD 16:8 (right, n=14). Consecutive days are plotted on successive lines. **C.** Behavioral activity phase (alpha) measured during 10 days of LD 12:12 was compared to alpha during the last 10 days of LD 8:16 (left) or LD 16:8 (right) and the first, second and third day of DD (mean ± SD). A significant compression of alpha was found during exposure to LD 8:16, which retained during the first 3 days of DD. A significant decompression alpha was caused by exposure to LD 16:8, which retained during the first 3 days of DD.

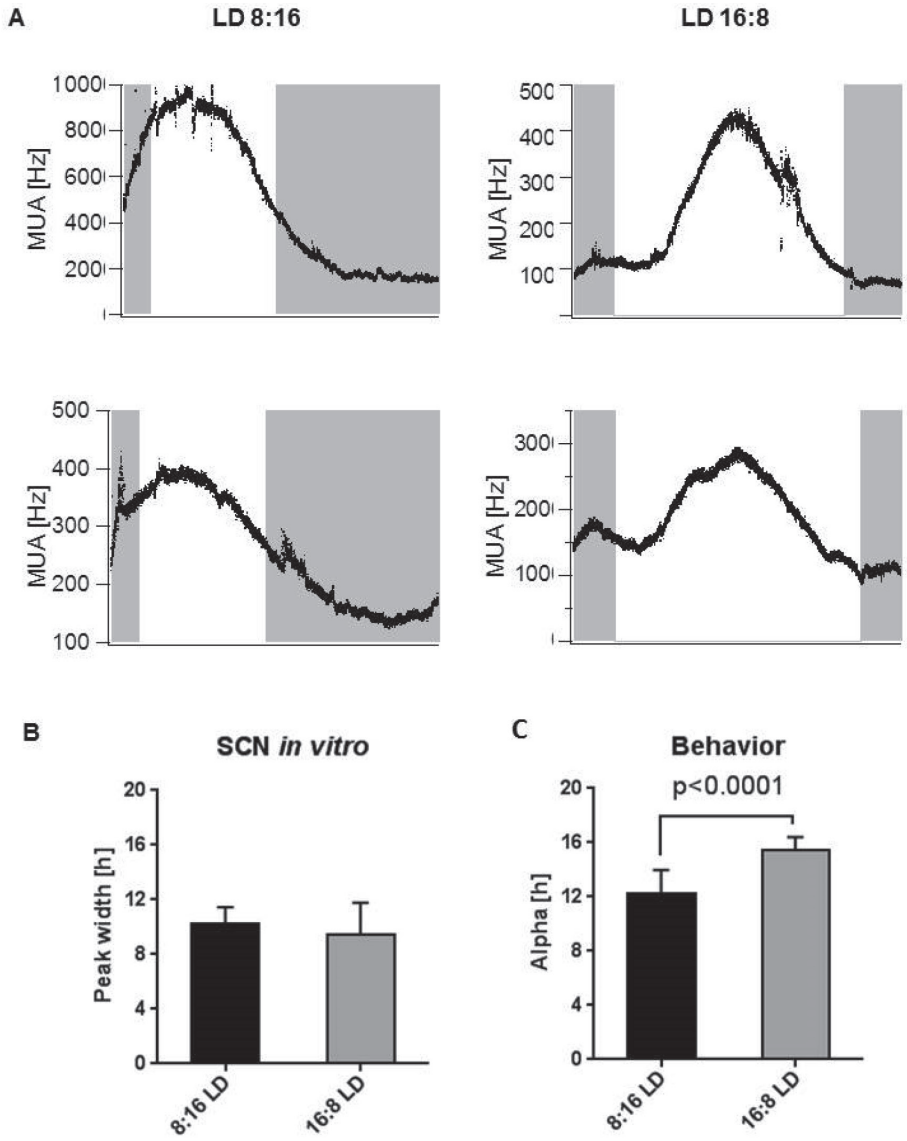

Figure 4. SCN multiunit electrical activity (MUA) *in vitro* after short and long days. A. Raw traces of SCN electrical activity from animals kept in LD 8:16 (left) and LD 16:8(right) photoperiod measured *in vitro*. The white-grey background represent the light-dark regime to which the grass rats were entrained and the y-axis shows MUA in Hz. SCN discharge rate of animals after short and long days follow a unimodal circadian pattern, with maximal electrical activity at midday. **B.** Elevated MUA levels (measured by the peak width of the waveform) were present for a duration of 10.3±1.2 h (n=4) after LD 8:16 and for a duration of 9.5±2.3 h (n=4) after LD 16:8, and were not significantly different (p=0.43). **C.** Behavioral activity phase (alpha) on the first day of constant darkness after exposure to LD 8:16 or LD 16:8 are significantly different (12.2±1.8, n=14 versus 15.4±1.0, n=14 respectively, p<0.0001).

Discussion

The aim of this study was to investigate the regulation of behavioral patterns by the SCN's electrical activity in a diurnal animal. Therefore, we recorded SCN electrical activity in grass rats with full diurnal and crepuscular behavioral phenotypes *in vivo* and *in vitro*. Additionally, we assessed SCN electrical activity patterns of SCN isolated from grass rats with photoperiod-induced changed behavioral patterns. Interestingly, *in vitro* recordings of SCN discharge revealed unimodal electrical activity rhythm in the SCN of both groups, whereas *in vivo* recordings revealed bimodal rhythms in crepuscular rats. Moreover, photoperiodic-induced compressions or decompression of the behavioral activity phase did not correlate with changes in SCN electrical activity patterns found *in vitro*. The results indicate that in day-active grass rats behavioral phenotypes (i.e. unimodal or crepuscular) nor photoperiodic-induced changes correspond with SCN electrical activity *in vitro*. As the SCN's electrical activity pattern *in vivo* reflected the behavioral activity patterns in all cases, we expect that an interplay between the SCN and extra-SCN areas is of large importance in diurnal species.

SCN electrical activity pattern *in vivo*

By the use of implanted microelectrodes we were able to measure the electrical activity of the SCN in freely-moving *A. ansorgei*, while monitoring their behavioral activity with passive infrared detectors. We successfully performed long-term measurements of SCN electrical activity in 4 grass rats, of which one that displayed a true diurnal behavioral phenotype and 3 that displayed a crepuscular phenotype. The recording from the full diurnal rat shows a unimodal circadian pattern that is higher during daytime and low during night time. This finding is similar to previous *in vivo* measurements of the SCN's discharge rate in the diurnal chipmunk (*Eutamias sibiricus*) (9) and nocturnal rodents such as hamsters (13), rats (14,15) and mice (6,16). We successfully performed long-term measurements of SCN electrical activity in 3 grass rats with crepuscular behavioral phenotypes. These grass rats displayed typical elevated levels of behavioral activity around dawn and dusk. In the crepuscular *A. ansorgei*, SCN electrical activity rhythms shows two distinct peaks, of which one around dawn and one around dusk. The peak times of the bimodal SCN electrical activity waveform correspond to the time of maximal behavioral activity. Our findings show that the SCN electrical activity rhythm is different in grass rats with distinct behavioral phenotypes, and indicate an interplay between the SCN and the periphery.

Studies in which behavioral patterns of mice were altered revealed that behavioral patterns correlate the SCN electrical activity waveform (5). In particular, SCN electrical activity levels are inversely related to the intensity of behavioral activity,

indicating that SCN electrical activity level caries information that determines the temporal profile of behavioral activity (6). Our findings indicate that in diurnal and nocturnal animals different mechanisms determine behavioral patterns.

Endogenous electrical activity rhythm of SCN from day-active grass rats with full diurnal or crepuscular behavioral phenotypes

To test whether behavioral patterns are regulated by the SCN, endogenous firing rate rhythms were measured in SCN from grass rats with full diurnal or crepuscular behavioral phenotypes (Fig.2A). *In vitro* electrophysiological measurements in the SCN from both groups of animals revealed unimodal sinusoidal electrical activity patterns, regardless of the behavioural phenotype (Fig.2B). Our *in vitro* measurements on the isolated SCN from diurnal grass rats show robust circadian oscillations in firing rates, with high electrical activity levels during subjective daytime and low levels during subjective night time. This result is in agreement with electrophysiological and molecular studies of SCN rhythmicity in nocturnal and diurnal animals. *In vitro* recordings showed that the isolated SCN from nocturnal animals generate robust endogenous rhythms (7,8,17), however, to date no studies reported about the endogenous circadian electrical activity rhythm of the SCN from a diurnal animal.

The circadian phase of the SCN electrical activity rhythm was determined by examination of the time of maximal SCN discharge. SCN's maximum discharge rate from day-active grass rats with an unimodal behavioral phenotype occurred around midday. Likewise, SCN from grass rats with a crepuscular behavioral phenotype showed a peak in firing rate close to midday and were not significantly different in phase (Fig.2C). These results show that in day-active grass rats, behavioral patterns (unimodal or crepuscular phenotype) are not regulated by the endogenous SCN electrical activity rhythm.

Photoperiod-induced behavioral adaptations in the day-active grass rat

By exposing grass rats to short (8 h light and 18 h darkness, LD 8:16) or long photoperiods (16 h light and 8 h darkness, LD 16:8) respectively we manipulated the behavioral activity and rest phase, and questioned whether these were accompanied by alternations of the SCN electrical activity profile in vitro, as is the case in nocturnal animals (11,18). We were able to successfully manipulate the behavioral activity phase by subjecting the animals to short or long photoperiods. Within 30 days of exposure to long days, grass rats displayed a decompression of the active phase to the long photoperiod, which means that the duration of the active phase corresponds to the duration of light per day (16.2 ± 0.4 h and 16 h, respectively). Accordingly, the behavioral activity phase displayed under the long photoperiod was significantly longer than found under LD 12:12. Grass rats exposed to a short

photoperiod displayed an activity phase that was significantly shorter than found under LD12:12, however the compression of the behavioral activity phase was incomplete to match the external LD cycle. Previous studies with different species of grass rats (*A. ansorgei* and *A. niloticus*) also reported about incomplete compression of the behavioral activity phase during exposure to short photoperiods (19-21). Moreover, in day-active *A. niloticus* short photoperiod (LD8:16) does not induce changes in the behavioral pattern, and accordingly no changes in PER1 and PER2 expression patterns in the SCN (19).

To check whether the photoperiodic-induced changes of the behavioral activity phases would retain in the absence of light input, we exposed grass rats from short or long days to constant darkness. Measurements of behavioral activity during the first three days of constant darkness revealed that the photoperiod-induced compression or decompression were retained during the first three days of constant darkness indicating the presence of photoperiodic memory in diurnal grass rats, and encoding of the period by a central mechanism.

In vitro electrical activity rhythm of SCN from the day-active grass rat after a long and short photoperiod

To assess the role of SCN electrical activity in the regulation of the duration of the behavioral activity phase, we performed electrophysiological measurements in the isolated SCN from grass rats adapted to short and long days. The recordings showed circadian rhythms in SCN discharge rate after both long and short photoperiods, with maximal firing during subjective daytime and minimal firing during the subjective night. We evaluated whether compression or decompression of the behavioral activity phase correlated with waveform changes in SCN electrical activity waveform. In grass rats with compressed behavioral activity phase (after short days) the peak width of the SCN waveform was not different from the peak width found in animals with a decompressed activity phase (after long days). The results indicate that in day-active grass rats, the external cycle is not endogenous encoded by the SCN. Grass rats exposed to a long photoperiod displayed complete behavioral adaptation and behavioral activity onset occurred close to light onset.

Previous *in vivo* recordings of SCN electrical activity in freely moving mice revealed that behavioral transitions from rest to activity – and vice versa – occur at the mid-point in the declining and increasing slopes in SCN activity, respectively (5), and this correlations is retained under different photoperiods, indicating a role for the SCN in photoperiodic behavioral adaptations. In nocturnal animals, short photoperiods are accompanied by a narrow peak width of the SCN electrical activity waveform realized through a compression of individual cellular activity profiles, while long photoperiods result in broad peak width of the SCN rhythm as a result of desynchrony among individual cells (3,11,22,23). We show that in the day-active grass

rat the duration of the behavioral activity phase does not correlate with the peak width of the SCN electrical activity rhythm measured *in vitro*, suggesting fundamental differences in the control of behavioral patterns in diurnal and nocturnal animals.

Involvement of extra-SCN areas in the regulation of behavioral activity patterns

We find that SCN electrical activity rhythms *in vivo* are unimodal or bimodal in full diurnal or crepuscular grass rats respectively, while SCN rhythms *in vitro* are similar between the groups, indicating that the organization of temporal activity patterns do not arise exclusively at the level of the SCN. Instead we expect that in diurnal animals the SCN is more subject to input from intrinsic and extrinsic factors. Moreover, photoperiodic-induced compressions or decompression of the behavioral activity phase did not correlate with changes in SCN electrical activity patterns *in vitro*, which suggest that the duration of the behavioral activity phase is also not intrinsically encoded for at the level of the SCN. Several studies conducted have pointed to the intergeniculate leaflet (IGL) as a potential modulator of behavioral activity patterns. For example, in nocturnal mice (24) and rats (25) and in day-active grass rats (26) and Octodon degus (27) bilateral ablation of the IGL results in a change in circadian behavioral patterns. *In vivo*, the IGL feeds back to the SCN with neuropeptide Y (NPY), and mice that lack NPY show no photoperiod-induced changes in behavioral patterns during simulated natural photoperiods (28). Interestingly, in the day-active grass rat patterns of wheel running activity are related to Fos expression in the IGL (29). These findings indicate an important role for the IGL in the modulation and fine tuning of temporal behavior in response to seasonal changes in day length.

Conclusion

Studies performed in nocturnal rodents have provided strong evidence that the SCN's electrical activity waveform regulates behavioral activity patterns. We show that in day-active grass rats SCN electrical activity patterns *in vivo* do correlate with the behavioral phenotype, while endogenous SCN electrical activity patterns *in vitro* do not. The findings indicate that in day-active grass rats the regulation of behavioral activity arises from an extra-SCN area, which feeds back to the SCN. In addition, the results point to fundamental differences in intrinsic contribution of the SCN in the regulation of behavioral activity patterns in nocturnal and diurnal animals. On the basis of the present findings we expect that behavioral activity leads to stronger modifications of SCN electrical activity, and therefore SCN output, in diurnal than in nocturnal species.

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8

THE SUPRACHIASMATIC NUCLEI AS A SEASONAL CLOCK

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Abstract

In mammals, the suprachiasmatic nucleus (SCN) contains a central clock that synchronizes daily (i.e., 24-hour) rhythms in physiology and behavior. SCN neurons are cell-autonomous oscillators that act synchronously to produce a coherent circadian rhythm. In addition, the SCN helps regulate seasonal rhythmicity. Photic information is perceived by the SCN and transmitted to the pineal gland, where it regulates melatonin production. Within the SCN, adaptations to changing photoperiod are reflected in changes in neurotransmitters and clock gene expression, resulting in waveform changes in rhythmic electrical activity, a major output of the SCN. Efferent pathways regulate the seasonal timing of breeding and hibernation. In humans, seasonal physiology and behavioral rhythms are also present, and the human SCN has seasonally rhythmic neurotransmitter levels and morphology. In summary, the SCN perceives and encodes changes in day length and drives seasonal changes in downstream pathways and structures in order to adapt to the changing seasons.

1. Introduction

The rotation of the Earth on its axis, combined with the Earth's annual revolution around the Sun, causes both daily and seasonal fluctuations in photoperiod, light intensity, temperature, and food availability. Many organisms have evolved an innate clock that produces circadian rhythms (i.e., rhythms with a period (revolution time) of approximately 24 hours) that enables them to anticipate and accommodate these cyclic changes in the environment. The internal clock is an adaptation to the environment, and it increases the organism's likelihood of survival (i.e., the organism's fitness) (1). Animals that are synchronized to both daily and annual cycles can anticipate and prepare for changes in food availability and ambient temperature, as well as the presence of predators, mating opportunities, and social interactions. Most animals have a clear 24-hour rhythm in terms of their physiological functions, including body temperature, hormone production and secretion, behavioral activity, and sleep. Besides a circadian rhythm, many animals also display a seasonal rhythm. Seasonal breeding animals such as squirrels, hamsters and sheep experience drastic changes in behavior, reproductive physiology and metabolism. Other animals, like mice and rats display altered behavioral activity patterns in response to changing day lengths (Fig. 2 A and F).

In mammals, the central clock that synchronizes these 24-hour rhythms is located in the suprachiasmatic nucleus (SCN), a bilateral structure comprised of approximately 10,000 neurons (2). The SCN is located in the ventral periventricular zone of the anterior hypothalamus, dorsal to the optic chiasm, in close proximity to the third ventricle. The SCN is required for the generation of overt circadian rhythms, and is synchronized to the external day-night cycle by light (3). Light information reaches the SCN through a monosynaptic pathway from the retina to the SCN; this pathway is known as the retinohypothalamic tract (RHT). Thus, the SCN is the first and most rostral structure in the brain to receive photic information on photoperiod. Because of its central role in detecting and encoding photic information, the SCN is indispensable for regulating daily and seasonal rhythms.

The molecular mechanism that underlies rhythm generation is based upon interconnected positive and negative feedback loops that regulate the transcription of core clock genes and the function of these genes' protein products. The transcription factors Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-like protein 1 (Bmal1) promote the transcription of target genes, including the Period (*Per1*, *Per2*, and *Per3*) and Cryptochrome (*Cry1* and *Cry2*) gene families. In turn, the Per and Cry proteins repress CLOCK/Bmal1-mediated gene transactivation. The nuclear receptors ROR (α, ß, and γ), PPARα, and REV-ERB (α and ß) comprise an additional regulatory loop. Results obtained from experiments using dissociated SCN cells revealed that individual cells have a circadian rhythm

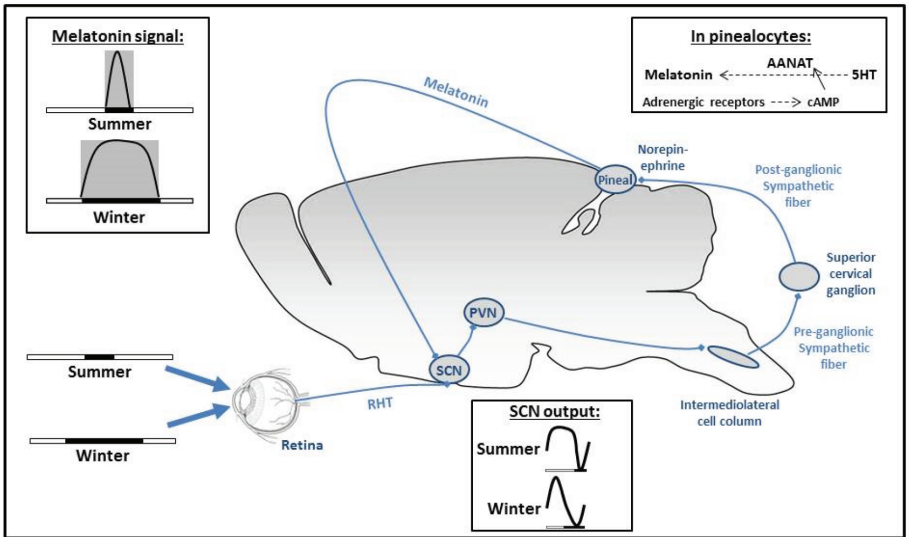

Figure 1. Schematic overview of the pathway regulating seasonal melatonin secretion. Light synchronizes the master clock with the external day-night cycle, and photic information received by the retina reaches the suprachiasmatic nuclei (SCN) via the retinohypothalamic tract (RHT). The external photoperiod influences the electrical activity of the SCN, thereby enabling the SCN to code for long summer days and short winter days. This photoperiodic information is then transmitted from the SCN to the pineal gland via a polysynaptic efferent pathway that travels through the paraventricular nucleus (PVN) to the intermediolateral cell column of the thoracic spinal cord, to the superior cervical ganglion, and finally to the postganglionic adrenergic fibers that innervate the pineal gland. Norepinephrine is released from these sympathetic nerve endings at night, stimulating postsynaptic β1 and α1 adrenergic receptors on the pinealocytes, causing a large increase in intracellular cAMP levels. This increase in cAMP activates serotonin *N*-acetyltransferase (AANAT). Oscillating levels of activated AANAT result in the rhythmic synthesis and secretion of melatonin. During short summer nights (i.e., long photoperiod), the nocturnal melatonin signal is compressed temporally; in contrast, the signal is relatively prolonged during long winter nights (i.e., short photoperiod). These photoperiod-induced changes in melatonin secretion have wide-reaching effects on seasonal animal physiology and behavior.

in their firing rate, with a wide range of intrinsic periods (ranging from 22 hours to 28 hours) (4-6). These results gave rise to the notion that individual SCN neurons are autonomous single-cell oscillators driven by their intrinsic molecular feedback loops (6).

In the past decade, it has become increasingly evident that the SCN's ability to regulate seasonal rhythms is dependent upon the plasticity of the SCN's neuronal network. By adjusting the phase relationship among single cell oscillators, the SCN can code for short winter days and long summer days, respectively. Thus, the SCN's ability to encode photoperiod information requires the presence of a

functional neuronal network. Hence, this is in contrast, with the SCN's ability to produce circadian rhythms, which is a cell autonomous property. By encoding photoperiod information, the SCN creates an internal representation of day length, and this representation is transmitted to other brain nuclei, including the pineal gland, which plays a major role in the regulation of seasonal reproduction cycles by releasing melatonin. A schematic overview of the pathway regulating melatonin secretion is shown in figure 1. In this chapter, we will review the changes that occur within the SCN under the influence of a changing photoperiod. We will first describe the characteristic light response properties of SCN neurons, that are essential to measure the length of the day. Next, we will discuss the SCN's heterogeneous organization and the role of the SCN in storing photoperiod information. We will then review several of the SCN's efferent pathways that are involved in seasonal responses, including the pathways that lead to the pineal gland, which plays a key role in regulating breeding cycles. Finally, we will review the evidence showing that the human clock has seasonal rhythmicity.

2. The SCN as a luminance detector

Irradiance detection is one of the SCN's primary functions (7;8). By detecting changes in ambient illumination levels, the clock becomes properly phased relative to the external day-night cycle and is therefore attuned to gradual changes in day length; this information is then used as an indication of the changing seasons. The SCN's ability to respond in a sustained fashion (i.e., without adaptation) to environmental light is nearly unique to the SCN and is typical of some of the non-image-forming visual centers in the brain (9;10). Irradiance detection is based on the complementary actions of melanopsin, rods, and cones (11-14). Melanopsin alone is sufficient for photic entrainment (15;16), and in the absence of rods and cones, sustained responses can be recorded from ip-retinal ganglion cells (ipRGCs) and cells in the SCN (17-19). In the absence of melanopsin, however, sustained responses are preserved in the SCN, and although the SCN's phase-shifting capacity is reduced, animals entrain normally to the light-dark cycle (20;21). These findings underscore the presence of overlapping tasks of rods, cones, and melanopsin.

Recordings within the SCN have revealed the presence of a subpopulation of cells that are acutely affected by light stimuli. In nocturnal rodents, including rats, hamsters and mice, approximately 25% of the SCN's cells are excited by light stimuli, and a smaller population has a reverse response and is inhibited—or silenced completely—by light (22-25). In diurnal animals, the percentage of light responsive cells is lower, and relatively more cells are light suppressed (26;27).

The magnitude of the change in the discharge rate of both light-activated and light-inhibited SCN neurons is a function of light intensity (22;24). The intensity-response

curve is sigmoidal with a working range centered around the light intensities that occur at dawn and dusk. This working range is relatively narrow compared to the range of light intensities that actually occurs in the environment; consequently, changes in environmental illumination are translated into a signal that indicates "day" versus "night", whereas the SCN can discriminate only poorly between light intensities that occur throughout the day (for example, to determine whether it is a sunny day or a cloudy day). The SCN's ability to detect changes in illuminance is a prerequisite to determine the length of the day and thus changes in photoperiod.

3. SCN network plasticity

The combined electrical activity of individual SCN neurons is integrated to produce electrical activity oscillations at the SCN network level. This output signal has a sinusoidal-like waveform pattern and oscillates with ~24-hour precision. The peak of the SCN's electrical output is during the day, and the trough is during the night. This rhythm is typical of the SCN and is present in both diurnal and nocturnal species. *In vivo* electrophysiology recordings in the SCN of freely moving mice revealed that transitions in behavior from rest to activity—and vice versa—occur at half-maximal levels of the SCN's electrical activity rhythm (28).

Compelling evidence suggests that plasticity in the SCN's neuronal network plays a major role in the SCN's ability to adjust to changes in the photoperiod. *In vivo* recordings of SCN discharge rate in freely moving mice showed that after the animals are moved to continuous darkness, the SCN's electrical activity waveform is retained for many circadian cycles (28;29). Thus, the SCN has a "memory" for photoperiod. During both long and short photoperiods, increasing electrical activity triggers the offset of behavioral activity when it reaches the 50% level and decreasing electrical activity triggers the onset of behavioral activity when the 50% level is reached. Thus, the SCN's waveform—and in particular, the peak width of the waveform—stores photoperiodic information (28). Waveform changes are preserved and measurable *in vitro* (30;31). Following a change to a short photoperiod, the peak of the SCN's electrical activity pattern is compressed (i.e., the peak width becomes narrower), whereas changing to a long photoperiod broadens the peak width (30). *In vitro* single-cell recordings from acute SCN slices obtained from both mice and rats that were entrained to a 12-hour photoperiod revealed that individual cells are active for a relatively short period of time (*ca*. 4-5 hours) (32;33). The majority of neurons were active throughout midday, some neurons were active at dawn and dusk, and relatively few neurons during the night (29;32). These findings revealed that the electrical activity patterns of individual cells differ from the electrical activity waveform of the entire SCN network, and indicate that the waveform of the SCN rhythm is determined by the temporal distribution of the single-cell activity patterns (29;32;34;35).

When entrained to a short photoperiod, the individual electrical activity patterns have a narrow phase distribution, meaning that the peak in their activity is close in time (i.e. around midday), whereas the patterns are more widely distributed when entrained to a long photoperiod. The narrow phase distribution of subpopulation activity patterns in a short photoperiod explains the compression of electrical activity waveforms measured at the population level in the SCN (29) (Fig. 2 G-J). Likewise, the electrical activity in the SCN of mice entrained to a long photoperiod has a decompressed peak SCN waveform, which can be explained by the wide distribution of subpopulation activity patterns (29;30) (Fig. 2 B-E). Importantly, the individual electrical activity patterns in short photoperiods are remarkably similar to the activity patterns in long photoperiods (29;29;32;34;35). Thus, the electrical activity pattern at the SCN population level is determined by the phase relationship between the individual SCN neurons (29;32;34;35). Furthermore, the single-cell electrical activity profiles in the dorsal SCN differ between long photoperiods and short photoperiods while this difference is not present in the ventral SCN (35). Computational studies found that differences in single-unit activity profiles in the dorsal SCN are not sufficient to explain the waveform changes at the SCN network level (35;36). Moreover, molecular studies revealed that the single-cell expression patterns—measured by measuring *Per1* expression—do not differ between long and short photoperiods (37). Together the studies support the hypothesis that the phase distribution of single cells plays a key role in seasonal adaptation by the SCN (35). Figure 2 provides an illustrative overview of the seasonal information processing by the circadian system.

Molecular and electrophysiology studies revealed differences in regional organization within the SCN; molecular expression patterns are more region-specific, whereas electrical activity profiles are more homogenous. Anatomically, the SCN can be divided in two major sub-regions, namely the ventrolateral and dorsomedial regions (38). The ventrolateral SCN contains vasoactive intestinal polypeptide (VIP)-expressing neurons, whereas the dorsomedial SCN contains predominantly arginine vasopressin (AVP)-expressing neurons (7). γ-aminobutyric acid (GABA) is the most common neurotransmitter in the SCN and is expressed in both sub-regions (39). In the past two decades, it has become increasingly clear that several neurotransmitters in the SCN, including VIP, GABA, and gastrin-releasing peptide (GRP), are involved in the synchronization of individual neurons. Below, we will discuss the sub-regional photoperiodic differences in clock gene profiles and the importance of neurotransmitters in seasonal encoding of the SCN.

3.1.1 Rostro-caudal axis

As mentioned above, anatomically the SCN can be divided in a ventrolateral and dorsomedial region, however, in seasonality also the rostral and caudal SCN

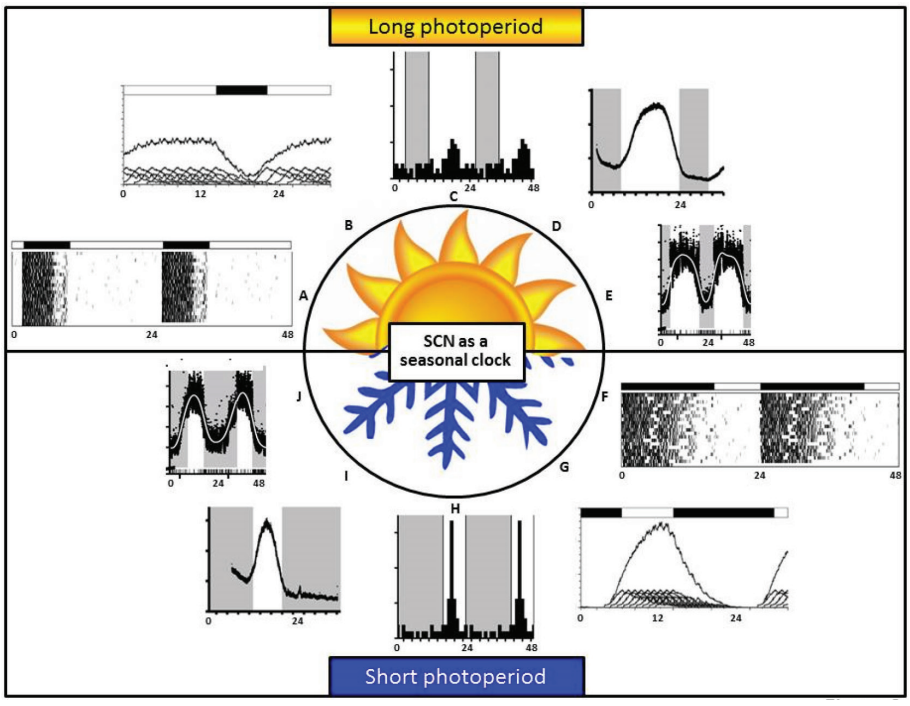

Figure 2. The SCN's ability to regulate seasonal rhythms is dependent upon the plasticity of the SCN's neuronal network. The graphs should be read in a clockwise manner.
In a long photoperiod (upper panel), animals display compressed activity patterns in accordance with the light-dark cycle (A; double plotted actogram). In long days, there is relatively weak phase synchronization among the individual SCN neurons and subpopulations of SCN neurons; accordingly, the distribution of neuronal activity patterns is wider (B; average single unit activity plotted as a function of time in h and C; double plotted histogram of SCN neuronal subpopulation activity peak times plotted as a function of time in h). Both *in vitro* (D; multiunit activity in Hz plotted as a function of time in h) and *in vivo* (E; multiunit activity in Hz plotted as a function of time in h) measurements of electrical activity in the SCN of mice entrained to a long photoperiod reveal a decompression of the peak in the SCN waveform, consistent with the wide distribution of subpopulation activity patterns observed in long days. In short photoperiods (lower panel), animals have long bouts of nocturnal activity (F double plotted actogram). Electrical recordings of small populations of SCN neurons show that in short days, these subpopulations are relatively phase-synchronized. Consequently, the distribution of single-cell activity is narrow and reveals a clear peak in individual activity at midday (G; average single unit activity plotted as a function of time in h and H; double plotted histogram of SCN neuronal subpopulation activity peak times plotted as a function of time in h). The narrow phase distribution of the patterns of subpopulation activity in a short photoperiod causes a compression of the electrical activity waveform, measured at the SCN network level both *in vitro* (I; multiunit activity in Hz plotted as a function of time in h) and *in vivo* (J; multiunit activity in Hz plotted as a function of time in h). Adapted with permission from (29;32) (Copyright (2003) National Academy of Sciences, U.S.A. and Elsevier).

can be distinguished from each other and exhibit differences in their response to change in day length (40-43). For example, Hazlerigg and colleagues reported a difference between the rostral and caudal expression patterns of Per2, Rev-erbα, and D-site albumin promoter binding protein (Dbp) in the SCN of Siberian hamsters (*Phodopus sungorus*, also known as the Djungarian hamster) following exposure to a long photoperiod (16 hours light:8 hours dark) (42). Although the peak time of RNA expression in the rostral SCN was unaffected—or delayed slightly relative to midday—following exposure to a long photoperiod, the peak expression of all three genes was advanced in the caudal SCN. In contrast, the expression patterns of these genes were similar in the rostral and caudal SCN of Siberian hamsters that were previously housed in a short photoperiod (8 hours light:16 hours dark), and RNA levels peaked at approximately the same time in both sub-regions (42). Furthermore, SCN gene expression studies demonstrated that photoperiod regulates SCN gene expression of the E-box-regulated genes (Per1, Per2, Cry1, Rev-erbα, AVP and Dbp), while Bmal1 and Cry2 are not affected (44). Based on these findings, a multiple-oscillator model was proposed in which the caudal and rostral SCN encode information regarding the time at which dawn and dusk occur, respectively. Thus, as the photoperiod is lengthened, the caudal SCN tracks dawn, and the rostral SCN tracks dusk (40). Long-term *in vitro* measurements of circadian rhythms in the clock gene *Per1* in the SCN of mice that were previously exposed to either a long, medium, or short photoperiod revealed three oscillating SCN sub-regions; one sub-region is located in the caudal SCN, and the other two sub-regions are located in the rostral SCN (41). The circadian oscillation in *Per1* expression in the caudal SCN was phase-locked to the offset of behavioral activity in all three photoperiods; in contrast, the oscillation of the rostral SCN was phase-locked to the onset of behavioral activity. The *Per1* expression pattern in the rostral SCN was unimodal in short and medium photoperiods; however, in long photoperiods, a bimodal pattern emerged, suggesting the presence of a second oscillator in the rostral SCN (41).

3.1.2 Ventral versus dorsal SCN

Because nearly all light-responsive cells are concentrated in the retinorecipient ventrolateral SCN (i.e., the "core" SCN), this sub-region is acutely affected by light (45-48). Light increases the expression of *c-fos*—measured as *c-fos* mRNA and c-Fos protein immunoreactivity (c-Fos-ir)—in the ventrolateral SCN in rats, mice and hamsters (49-52). Moreover, the ventral SCN has light-induced alterations in clock gene expression (53-57) and acute responses in electrical activity (55;58-62). The up-regulation of *c-fos* expression following a light pulse is dependent on the circadian phase (i.e. the time of the cycle) and occurs exclusively during subjective night (63-65). In rats that are first entrained to a long photoperiod and subsequently housed in continuous darkness, the interval for *c-fos* mRNA expression and c-Fos-ir

in the ventral SCN in response to a light pulse was significantly shorter than in rats from short photoperiods (63). The after effects of photoperiod on the interval of *c-fos* expression indicates that the ventral SCN has a memory of the previous photoperiod (66). Moreover, in hamsters a striking difference in c-Fos expression in the ventral SCN during the dark phase was found between hamsters housed in a long and short photoperiods (43).

The dorsomedial sub-region of the SCN (i.e., the "shell" of the SCN) does not receive direct light input (49;57;67-70); instead, this sub-region receives non-photic input from the cortex, basal forebrain, and hypothalamus (49;67;71). To synchronize with the external light-dark cycle, the dorsomedial SCN is dependent on projections from the ventral SCN. The functional difference between the various sub-regions of the SCN becomes clear in the case of temporal asynchrony between the SCN and the external light-dark regimen, which is triggered by exposure to a shift in the external cycle (72;73). Although the ventral SCN adapts rapidly to a new external cycle, the dorsal SCN requires several days to resynchronize (43;74;75). Accordingly, the dorsal SCN lacks a light-induced up-regulation of *c-fos* mRNA levels; however, the dorsal SCN has spontaneous rhythmic expression of *c-fos* and the protein product c-Fos (70). This rhythmic expression of *c-fos* in the dorsal SCN persists in continuous darkness for at least two cycles, in contrast to the light-induced *c-fos* expression rhythm in the ventral SCN. The spontaneous *c-fos* expression rhythm in the dorsal SCN may represent rhythmic intrinsic neuronal activity that is driven by a molecular feedback loop (70). Rhythms in *c-fos* expression—and c-Fos protein levels—adapt to the external photoperiod. In rats entrained to a long photoperiod, the dorsal SCN had a longer c-Fos-ir interval compared to the dorsal SCN of rats that were entrained to a short photoperiod (76). The extension of the c-Fos-ir interval was attributed solely to an earlier morning onset of high c-Fos-ir expression in long days (approximately 7 hours) compared to short days, as the evening decline in c-Fos levels occurred at approximately similar times under both light regimens (76). Thus, the seasonal adaptation of *c-fos* mRNA and c-Fos protein patterns in the dorsal SCN reflects seasonal adaptation of the dorsal SCN's molecular clock machinery. Like the ventral SCN, the dorsal SCN also has photoperiod memory (76). Thus, even though the ventral SCN receives direct light input, the dorsal SCN is indirectly affected by it and both sub-regions adjust to the photoperiod in a similar time frame (66;76). It is difficult to reconcile the rostral-caudal differences in the SCN observed under different photoperiods with the anatomical organization of the SCN, which is mainly specified along the ventro-dorsal axis.

3.1.3 Neurotransmitters involved in seasonal encoding

Several neurotransmitters are involved in synchronization and in the coupling between SCN neurons; therefore, each of these transmitters may play a key role

in seasonal encoding of the SCN. The primary neurotransmitter in the ventral SCN is VIP. In the ventral SCN, VIP-expressing cells receive light information from the retina, and they project to the dorsal SCN (2;77). The *in vivo* and *in vitro* application of VIP can mimic the light-induced responses of the SCN (78;79), and applying VIP to SCN neurons isolated from VIP-deficient mice can restore synchrony in the clock neurons (80;81). Furthermore, the absence of VIP or its receptor (VIP2R, also known as VPAC2) attenuates the SCN's electrical activity (82) and molecular rhythmicity (80). In mice, the loss of either VIP or VPAC2 causes behavioral disruptions such as the loss of a coherent circadian rhythm in continuous darkness, as well as changes in the ability to entrain to various light-dark cycles (81;83-85). The importance of VIP in seasonal adaptation of the SCN became obvious from *in vivo* electrophysiological SCN measurements in freely–moving VIP knockout mice. VIP knockout mice housed in a long, medium or short photoperiod showed no differences in SCN peak width after release in darkness and hence, lost the ability to encode photoperiodic information (86).

The GRP-expressing SCN cells are located in the ventral sub-region of the SCN and play a role in transducing light-related information to other regions in the SCN (87;88). The *in vitro* application of GRP to SCN slices mimics the phase-shifting response to light (89) and can increase synchronization among SCN neurons in SCN slices from VIP receptor–deficient mice (80;90). Thus, GRP may be involved in both synchronization and coupling between SCN neurons, and its role should be further explored.

The majority of neurons in the dorsal SCN contain the neuropeptide AVP. The expression of AVP (measured as mRNA and peptide production) is rhythmic (91). Moreover, the rhythm of AVP expression is driven by the intrinsic molecular feedback loop of the core clock machinery. The rhythm of AVP expression is also subject to changes in photoperiod. The SCN of rats housed in a long photoperiod had significantly longer AVP expression interval compared to the SCN of rats housed in a short photoperiod (76;92). Furthermore, SCN slices obtained from hamsters housed in a long photoperiod reached higher peak levels of AVP expression than slices obtained from hamsters housed in a short photoperiod (92). Finally, the *in vitro* application of AVP restored both rhythmicity and synchrony in SCN obtained from VIP-deficient mice (80), and these effects may have been mediated by changes in intracellular calcium levels (93).

The role of GABA in synchronization in the SCN was examined in desynchronized SCN (obtained from mice that were entrained to an extremely long photoperiod, i.e., 20 hours). In SCN slices were $GABA_A$ signaling was blocked desynchrony among the SCN cells was maintained, while untreated SCN slices regained synchrony (94). This indicates that $GABA_A$ signaling promotes resynchronization in long days (94). GABA is the most prevalent neurotransmitter in the SCN, and the SCN has both classic

GABAergic inhibition and GABAergic excitation (73;95-98). *In vitro* experiments revealed that GABAergic excitation is functionally important for synchronizing the dorsal and ventral SCN (73). Recently, the influence of photoperiod on the ratio between GABAergic excitation and GABAergic inhibition was examined. Remarkably, in the SCN of mice that were housed previously in long days, slightly more GABAergic neurons were excitatory (40%) than inhibitory (36%); in contrast, in the SCN of mice that were housed in short days, a higher percentage of GABAergic neurons were inhibitory (52%) than excitatory (28%) (99). The ratio between excitatory and inhibitory GABAergic neurons changed by blocking the Cl^- cotransporter NKCC1 using bumetanide, which suggests the involvement of NKCC1 in seasonal adaptation. Whether photoperiod also affect GABAergic inhibitory-excitatory balance in other brain regions should be investigated in future studies. The finding that photoperiod alters the balance in dopamine and somatostatin underscores the potential large influence of photoperiod on neurotransmitter signaling in the brain (100).

3.2 Phase shifts

In early studies, Elliott and Pittendrigh found that light pulses triggered a larger shift in running-wheel activity in hamsters entrained to short days as compared to long days (101). Later studies performed in mice and hamsters have supported these findings (102-104). Moreover, the effect of light on the SCN can be mimicked *in vitro* by applying a glutamate receptor agonist. *In vitro*, an NMDA pulse caused a significantly larger delay in the SCN of mice from a short photoperiod compared to the SCN of mice from a long photoperiod (102). In addition, after advancing the light-dark cycle, the high-amplitude SCN rhythms in mice entrained to a short photoperiod advanced significantly more than the low-amplitude SCN rhythms in mice entrained to a long photoperiod (105).

These findings are unexpected in view of the current knowledge that the amplitude of the SCN rhythm is larger in short days than in long days. The limit-cycle oscillator theory is often used to model the phase-shifting behavior of oscillators. A special property of the limit cycle is that the original rhythm (period) is restored when it is perturbed, and predicts that in general oscillators with high amplitude shift to a lesser degree than low-amplitude oscillators in response to an external perturbation of a given magnitude (106-108). Based on this prediction, one would have expected that low-amplitude SCN rhythms—as occur in long days—would have a larger phase shift than high-amplitude SCN rhythms.

One possible way to reconcile this discrepancy between theory and experimental observations is to take into consideration the differences in synchronization in long and short days (102). In a more synchronized SCN neuronal population (i.e., in short days), the majority of the individual cells will respond with a common phase-shifting response to an external perturbation, which will lead to a large shift at the

ensemble network level. If the neuronal population of the SCN is less synchronized, the individual cells will have divergent phase-shifting responses, resulting in a smaller shift in the SCN network. Simulation studies have recapitulated precisely the experimental observations and demonstrated that the synchrony among individual cells determines the phase-shifting response of the SCN network (102). Thus, although the limit-cycle oscillator theory may well predict the behavior of an *individual* oscillator, it cannot predict the behavior of a *network* of oscillators

4. SCN efferent pathways

4.1 Peripheral clocks

In vertebrates, most peripheral cell types contain endogenous circadian oscillators (109-113). For example, tissue explants obtained from heart, lung, and liver have revealed oscillations in the expression of their clock genes (109;110). The molecular mechanism underlying these peripheral clocks is the same mechanism that underlies the central clock. However, non-transcriptional oscillations could also play a role as shown by peroxiredoxins that undergo ~24-hour redox cycles in erythrocytes (114).

The SCN synchronizes the peripheral clocks via humoral and neuronal outputs (115-117), and when input from the SCN is lost, the peripheral clocks rapidly become desynchronized (109;110). Synchronization of peripheral clocks to the light-dark cycle is essential for maintaining seasonal physiology. Exposing rats to a different photo affects the expression of *Per1* in the liver and *Per2* in the lung and heart (118). Exposing Syrian hamsters (*Mesocricetus auratus*, also known as the golden hamster) to either short or long days significantly changes the circadian expression of *Per1* in the lung and heart, as well as the clock-controlled gene *Dbp* in the heart (119). The expression profiles of clock genes in the SCN and peripheral tissues are differentially altered by photoperiod (119;120). The rhythm of clock gene expression in the SCN adjusts to the transition from long to short days primarily by phase-advancing the expression decline; in contrast, the expression of Rev-erbα in the liver—as well as the rhythm of locomotor activity—adjusts to the same transition by advancing the onset of activity. Exposing an animal to short days for several months causes the animal to revert to a long photoperiod–like physiology. Although the rhythm of clock expression in peripheral tissues reflects long day–like behavior, the expression profile of clock genes in the SCN continues to reflect short days, resulting in a clear dissociation between the SCN and the peripheral clocks (119). This functional dissociation suggests that regulation of the peripheral clocks by the photoperiod is not driven solely by the SCN and/or melatonin and is likely under the control of other factors such as food intake and/or locomotor activity. Indeed, placing animals on a restricted feeding regimen can entrain the liver independent of the SCN and the light-dark cycle (121;122).

4.2 Neural and humoral pathways

The SCN signals to both intrahypothalamic and extrahypothalamic target areas via efferent neural pathways. Anatomical studies using anterograde and retrograde tracers in the rat brain identified the following six primary efferent projections from the SCN (123-127): (*i*) fibers that terminate in the zone between the SCN and the paraventricular nucleus (PVN), as well as the periventricular nucleus and anterior hypothalamic area (with some axons continuing dorsally and terminating in the mid-rostrocaudal parts of the PVN, and some axons terminating caudally in the dorsomedial nucleus); (*ii*) rostrally directed fibers that terminate in the ventral parts of the medial preoptic area and anteroventral periventricular nucleus; (*iii*) anterodorsally oriented fibers that traverse the medial preoptic nucleus (including adjacent regions) and terminate ventrally in the intermediate lateral septal nucleus; (*iv*) fibers that run caudal to the third group and terminate in the preoptic continuation of the bed nucleus of the stria terminalis, as well as in the parataenial nucleus and rostral part of the PVN; (*v*) laterally directed fibers that traverse the optic tract and terminate in the ventral lateral geniculate nucleus; and (*vi*) fibers that extend posteriorly through the anterior hypothalamic and retrochiasmatic areas and terminate between the arcuate nucleus and ventral parts of the ventromedial nucleus, as well as in adjacent parts of the lateral hypothalamic area.

In Siberian hamsters, obstruction of the dense efferent projections that originate in the dorsal tips of the SCN and traverse dorsomedially and dorsocaudally does not block short-day melatonin signals, suggesting that these projections are not the route that the SCN uses to transmit photoperiod information to other areas of the central nervous system (128). Other projections that might be responsible for transmitting the short-day signal include the rostrally and caudally traversing fibers along the ventral surface of the brain (128). An additional pathway may involve humoral output from the SCN. The SCN's exocrine function has been suggested based on experiments in which SCN transplants restored circadian activity rhythms in SCN-lesioned animals and animals that were arrhythmic due to other factors (129-132). Encapsulating the transplanted SCN, which prevents neural outgrowth, did not prevent the restoration of circadian rhythmicity, suggesting that humoral signals diffuse from the transplanted SCN (133). Moreover, the structural integrity of the SCN seems unnecessary for maintaining rhythmicity, as even micropunches are sufficient to restore activity patterns (134). Even though locomotor rhythm can be restored by SCN transplants, the transplants are unable to restore the reproductive response to day length (131), suggesting that neural efferent pathways are necessary for transmitting information regarding day length from the SCN.

4.3 Melatonin

4.3.1 Melatonin signaling

The SCN conveys photoperiodic information to the pineal gland via a polysynaptic efferent pathway as shown in figure 1. This pathway runs from the paraventricular nucleus of the hypothalamus, to the intermediolateral cell column of the thoracic spinal cord, to the superior cervical ganglion, and finally to the postganglionic adrenergic fibers that innervate the pineal gland. During night time, norepinephrine is released from these sympathetic nerve endings and stimulates postsynaptic ß1 and α1 adrenergic receptors on pinealocytes, triggering a large increase in intracellular cyclic adenosine monophosphate (cAMP) levels (135). This increase in cAMP activates serotonin *N*-acetyltransferase (also known as arylalkylamine *N*-acetyltransferase, or AANAT), a key regulatory enzyme in the melatonin biosynthesis pathway (136). Oscillations in the level of AANAT cause the rhythmic synthesis and secretion of melatonin (137). In both diurnal (i.e., day-active) as well as nocturnal (i.e., night-active) animals, melatonin levels are high during the dark phase. Upon exposure to light, AANAT levels decrease rapidly, in turn reducing melatonin levels. When entrained to a long photoperiod, the light-induced decrease in AANAT is advanced by an earlier dawn, whereas the dark-induced increase in AANAT is delayed by a later dusk. As a result, the duration of the nocturnal melatonin signal is compressed during the relatively short nights in the summer and expanded during the long winter nights (138). Thus, the temporal pattern of melatonin levels serves as a humoral signal conveying information regarding both the time of day and day length. Photoperiod-induced changes in melatonin secretion have wide-reaching effects on seasonal animal physiology and behavior, as the MT1 and MT2 melatonin receptors are distributed widely throughout the body, including the central nervous system, heart, endocrine system, and immune system. Because the SCN expresses high levels of melatonin receptors, melatonin serves as a feedback system on the clock (139-141). Melatonin has been shown to inhibit glucose uptake by the SCN as well as inhibit SCN single-unit activity during the late subjective day (142-144). Furthermore, melatonin can mediate a phase advance in the central clock at the late subjective day and late subjective night (145). However, the precise role of this hormone in the circadian system is still a subject of debate. Pinealectomy (removal of the pineal gland) has only limited effects on mammalian circadian rhythmicity (146); moreover, C57Bl/6 mice—a melatonin-deficient inbred mouse strain (147)—have normal locomotor activity rhythms (148;149). In contrast, giving daily injections of melatonin can restore—at least partially—circadian activity patterns in animals housed in continuous conditions (150-154), but only if the SCN is intact (150).

4.3.2 The seasons and melatonin

Melatonin is secreted at night, and the duration of melatonin production reflects the length of the dark period (i.e., the scotoperiod); thus, daily melatonin secretion is prolonged in the winter (when nights are longer)as compared to the summer (155). In the laboratory setting, seasonal responses can be evoked by the administration of exogenous melatonin (156). For example, long-lasting pulses of melatonin in pinealectomized Siberian hamsters elicit a short day–like response, thus mimicking winter even in hamsters that are housed in a long photoperiod (157). This result has been confirmed in hoofed animals with intact melatonin signaling. Prolonging the melatonin signal by giving oral or intramuscular melatonin to sheep and deer housed in long days triggers a short day–like reproductive response (158-162). Using radiolabeled melatonin as a tracer, several high-affinity binding sites for melatonin have been identified in the Siberian hamster brain; these sites include the paraventricular nucleus , the stria medullaris, the nucleus reuniens, the pars tuberalis (PT), and the SCN (139;163). Quantitative autoradiography has revealed that the density of melatonin receptors in the SCN of Syrian hamsters is lower in short photoperiods than in long photoperiods (164). The SCN of hedgehogs, however, has a similar number of melatonin-binding sites under both long and short photoperiods (165). Moreover, unlike in the Syrian hamster, changing the photoperiod does not affect melatonin receptor density in the SCN of Siberian hamsters, although the amplitude of the melatonin peak is higher under short photoperiods than under long photoperiods (164;166). Consistent with this finding, Siberian hamsters lack a circadian rhythm in melatonin binding in the SCN (164;166). In contrast, in the rat SCN, melatonin receptors fluctuate daily, with increased receptor density during the day and decreased density at night (167;168). In summary, photoperiod affects the level of melatonin-binding sites in the SCN of Syrian hamsters, but not in the SCN of hedgehogs or Siberian hamsters, while the effect of photoperiod in the rat has yet to be investigated.

The pars tuberalis (PT) in the pituitary gland expresses the highest density of melatonin receptors of any tissue and is the most conserved site of expression across mammalian species. The PT plays a role in mediating some of the seasonal photoperiod-induced responses in mammals, including the photoperiod-induced regulation of prolactin (169;170). In short photoperiods, the density of melatonin receptors is reduced in the PT of both Syrian hamsters and Siberian hamsters (164). This short photoperiod–induced decrease in melatonin-binding capacity is directly determined by the duration of the nocturnal melatonin signal (171-173). Thus, the short photoperiod–induced reduction in melatonin receptor density in the PT of both Syrian hamsters and Siberian hamsters—and in the SCN of Syrian hamsters—is likely due to a long-term, gradual melatonin-induced inhibition of melatonin receptor gene expression (164).

5. Seasonal physiology and the SCN

5.1 Hibernation and the SCN

Hibernation is an energy-conserving strategy used by many species to cope with annually recurring periods of cold and/or food shortage. For most mammalian hibernators, the hibernation season is marked by prolonged intermittent periods of hypometabolism that generally last 4-10 days, with brief recurrent returns to euthermic body temperature lasting approximately 6-24 hours (174). When hibernating (i.e., in a state of deep torpor), body temperature, cardiac function, and respiratory rate are reduced, muscle tone is more relaxed, and the animal adopts a heat-conserving "curled-up" body position.

The hibernation season for the golden-mantled ground squirrel (*Spermophilus lateralis*) lasts approximately six months (from autumn through winter) and is spent in an underground hibernaculum. This period of deep torpor interrupted by spontaneous periods of arousal eventually turns to a state of continuous arousal during spring and summer. The body weight of these squirrels also follows a seasonal rhythm: in the summer, they increase their food intake and fat mass, and in the winter these fat reserves are depleted gradually despite the animal's reduced energy expenditure. When these squirrels are transferred to a constant photoperiod and temperature condition, this annual rhythm in body weight change persists (175). Lesioning the SCN can disrupt the animal's natural annual rhythm in body mass, but only when the animal is exposed to temperatures similar to temperatures experienced during the winter (176). When SCN-lesioned squirrels are housed at 23˚C, only ~20% of the animals display disruptions in their annual body mass rhythm (177;178). Interestingly, experiments that measured the uptake of radiolabeled 2-deoxyglucose in ground squirrels revealed that during hibernation, the SCN is more metabolically active relative to other brain regions (179); indeed, of the 96 brain structures that were studied, the only brain regions with a metabolic rate higher than the SCN during deep hibernation were the paratrigeminal nucleus (which integrates visceral and somatic information) and the cochlear nucleus (180). The uptake of 2-deoxyglucose by the SCN increased when the animal entered hibernation and remained high throughout the entire hibernation period (181). As the animal returned to its euthermic state, the uptake of 2-deoxyglucose by the SCN declined abruptly. In the SCN, expression of the light-responsive transcription factor c-Fos increases during deep hibernation and peaks during arousal from hibernation (182;183). Complete—or even partial—ablation of the SCN disrupts the animal's normal hibernation pattern and the weight loss that usually occurs during hibernation (176;184;185). These results suggest that the SCN plays an important functional role in hibernation. However, the SCN's role in hibernation might not be circadian per se, as the clock genes *Per1*, *Per2*, and *Bmal1*, as well as the clock-controlled gene *AVP*, do not oscillate in the SCN of hibernating

European hamsters (*Cricetus cricetus*, also known as the common hamster or black-bellied hamster). Moreover, hibernating marmots and European hamsters do not have rhythmic plasma melatonin levels or rhythmic AANAT expression (183;186). However, because these experiments were performed at different ambient temperatures and in a variety of species and/or strains, the results are difficult to compare. Nevertheless, although its exact role remains unclear, the SCN seems to be required for establishing annual hibernation patterns and the related rhythmic fluctuations in body weight.

5.2 Seasonal breeding

Seasonal breeders adapt their reproductive cycle to the changing seasons in order to maximize reproductive success and offspring survival. Many species of birds, voles, mice, and hamsters breed in the spring and have a gestation period of only several weeks; thus, these species are classified as so-called "long-day breeders". On the other hand, goats, sheep, and deer breed in the autumn and have a gestation period of approximately six months; thus, these species are classified as "short-day breeders". The offspring of both long-day breeders and short-day breeders are born in the spring and/or summer, when food is abundant. These seasonal breeders primarily use the changing photoperiod as a breeding calendar, as this is the most reliable seasonal environmental cue to indicate the time of year. Seasonal reproduction is regulated by both luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which together regulate seasonal changes in gonad size. Exposing male hamsters to a short photoperiod causes gonadal regression, which is accompanied by the cessation of spermatogenesis and a decline in testosterone secretion. Photoperiod also affects the body weight of hamsters, although the effect differs among species. For example, in short days, Syrian hamsters gain weight, whereas Siberian hamsters lose weight (187-189). This opposite effect of photoperiod on weight change in two different hamster species represents different strategies used to cope with food shortage during the winter months (for review, see Ref. (190); Syrian hamsters accumulate fat prior to the winter, and that added fat is then utilized in the winter; in contrast, Siberian hamsters reduce their food intake prior to the winter in order to acclimate their energy requirements to accommodate the reduced food availability during the winter months.

5.2.1 Seasonal breeding and the SCN

Exposure to artificial light during the subjective night suppresses the circadian rhythms of activity and SCN output (191-194). Even ecologically relevant levels of seemingly dim light—similar to the levels of light pollution produced in urban areas (i.e., approximately 5 lux)—can affect the circadian system in rodents (for review, see Ref. (195). For example, these low levels of illumination during the night prevent

short-day responses in reproduction and body mass in Siberian hamsters (196). In addition, lesioning the SCN disrupts the photoperiod-induced responses to short day exposure in both Syrian hamsters and Siberian hamsters (187;197;198). Melatonin secretion is also disrupted by lesioning the SCN; however, the lack of photoperiod-induced response after SCN lesioning cannot be attributed exclusively to disrupted melatonin signaling. The infusion of exogenous melatonin to mimic short days does not elicit short day–like responses in gonad size or body weight in SCN-lesioned Siberian hamsters (199). In contrast, the nocturnal infusion of melatonin does cause short-day–like gonad atrophy in SCN-lesioned Syrian hamsters (200).

The mink is a short-day breeder that becomes sexually inactive when day length exceeds 10 hours and reaches peak sexual activity in February. Complete destruction of the SCN in minks abolishes the short day–evoked increase in testicular activity, comparable to the studies performed in long-day breeders (201). Testicular regression in summer is not triggered by day length and, in line with this, is not abolished by lesioning the SCN (201;202). Thus, in the mink, the SCN regulates testicular activity under short day conditions, but does not regulate testicular regression during long days.

5.2.2 Prolactin

Most species of seasonally breeding mammals have a seasonal pattern of prolactin secretion, with peak secretion occurring in the spring and/or summer and the nadir occurring in the autumn and/or winter (for review, see Ref. (203). Prolactin (also known as luteotropic hormone or luteotropin) is a polypeptide hormone that is both synthesized in and secreted from lactotrophs, specialized cells in the anterior pituitary gland. Prolactin is best known for its role in stimulating the production of milk in female mammals; however, more than 300 separate biological activities have been attributed to prolactin involving reproduction, immune responses, angiogenesis, osmoregulation, metabolism, and behavior. In addition to seasonal fluctuations, prolactin secretion follows a diurnal cycle and the ovulatory cycle. In rats, stimulating the uterine cervix (either through mating or by artificial stimulation) induces the twice-daily secretion of prolactin secretion (204-206). The diurnal surge in prolactin secretion begins in the afternoon (between 1 pm and 3 pm), peaks in the early evening (between 5 pm and 7 pm), and returns to basal levels by midnight. The nocturnal surge begins at 1 am, peaks in the early morning (between 3 am and 7 am), and returns to basal levels by 11 am. The ovulatory cycle of prolactin secretion in female rats includes a pre-ovulation surge in prolactin in the afternoon of the proestrus phase, and this surge coincides with an estradiol-induced surge in luteinizing hormone.

Surges in prolactin secretion occur in response to hypothalamic dopaminergic inhibition and in response to stimulation by hypothalamic-releasing neurohormones such as oxytocin and thyrotropin-releasing hormone. Several studies suggest that prolactin secretion is controlled by the coordinated activity of neuroendocrine dopamine and oxytocin, both of which are controlled by daily output from the SCN. Exposing rats to continuous light, which inhibits SCN output by ~50% (191), suppresses the abovementioned cervix-stimulated prolactin surge (207;208). Lesioning the SCN abolishes both the mating-induced prolactin surge and the cervical stimulation–induced prolactin surge (209-212). Furthermore, knocking down the clock genes *Per1*, *Per2*, and *CLOCK* within the SCN, thereby disrupting the circadian rhythmicity in behavior and physiology, blocks the proestrus and estradiol-induced prolactin surges (213). Inhibiting the neurotransmitter VIP in the SCN by injecting antisense oligonucleotides disrupts both the prolactin surge induced by cervical stimulation and the estradiol-induced surge in ovariectomized rats (214-216). In addition, administration of vasopressin to the medial preoptic area during a prolactin surge suppresses this surge (217). The SCN transmits circadian information via vasopressin-containing fibers to estrogen receptor–containing neurons in the medial preoptic area and/or via VIP-containing projections to dopaminergic and/or oxytocinergic neurons in the paraventricular nucleus and the periventricular nucleus, thereby controlling the timing of the circadian surge in prolactin (and likely the seasonal surge in prolactin as well). Thus, the SCN plays a role in regulating prolactin, thereby coordinating seasonal physiology.

6. The seasons and the human SCN

In human populations, a wide variety of seasonal rhythms have been reported (218;219), including seasonal fluctuations in birth rate (220), blood pressure (221), and sleep (222); for review, see Ref. (223). On the other hand, other studies failed to detect significant differences in human physiology as a result of changing photoperiod (for review, see Ref. (224). For example, measuring plasma melatonin levels in healthy urban subjects revealed no difference in the duration of elevated night melatonin levels between the summer and winter seasons (225). However, the melatonin rhythms were phase-delayed by approximately 1.5 hours in the winter compared to the summer. It is likely that the use of artificial light in modern society limits the ability of the circadian system to synchronize to naturally occurring changes in day length, thereby attenuating our photoperiod-induced seasonal physiology (226). Furthermore, one-fourth of the world's population practices daylight saving time (in which the clock is changed by one hour twice a year), which disrupts the seasonal adaptation of the human circadian system to the changing photoperiod when the clocks are advanced in the spring (i.e., at the start of summer time) (227).

A few studies reported seasonal changes in the human SCN. For example, a study of postmortem brain specimens found that serotonin (5-HT) levels have seasonal fluctuations, particularly in the hypothalamus (228). Specifically, the concentration of 5-HT peaked in October-November and reached a nadir in December-January. Consistent with this finding, similar seasonal patterns have been reported for L-tryptophan (a precursor of serotonin) in the blood (229), as well as 5-HT and its metabolites in the cerebrospinal fluid (230). Another study using single-photon emission computed tomography (SPECT) found a seasonal rhythm in the availability of serotonin transporter binding sites in the hypothalamus of healthy female subjects (231). Brain-derived neurotrophic factor (BDNF), which interacts closely with 5-HT in both neuronal functioning and plasticity, also fluctuates seasonally in humans (232). The reductions in 5-HT concentration and transporter binding sites during the winter coincide with an increased prevalence of depression during this same period. 5-HT is strongly involved in the regulation of circadian rhythms by the SCN. For example, the SCN receives robust serotonergic projections from the midbrain raphe nuclei, and changes in 5-HT affect circadian behavior and neuroendocrine rhythms in rodents. The seasonal fluctuations in 5-HT levels may reflect changes in serotonergic projections to the SCN, which induce seasonal rhythmicity in both physiology and behavior.

Seasonal changes in SCN morphology have been identified using postmortem brain tissue obtained from young subjects (males and females 6-47 years of age). In early autumn (i.e., from August through October), the number of vasopressin-containing and VIP-containing neurons was significantly higher than in late spring/early summer (i.e., from April through June) (233-235). The SCN-PVN axis is essential for relaying photoperiodic information to other parts of the body. The volume of the PVN also fluctuates seasonally, peaking in the spring (236). In contrast, other brain regions that are not involved in the temporal organization of biological processes do not fluctuate seasonally, suggesting that seasonal changes in morphology are limited to brain regions that play a role in the transmission of photoperiod-related information. As one ages, the seasonal fluctuation in SCN morphology is progressively reduced (237); for example, the annual fluctuation in vasopressin-containing neurons in the SCN diminishes in elderly people (i.e., in individuals 50-95 years of age). This observation is consistent with other perturbations in the circadian system observed in aging humans and other mammals, including fragmented activity patterns and a decline in neuronal activity in the SCN (238-240).

7. Conclusions

It is becoming increasing clear that in addition to orchestrating circadian rhythmicity, the SCN also plays an important role in seasonal physiology. The unique light

response characteristics of SCN neurons enables these cells to measure the length of the day (i.e., the photoperiod). This information is then encoded by the SCN by adjusting the level of phase synchrony among the individual SCN neurons. At the molecular level, the regional organization is clearly heterogeneous, whereas at the level of electrical activity, changes in phase synchrony are integrated and measurable throughout the SCN. The ensemble electrical activity of the entire SCN reflects the length of the day. Thus, the ability of the SCN to encode photoperiod information is dependent on the integrity of the neuronal network. Seasonal changes in certain physiological functions are regulated by structures that lie downstream of the SCN. The SCN projects to intrahypothalamic and extrahypothalamic target areas, and it projects indirectly to numerous endocrine systems in our body. Despite the widespread use of artificial light, many studies report the presence of seasonal rhythms in humans, although other studies found no evidence of such rhythms. In the human SCN, seasonal rhythms are well documented. Whether seasonal rhythms are important to humans, and whether they contribute to our health and/or fertility, are open questions that must be addressed.

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9

GENERAL DISCUSSION

General discussion

A fundamental property of the circadian system is that it synchronizes to the environmental day-night cycle. The master clock of the body, which resides in the suprachiasmatic nuclei (SCN) of the hypothalamus, is a self-sustained oscillator that regulates circadian rhythms in the brain and in peripheral organs. The SCN are susceptible to resetting time cues in order to remain in synchrony with the daily external cycle. Light information detected at the level of the retina provides major input to the SCN, however also internal cues such as the animal's own behavioral activity can affect the SCN. Besides its role as a daily clock, the SCN also function as a seasonal clock by adjusting its oscillation pattern to the duration of light per day (i.e. photoperiod). Both the ability of the SCN to adapt to a new light-dark cycles and to adapt to a new photoperiod requires plasticity of the SCN neuronal network.

Robustness of the SCN's output and synchrony among SCN neurons

Synchronization of the SCN electrical activity rhythm to the external light-dark cycle is primarily driven by light. The effect of light on the SCN rhythm is dependent on the SCN's phase; a light pulse at the beginning of the light causes a phase delay of the rhythm, while a light pulse at the late night results in phase advance (1). After a phase advance or delay, the rhythm of the SCN resumes with the same velocity as before the perturbation. Because this behavior is typical for a limit cycle oscillator, limit cycles have been extensively utilized to predict the behavior of the SCN (2-6). A prerequisite of a limit cycle oscillator is that its phase-shifting capacity depends on the amplitude of the oscillation. The theory predicts that high-amplitude oscillations have less phase-shifting capacity than low-amplitude oscillations. The amplitude of the SCN discharge can be manipulated by adaptation to short or long photoperiods. In a short photoperiod, the peak of the SCN's electrical activity pattern is more compressed (i.e. more narrower) and the amplitude of the SCN rhythm is higher. On the other hand, in a long photoperiod, the peak of the SCN's electrical activity pattern is more decompressed (i.e. more broad) and the amplitude of the SCN is lower (7-10). The photoperiodic-induced alterations are preserved in the isolated SCN allowing ex vivo analysis of the involved mechanisms (11,12).

Based on limit cycles, it was expected that the SCN from a long photoperiod will show a large phase shift and that SCN from a short photoperiod (resulting a high amplitude oscillation) will show a small phase shift after a perturbation of the same magnitude. In chapter 2 we explored this hypothesis by investigating the phase-advancing capacity of the SCN with a high or low amplitude oscillation by subjecting mice from short and long photoperiods to a 4 hour phase-advancing shift of the light-dark cycle. *In vitro* electrical activity recordings revealed higher amplitude rhythms in SCN from short days as compared to long days. Following a 4 hour phase-advance

of the light-dark regime the SCN from short day mice displayed a significantly larger phase advance than SCN from long day mice. This finding is in line with previous studies on the phase-delaying capacity of high- and low-amplitude SCN oscillations (13), and contradicts the predictions of the limit cycle model.

The discrepancy between the empirical data and the limit cycle model could be explained by properties of the SCN that arise at the level of the SCN network organization. In a highly phase-synchronized neuronal population (as in short days), the perturbation will presumably cause consistent phase shifting responses in the population of neurons, and the sum of the individual phase-shifting responses will result in a large magnitude shift at the level on the SCN ensemble. On the other hand, in a phase-desynchronized neuronal population (as in long days) the perturbation will cause inconsistent phase shifting responses throughout the population, and the sum of the individual phase shifts will result in a small magnitude response at the SCN tissue level. In order to test this model experimentally it is of great importance to construct phase response curves for individual SCN neurons, however with the current technology this is not yet possible. Supporting evidence is already provided by *in vitro* studies that showed differential phase shifting responses of SCN neurons after a pulse with N-methyl-D-aspartate (14).

Studies performed with SCN explants revealed that SCN with normal physiological coupling (i.e. normal phase-synchrony and normal amplitude) among the individual SCN neurons were not able to synchronize to temperature cycles of 22 hour, while SCN with weakened coupling (i.e. less phase-synchrony and lower amplitude) by TTX or MDL were able to entrain (5). These data fit with limit cycle oscillator characteristics, and seem in at first glance contradictory to our results (chapter 2,(15)). The most important differences between a stimulation with temperature or light is the proportion of neurons that is affected by the stimulus. The SCN is a heterogeneous tissue, and only about 30% of the SCN neurons exhibit acute light-responsiveness (16-19). When SCN explants receive a temperature pulse, the total SCN neuronal population is directly affected. In chapter 3 of this thesis we investigated the influence of temperature and light on SCN populations with different intrinsic coupling among the individual SCN neurons (thereby changing the amplitude of the SCN ensemble). Mathematical modelling (Poincaré and Goodwin model) enabled us to stepwise change the proportion of neurons that are susceptible to the stimulus, and to very accurately check the effect of coupling strength on the phase shifting capacity of the SCN, which is impossible with empirical techniques. The results show that when 100% of the SCN neurons are responsive to the stimulus, a negative linear relationship exists between coupling strength (amplitude strength) and the phase-shifting capacity. On the other hand, when a fraction of the population is responsive to the stimulus, a positive linear relationship is present between coupling strength (amplitude strength) and the phase-shifting capacity until a certain threshold point

for the coupling strength (20). This study highlights the importance of the coupling strength within the SCN for the phase shifting capacity of the SCN ensemble.

With aging, humans experience changes in the circadian timing system, which are manifested as a reduction in the behavioral activity pattern and in disruptions of sleep-wake rhythms (21-24). Given that the SCN output is strongly associated with behavior and physiology, these age-related disturbances in circadian rhythmicity could be caused by age-related disorders at the level of the SCN. Supporting evidence for this hypothesis comes from animals studies. *In vivo* and *in vitro* measurements of the SCN electrical activity output revealed a reduced circadian amplitude in older mice compared to young mice (25-28). Interestingly, reduced SCN rhythm amplitude is not the result of a loss of SCN neurons, but by an altered pattern of electrophysiological activity of the individual SCN neurons (25,29-33). Electrical activity measurements in SCN from aged animals revealed that activity patterns of the individual neurons are less synchronized, and even have anti-phasic activity (25). Results from computational studies indicated that decreased coupling in the aged SCN can lead to reduced synchrony among SCN neurons (26). Indeed, in elderly people a decrease of several neurotransmitters (i.e. coupling agents) within the SCN is reported (34,35). Our studies in mice (chapter 3 and 4) revealed that SCN with low amplitude rhythmicity (low coupling) have a smaller phase shifting capacity than SCN with high amplitude rhythmicity (high coupling) (15,20). These findings suggest that older individuals will have more difficulties with adaptions to new light-dark cycles (jetlag), photoperiodic changes (seasons) and shift-work. Light therapy and physical exercise can be employed as non-invasive therapies to boost the output of the SCN in elderly.

Circadian photo-entrainment

Photic-entrainment is accomplished by light signals that are transmitted through the RHT to the SCN. Light signals trigger the release of glutamate and PACAP, which activates NMDA and/or AMPA receptors and PACAP receptors respectively, and leads to a tonic increase in electrical activity levels in the SCN (36-41). Moreover, the magnitude of light-induced increase in SCN neuronal activity is highly correlated with the light-induced phase shift of the behavioral activity rhythm (42).

In the retina, light is sensed by rod- and cone photoreceptors, and by photosensitive retinal ganglion cells (pRGCs) that contain the photopigment melanopsin. In addition to their intrinsic sensitivity to light, pRGCs receive indirect light input from the rod- and cones. Mice lacking melanopsin are able to entrain to light-dark cycles, demonstrating that melanopsin is not required for photo-entrainment (43,44). However, mice that retained only melanopsin as a functional photoreceptor and lacked rods and cones, can also still entrain to light-dark cycles (45). In chapter 4 we investigated the role of rod-and cone photoreceptors in the light

response of the SCN. Mice were implanted with a micro-electrode aimed at the SCN, and after recovery these animals were exposed to light pulses of different intensities of UV (365nm), blue (467nm) and green (505nm) light. In wild-type mice, UV, blue or green light exposure induced a transient on-response, a sustained response and a transient off-response in SCN firing rate. The SCN response characteristics to the different wavelengths of light were similar to the response characteristics previously described for white light pulses (39).

To test whether melanopsin has a crucial role in light responses in the SCN, SCN recordings were performed in melanopsin knockout mice while applying light pulses of UV, blue and green light. Mice that lacked melanopsin showed light responses that were indistinguishable from those displayed in wild-type mice, with regard to the magnitude of the light response and the response latency. This result, indicating that melanopsin is not critical for light-responses in the SCN, was surprising as the ability to mediate tonic SCN light responses was assumed to arise from melanopsin-based phototransduction (36,37,46,47). The results presented in chapter 4 indicate irradiance encoding in the SCN in the absence of melanopsin, which suggest a role for rod- and/or cone photoreceptors in circadian photo-entrainment(40).

The relative contribution of rod-and cone photoreceptors to the light responsiveness of the SCN was examined in the studies presented in chapter 5. Mutant mice having only functional cone photoreceptors ("cone-only" mice) were subjected to similar *in vivo* electrophysiological experiments as described in chapter 4. We observed activations of SCN neuronal activity in response to 1 minute light pulses of UV or green light in the "cone-only" mice. To check whether cone-mediated light responses of SCN neuronal activity is sufficient to drive circadian entrainment, we subjected the cone-only mice to light-dark cycles of only UV or green light. Some, but not all, cone-only mice were able to entrain to light-dark cycles of UV and green light. What brings about the inter-individual differences in entrainment of the cone-only mice remains to be elucidated, and may arise from developmental variability in retinal organization in this mutant mouse strain. However, most of the "cone-only" mice displayed stable entrainment by the use of solely cones, which indicates the involvement of the cone photoreceptor in circadian photo-entrainment. Our data are supported by behavioral studies performed with mice that lacked mid-wavelength (MW) cones. The MW-coneless mice showed significantly reduced behavioral phase shifting response in response to 5 and 15 minute light pulses of 530 nm and to 1 and 5 minute light pulses of 480 nm (48,49). Also, the induction of *Per1* and *Per2* in response to light pulses of 480 and 530 nm was attenuated in mice that lacked MW cones (48), suggesting that cones have a role in light-mediated behavioral phase-resetting. Our results show that melanopsin as well as cone photoreceptors can drive acute light responses in the SCN. The relative contribution of the different photoreceptors to the light response in the SCN remains to be determined, and is most likely dependent

on the light intensity and spectral composition. In a bright environment, melanopsin will contribute to a large extent to SCN light responses, while in dim light conditions SCN light responsiveness will be largely dependent on classical photoreceptors. This information is important for humans that expose themselves for example to dim light at night.

The human retina is equipped additionally to rod photoreceptors and ipRGCs with a three-cone photopic system of cones that are maximally sensitive to light in the blue (S cone), green (M cone) or red (L cone) portion of the visual spectrum. Light activation of SCN neurons can acutely suppress the synthesis of the hormone melatonin, which is released during the (subjective) biological night and is often referred to as the "sleep-hormone" in humans. Studies performed in humans revealed that circadian phase resetting, melatonin suppression, and alertness are most sensitive to short-wavelength light in the blue portion of the visual spectrum (~480nm) (46,50), which maximally activates melanopsin (51-55). Interestingly, Gooley and coworkers showed that exposure to green light (555 nm) for 6.5-hours can also suppress melatonin, and that green light can phase shift circadian rhythms in humans (56). Examination of the melatonin suppression response revealed that during the first quarter of light exposure, green and blue light were equally effective to suppress melatonin, whereas from the second quarter on green light was relatively less effective compared to the blue light (56). The response kinetics indicated that melatonin suppression caused by the green light was mediated by a photopigment that has a weak sensitivity to blue light, which indicates that is unlikely that the response to green light arises at the level of the melanopsin photoreceptor (56) and points to a cone-mediated light response. Moreover, irradiance phase-resetting response curves showed that low intensities of 555 nm light resulted in much greater phase-shifts of the melatonin rhythm than 460 nm light, and as cones are more sensitive to light than melanopsin, this results suggests that the phase-resetting of the melatonin rhythm by green light was mediated by M cones (56). The findings that M cones can mediate melatonin responses in humans are in line with our results in mice, which show that M cones can drive SCN light responses and photo-entrainment (chapter 5).

Circadian rhythm sleep disorder (57-59), seasonal affective disorder (SAD) (60,61), dementia (62,63) and Huntington's Disease (64) are a few of the large range of human disorder were light therapy is currently used in order to reduce the symptoms. Also, light can be used as an alerting stimulus to counteract sleepiness of people that work during the night shift (53,54,65) or to advance circadian rhythms in humans with a late chronotype ("evening-types") (66,67). Our results obtained in mice revealed that cones are able to mediate light responses in the SCN (chapter 5 and 6), and provide insights in the mechanism of cone-mediated circadian phase resetting in humans. The results indicate that light therapy for patients suffering from circadian rhythm associated disorders can be optimized by manipulating the duration, pattern and

spectrum of light to maximally stimulate melanopsin and classical photoreceptors. Moreover, the adjustments of lighting in schools and at the workplace can improve alertness of students and employers to most effectively stimulate photoreceptors.

Relationship between SCN output and behavioral activity

In nocturnal mice, SCN electrical activity waveform correlates to the pattern of behavioral activity, such that the occurrence of behavioral transitions is predictable from the half maximum levels of the SCN amplitude (68,69). SCN electrical activity can be considered as an important factor for overt behavioral rhythmicity. On the other hand, different types of behavioral activity, including wheel-running activity (70), social interactions (71) and sleep deprivation (72) can induce phase shifts of the SCN rhythm (73). Importantly, studies performed in mice revealed that behavioral activity can boost the amplitude of the SCN out, by suppressing the trough of the SCN waveform (74). Thus, a bidirectional interaction exists between SCN neuronal activity and behavioral activity.

To assess the importance of behavioral activity feedback in day-active animals, such as humans, we performed *in vivo* measurements of SCN electrical activity in the day-active grass rat while measuring their behavioral activity (chapter 6). The recordings revealed circadian rhythms in SCN electrical activity, with higher levels during day time and lower levels during the night. Analysis at a smaller time scale revealed that bouts of behavioral activity coincide with episodes of elevated levels of SCN electrical activity. To investigate the direction of the relationship between enhancements SCN electrical and behavioral activity, we performed *ex vivo* measurements of SCN electrical activity, to characterize the endogenous firing pattern of the SCN without feedback of other brain areas. The circadian firing pattern measured *ex vivo* followed a smooth unimodal circadian pattern, and was devoid of acute enhancements of SCN discharge rate, indicating that the variability observed *in vivo* arises from communication between the SCN and extra-SCN areas. Detrended fluctuation analysis showed that fractal patterns of SCN electrical activity measured *in vitro* were disrupted, suggesting that feedback to the SCN is required for fractal regulation of SCN electrical activity.

The results from chapter 6 show for the first time that in a day-active animal behavioral activity acutely increases the electrical activity of the SCN. Moreover, the results reveal that behavioral activity has opposite acute effects on the SCN discharge rate of nocturnal and diurnal species, leading to an enhancement of the amplitude of the SCN electrical activity rhythm in both species (provided that the animals are active during the biological correct time of day). This intriguing finding highlights the possibility that neurotransmitters involved with behavioral feedback to the SCN could have differential effects on SCN neurons of nocturnal and diurnal species. Support for this hypothesis are the effects of serotonergic activation on

light resetting of the SCN in the day-active *A. ansorgei* (75). The authors showed that injections of 5-HT receptor agonists induce small phase advances during the subjective night (75), while in nocturnal species 5-HT receptor agonists cause large phase advances only during the subjective midday (76-78). Also GABA is able to induce different responses in the SCN of nocturnal and diurnal rodents. Activating GABAA receptors in mice during the subjective day induces phase advances in nocturnal rodents, whereas the SCN of diurnal grass rats display a phase delay (79). The investigation of the direct effect of 5-HT or GABA on the firing rate of the SCN of diurnal rodent, for example by the administration of these substances to the isolated SCN *in vitro*, could elucidate the differential effect of behavioral activity on the SCN activity between nocturnal and diurnal species.

To explore regulation of temporal activity patterns by the SCN's electrical activity in a day-active rodent, we recorded SCN electrical activity in grass rats with full diurnal and crepuscular behavioral phenotypes *in vivo* and in vitro (chapter7). Additionally, we assessed SCN electrical activity patterns of SCN isolated from grass rats with photoperiod-induced changed behavioral patterns. Our *in vivo* recordings showed an unimodal electrical activity pattern in the SCN of a full diurnal grass rat and bimodal SCN discharge rate waveforms in crepuscular grass rats. Interestingly, *in vitro* SCN discharge rates followed unimodal rhythms in both groups. Moreover, photoperiodic-induced compressions or decompression of the behavioral activity phase did not correlate with changes in SCN electrical activity patterns found *in vitro*. The results indicate that in day-active grass rats behavioral phenotypes (i.e. unimodal or crepuscular) nor photoperiodic-induced changes correspond with SCN electrical activity *in vitro*. As the SCN's electrical activity pattern *in vivo* reflected the behavioral activity patterns in all cases, we expect that an interplay between the SCN and extra-SCN areas is of large importance in diurnal species.

Studies performed in humans revealed that physical exercise can accelerate the synchronization of sleep-wake rhythms to the external light-dark cycle (80-83) and it can improve health and well-being (84-89). Studies on the reciprocal relationship between SCN neuronal activity and behavioral activity have been largely conducted in night-active animals such as mice, rats and hamsters. These studies have provided insights in the potential of voluntary exercise as a non-invasive therapy for circadian rhythm associated disorders. For example, voluntary exercise in aged mice increases the amplitude of the SCN's output *in vitro* and improves resynchronization of the SCN and peripheral tissues to the light-dark cycle (90). Also, voluntary exercise improves circadian behavioral rhythmicity in a mouse model of Huntington's Disease (91). We demonstrated a beneficial influence of behavioral activity on the amplitude of the SCN rhythm in a day-active rodent. Our results support the notion that exercise can be used as a non-invasive therapeutic intervention for circadian rhythm disorders in humans.

Concluding remarks

Phase-synchronization among the single SCN neurons is plastic and influenced by environmental factors, such as the external photoperiod. In a short photoperiod, the SCN's electrical activity rhythm is robust due to highly synchronized single-cell activity patterns, while in a long photoperiod the SCN's electrical activity output is more dampened by reduced synchrony among individual cells. We showed that the phase-resetting capacity is larger in a highly synchronized SCN population (i.e. after short days) than in a desynchronized SCN (i.e. after long days). By the use of mathematics, we revealed that the phase-resetting behavior of the SCN depends on the proportion of neurons that receive the stimulus. The studies presented indicate that the function of the SCN clock depends not only on its intrinsic molecular machinery, but also on its organization at the network level.

Light is detected by the classical photoreceptors and melanopsin containing photosensitive retinal ganglion cells (pRGCs). In addition to their intrinsic photo perception, the pRGCs receive indirect light information from rods and cones, and project the integrated signal to the SCN. We found that all classes of photoreceptors can mediate light responses in the SCN, though the relative contribution depends on the wavelength (i.e.) and intensity of light. Thus, SCN photo-entrainment is not only determined at the level of the SCN, but is also dependent on the integration of spectral cues at the level of the retina.

Besides external factors such as light, the SCN is also influenced by internal feedback from extra-SCN areas. Our results demonstrate a beneficial influence of exercise on the amplitude of the SCN rhythm in a diurnal rodent, suggesting an enhanced robustness of the circadian system. In the day-active *A. ansorgei*, SCN rhythms coincide with behavioral activity patterns only in SCN with intact peripheral feedback, which highlights the importance of behavioral feedback even more.

Taken together, the studies described in this thesis provide evidence that the SCN clock is part of a larger brain network that includes the retina and areas involved in behavioral activity and sleep. At the integrated network level, the systems property emerge in an unpredictable way, underscoring the relevance of complex system level approaches in brain research.

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10

SUMMARY

NEDERLANDSE SAMENVATTING

LIST OF PUBLICATIONS

CURRICULUM VITAE

Summary

In mammals, the suprachiasmatic nucleus (SCN) contains a central clock that synchronizes daily (i.e., 24-hour) rhythms in physiology and behavior. Accumulating evidence suggests that the functional integrity of the SCN contributes to health, well-being, cognitive performance, and alertness. In line with this, deterioration of the 24-hour rhythm of the SCN is a risk factor for neurodegenerative disease, cancer, depression, and sleep disorders. In **chapter 1** we provide an overview of the literature on the biological clock of the suprachiasmatic nuclei (SCN), and we provide evidence for a role of synchronization of SCN neurons in health. The review summarizes the current state of knowledge on coupling mechanisms involved in (*i*) neuronal synchronization, (*ii*) the effects of light on the SCN and (*iii*) extra-SCN feedback systems. Additionally, we discuss how these mechanisms are affected in circadian disorders that occur with aging and disease.

The SCN's electrical activity ensemble follows a sinusoidal-like pattern that peaks during the day and is low during the night. The amplitude of the SCN's electrical activity rhythm is influenced by the external photoperiod, such that the amplitude increases in short days and decreases in long days. In **chapter 2** of this thesis, the effect of the photoperiod on the ability of the SCN to synchronize to the external light-dark cycle by shifts of the rhythm (i.e.phase shifting capacity) was investigated. Mice were subjected to a 4 hour phase advancing shift of the light-dark cycle after exposing them to a long or short photoperiod, and subsequently *in vitro* electrophysiological measurements were performed on SCN. Our *in vitro* electrical activity recordings revealed higher amplitude rhythms in SCN from short days as compared to long days. Following a 4 hour phase-advance of the light-dark regime the SCN from short day mice displayed a significantly larger phase advance than SCN from long day mice. We concluded that the amplitude of the circadian oscillation of the SCN determines its phase-advancing capacity.

The electrical activity displayed on the SCN tissue level consists of electrical activity patterns of single SCN neuronen. Single cells are active for a relatively short period (4 – 8 h). The majority of these neurons are active during the day and few are active during the night. Evidence obtained from electrophysiological and molecular studies indicated that plasticity of the phase distribution among single cell activity patterns underlies seasonal adjustments in SCN electrical activity rhythm amplitude and waveform. In **chapter 3** we determined, by the use of mathematical approaches, whether SCN phase shifting characteristics are dependent on synchrony among the SCN neurons and how the proportion of stimuli-receiving neurons determines the magnitude of the SCN shift. We found that when 100% of the SCN neurons are responsive to the stimulus, a negative linear relationship exists between the synchrony among the SCN neurons (amplitude) and the phase-shifting capacity.

On the other hand, when a fraction of the population is responsive to the stimulus, a positive linear relationship is present between the amplitude and the phase-shifting capacity until a certain threshold point. This mathematical study highlights the importance of interneuronal phase synchrony and coupling strength for the phase shifting capacity of the SCN ensemble. Also, the results indicate that the phase shifting properties of the SCN are strongly influenced by the heterogeneity of the neuronal network.

The major external stimulus affecting the SCN pacemaker is light. Light is detected in the outer retina by rod-and cone photoreceptors and in the inner retina by photosensitive retinal ganglion cells (pRGCs) containing melanopsin. The retina of mice contains the short wavelength cone that has a maximum sensitivity in the UV light range, the rod photoreceptor and the mid wavelength cone that both have a maximally sensitive to green light, and melanopsin that is most sensitive to blue light. All light information detected by cones, rods and melanopsin are integrated at the level of the pRGCs, and transmitted to the SCN via the retinohypothalamic tract (RHT). In the absence of rod- and cone photoreceptors pRGCs can still respond to light and this light response is sufficient to entrain the SCN. In **chapter 4** we tested whether melanopsin has a crucial role in generating light responses in the SCN, by performing *in vivo* SCN recordings in melanopsin knockout mice while applying light pulses of UV, blue and green light. Mice that lacked melanopsin showed light responses that were indistinguishable from those displayed in wild-type mice, with regard to the magnitude of the light response and the response latency. We concluded that melanopsin is not essential for the generation of light-responses in the SCN and that rod- and/or cone photoreceptors contribute to light-synchronization of the SCN.

Next, in **chapter 5**, we investigated to what extent cone photoreceptors contribute to photic entrainment of the SCN by performing electrophysiological measurements in the SCN of mice that have only cones as functional photoreceptors. We observed activations of SCN neuronal activity in response to 1 minute and 15 minute light pulses of UV or green light in the "cone-only" mice. To check whether cone-mediated light responses of SCN neuronal activity is sufficient to drive circadian entrainment, we subjected the cone-only mice to light-dark cycles of only UV or green light. Most of the "cone-only" mice displayed stable entrainment by the use of solely cones, which indicates the involvement of the cone photoreceptor in circadian photo-entrainment. Our results show that melanopsin as well as cone photoreceptors can drive acute light responses in the SCN.

SCN's rhythmicity is not solely influenced by photic input, but also by non-photic factors such as the animal's own behavioral activity level. In **chapter 6** the effect of behavioral activity on the amplitude of the circadian rhythm in SCN electrical discharge rate in a day-active animal was assessed by performing long term *in vivo* electrophysiological measurements in the SCN of the freely-moving day-active

grass rat (*Arvicanthis ansorgei*). The recordings revealed circadian rhythms in SCN electrical activity, with higher levels during day time and lower levels during the night. Analysis at a smaller time scale revealed that bouts of behavioral activity coincide with episodes of elevated levels of SCN electrical activity. To investigate the direction of the relationship between enhanced SCN electrical and behavioral activity, we performed *ex vivo* measurements of SCN electrical activity, to characterize the endogenous firing pattern of the SCN without feedback of other brain areas. The circadian firing pattern measured *ex vivo* followed a smooth unimodal circadian pattern which was devoid of acute enhancements of SCN discharge rate, indicating that the variability observed *in vivo* arises from communication between the SCN and extra-SCN areas. Detrended fluctuation analysis showed that fractal patterns of SCN electrical activity measured *in vitro* were disrupted, suggesting that feedback to the SCN is required for fractal regulation of SCN electrical activity. From this study we concluded that behavioral activity enhances SCN electrical activity in the day-active grass rat.

In nocturnal mice, the waveform of the SCN electrical activity correlates to the temporal behavioral activity pattern. To explore regulation of temporal activity patterns by the SCN's electrical activity in a day-active rodent, we recorded SCN electrical activity in grass rats with full diurnal and crepuscular behavioral phenotypes *in vivo* and in vitro (**chapter7**). Additionally, we assessed SCN electrical activity patterns of SCN isolated from grass rats with photoperiod-induced changed behavioral patterns. Our *in vivo* recordings showed an unimodal electrical activity pattern in the SCN of a full diurnal grass rat and bimodal SCN discharge rate waveforms in crepuscular grass rats. Interestingly, *in vitro* SCN discharge rates followed unimodal rhythms in both groups. Moreover, photoperiodic-induced compressions or decompression of the behavioral activity phase did not correlate with changes in SCN electrical activity patterns found *in vitro*. The results indicate that in day-active grass rats behavioral phenotypes (i.e. unimodal or crepuscular) nor photoperiodic-induced changes correspond with SCN electrical activity *in vitro*. As the SCN's electrical activity pattern *in vivo* reflected the behavioral activity patterns in all cases, we expect that an interplay between the SCN and extra-SCN areas is of large importance in diurnal species.

Besides their role as a daily clock, the SCN function as a seasonal clock and convey day length information to the pineal gland and other parts of the central nervous system. The SCN encode photoperiod information by changing the phase distribution of activity patterns of single SCN cells. Underlying the seasonal adaptations are changes in neurotransmitter profiles and clock gene expression patterns. The literature study presented in **chapter 8** discusses the unique light response properties of SCN neurons that are essential to measure the photoperiod. We discussed that the SCN's heterogeneous organization is required for storing

photoperiod information, and that efferent pathways are involved in orchestrating seasonal responses in physiology.

Finally, we evaluated the results of this thesis in **chapter 9** and discussed the fundamental findings in light of potential implications for human well-being and health. Collectively, the studies described in this thesis provide evidence that the SCN clock is part of a larger brain network that includes the retina and areas involved in behavioral activity and sleep. At the integrated network level, the systems property emerge in an unpredictable way, underscoring the relevance of complex system level approaches in brain research.

Nederlandse samenvatting

In alle zoogdieren, inclusief de mens, worden circadiane (circa = ongeveer, dia = dag) ritmen in fysiologie en gedrag gedirigeerd door de *circadiane klok*. De circadiane pacemaker bevindt zich in de suprachiasmatische kernen (SCN) van de hersenen. Onderzoek in het afgelopen decennium heeft aangetoond dat het correct functioneren van de SCN een positieve invloed heeft op de gezondheid, welzijn en alertheid van individuen. Omgekeerd heeft een storing van het circadiane systeem gevolgen voor de ontwikkeling van slaap-aandoeningen, depressie, neurodegeneratie en kanker. In dit proefschrift werd bestudeerd hoe de functionaliteit van de SCN wordt beinvloed door (*i*) SCN neuronale synchronisatie, (*ii*) de werking van licht op de SCN en (*iii*) terugkoppeling vanuit de periferie naar de SCN. **Hoofdstuk 1** van dit proefstuk geeft een overzicht van onderzoeken die een rol voor neuronale synchronisatie van SCN neuronen in gezondheid hebben aangeduid. Eveneens beschrijven we in dit hoofdstuk op welke manier neuronale koppelingsmechanismen aangedaan zijn wanneer er sprake is van circadiane aandoeningen die geassocieerd zijn met veroudering en ziektes.

De SCN hersenkernen zijn opgebouwd uit ongeveer 20.000 individuele neuronen. Deze neuronen produceren gezamenlijk een sinusgolfachtig signaal in elektrische activiteit, die zijn piek bereikt gedurende de dag en minimaal is gedurende de nacht. Uit eerder onderzoek is gebleken dat de amplitude van het ritme van de SCN wordt beïnvloed door de externe daglengte (fotoperiode). Korte fotoperiodes (winterdagen) verhogen de amplitude van het elektrische activiteit ritme van de SCN, terwijl lange fotoperiodes (zomerdagen) de amplitude verlagen. In **Hoofdstuk 2** hebben we onderzocht op welke manier het vermogen van de SCN om te herstellen van een *jetlag* wordt beinvloed door de voorafgaande fotoperiode (korte en lange dagen). Hiervoor hebben we muizen laten wennen aan korte of lange dagen waarna ze zijn blootgesteld aan een 4-uursvooruitschuivinging van het licht-donker regime, wat overeenkomt met een *jetlag* vooroorzaakt na een vlucht van Suriname naar Nederland. Vervolgens zijn er *in vitro* elektrofysiologische metingen gedaan op de uitgeprepareerde SCN. De *in vitro* metingen onthulde hogere amplitude ritmes in SCN elektrische activiteit in muizen uit korte fotoperiodes vergeleken met van muizen uit lange fotoperiodes. Na een 4-uursvooruitschuiving van het licht-donker regime lieten de SCN van muizen uit korte fotoperiodes een grotere verschuiving zien dan de SCN van muizen uit lange fotoperiodes. We concludeerde dat de fase-vooruitschuivende vermogen van de SCN klok bepaald wordt door de amplitude van de circadiane oscillatie.

Het elektrische activiteitprofiel op SCN weefsel niveau is opgebouwd uit de elektrische activiteitprofielen van de individuele SCN neuronen. De individuele cellen van de SCN zijn actief voor een relatieve korte periode (4 – 8 uur), waarvan het merendeel van de neuronen actief is gedurende de dag en een minderheid van de cellen

gedurende de nacht. Uit elektrofysiologische en moleculaire studies is gebleken dat plasticiteit van de fase-distributie onder de individuele neuronen ten grondslag ligt aan de seizoen aanpassing in amplitude van de SCN elektrische activiteit ritme en de golfvorm ervan. In **Hoofdstuk 3** hebben we met een wiskundige aanpak bepaald of de fase-verschuivende karakteristieken van de SCN afhankelijk zijn van samenhang onder de neuronen en op welke manier deze eigenschappen beïnvloed worden door de heterogeniteit van het SCN neuronale netwerk. We vonden dat wanneer 100% van de neuronen van de SCN reageren op de stimulus, een negatieve lineaire relatie bestaat tussen de mate van synchronisatie onder de individuele neuronen (sterkte van de amplitude) en fase-verschuivende capaciteit. Aan de andere kant, wanneer alleen een fractie van de neuronale populatie responsief is, bestaat er een positieve lineaire relatie tussen de mate van synchronisatie onder de SCN neuronen en de fase-verschuivende capaciteit tot een bepaald overgangspunt. Deze wiskundige studie benadrukt het belang van interneuronale synchronisatie en koppeling voor de fase-verschuivende vermogen van de SCN als geheel. Eveneens tonen de resultaten aan dat de fase-verschuivende eigenschappen van de SCN sterk beïnvloed worden door de heterogeniteit van het SCN neuronale netwerk.

Licht is de primaire externe stimulus verantwoordelijk voor het synchroniseren van het ritme van de SCN aan de externe licht-donker cyclus. Licht wordt gedetecteerd in het buitenste deel van het netvlies door fotoreceptoren bekend als staafjes en kegeltjes, en in het binnenste deel van het netvlies door foto-sensitieve retinale ganglion cellen (pRGCs) die melanopsine bevatten. Het netvlies van muizen bevat korte golflengte kegels die maximaal gevoelig zijn voor licht in het UV gebied, midden golflengte kegels en de staafjes die maximaal gevoelig zijn voor licht in het groene spectrum, en melanopsine wat gevoelig is voor blauw licht. Alle licht informatie die wordt gedetecteerd door de kegeltjes, staafjes en melanopsine wordt geintregeerd op het niveau van de pRGCs, en doorgestuurd naar de SCN via de retinohypothalamische tract (RHT). In de afwezigheid van staafjes en kegeltjes zijn de pRGCs nog steeds in staat om te reageren op licht en de lichtresponsen die worden gegenereerd door enkel de pRGCs zijn van voldoende sterkte om de SCN te kunnen synchroniseren aan de externe light-donker cyclus. In **Hoofdstuk 4** hebben we getest of melanopsine een cruciale rol speelt in het genereren van lichtresponsen in de SCN door het uitvoeren van *in vivo* metingen van SCN elektrische activiteit in de vrij-bewegende muis tijdens het toedienen van licht pulsen in het UV, groen of blauwe spectrum. Muizen die vanwege een genetische mutatie geen melanopsine hadden lieten vergelijkbare licht responsen zien als wild-type muizen met betrekking tot de grote en snelheid van de respons. We concludeerden dat melanopsine niet essentieel is voor het genereren van lichtresponsen in de SCN en dat de traditionele fotoreceptoren, staafjes en/of kegeltjes, betrokken zijn bij licht-synchronisatie van de SCN.

In **Hoofdstuk 5** hebben we vervolgens onderzocht in welke mate kegeltjes bijdragen aan de licht-synchronisatie van de SCN door het uitvoeren van elektrofysiologische metingen in SCN van muizen die door genetische modificaties alleen kegeltjes als functionele fotoreceptoren in de retina hadden. We observeerden een verhoging van SCN neuronale activiteit in reactie op 1 en 15 minuut licht pulsen in het UV en groene spectrum in de muizen met alleen kegeltjes als fotopigment. Om te controleren of de door kegeltjes gemedieerde lichtresponsen in SCN toereikend zijn voor het stabiel entraineren van het ritme van de SCN hebben we de muizen met enkel kegeltjes blootgesteld aan licht-donker cycli met lichtperiodes van alleen UV of groen licht. Het merendeel van de muizen met alleen kegeltjes als functionele fotoreceptor toonde stabiele synchronisatie aan het licht-donker regime, wat erop wijst dat kegeltjes betrokken zijn bij circadiane foto-entrainment. Deze resultaten tonen aan dat zowel melanopsine als kegeltjes acute lichtresponsen in de SCN kunnen produceren.

Het ritme van de SCN wordt niet alleen beïnvloed door licht informatie, maar bijvoorbeeld ook door terugkoppeling vanuit het lichaam naar de SCN. Uit eerder onderzoek met muizen is gebleken dat gedragsactiviteit een acute verlaging geeft van SCN neuronale activiteit. Muizen zijn nachtdieren en hebben hogere niveaus van gedragsactiviteit gedurende de donker-fase, welke samen valt met het dal in SCN neuronale activiteit. Wanneer de muis s ´nachts actief is, wordt het dal van het SCN elektrische ritme extra verlaagd, wat uiteindelijk een verhoging van de amplitude veroorzaakt. De verhoogde amplitude van het SCN ritme heeft weer een positief effect op het gedrag en op fysiologische ritmen. Dus, het elektrische activiteit ritme van de SCN en het gedragsritme van het dier hebben een positieve terugkoppeling naar elkaar, mits gedragsactiviteit in de donker-fase vertoond wordt. Waar in nachtdieren de ritmes in gedragsactiviteit en SCN neuronale activiteit in anti-fase zijn, oscilleren deze in dagdieren in fase. Dagdieren tonen verhoogde gedragsactiviteit niveaus gedurende de licht-fase van de dag, wanneer de SCN elektrische activiteit waardes ook hoog zijn. In **Hoofdstuk 6** is het effect van gedragsactiviteit op de amplitude van het circadiane ritme in SCN elektrische activiteit in een dagdier bestudeerd door middel van het uitvoeren van *in vivo* elektrofysiologische metingen in de SCN van vrij-bewegende dag-actieve gras rat (*Arvicanthis ansorgei*). De metingen lieten circadiane ritmes in SCN elektrische activiteit zien, met maximale waardes gedurende de dag en minimale waardes in de nacht. Analyse van de data op een kleinere tijdschaal onthulde dat episodes van gedragsactiviteit samen vallen met periodes van verhoogde SCN neuronale activiteit. Om het causale verband tussen de verhoogde SCN activiteit en gedragsactiviteit te onderzoeken hebben we *ex vivo* metingen van SCN elektrische activiteit uitgevoerd, wat ons in staat stelde om het endogene vuurpatroon van de SCN neuronen te meten in de afwezigheid van terugkoppeling van andere hersengebieden. De vuurfrequentie van de SCN neuronen

ex vivo vertoonde een circadiaan patroon die vrij was van acute verhogingen in elektrische activiteit. Dit resultaat suggereert dat de variabiliteit die geobserveerd *in vivo* voortkomt uit de communicatie tussen de SCN en andere hersengebieden. Wiskundige analyse (fractalanalyse) van de variabiliteit van de SCN vuurpatroon *in vivo* en *ex vivo* onthulde verstoorde elektrische activiteitspatronen *ex vivo*, wat suggereert dat terugkoppeling vanuit de periferie naar de SCN noodzakelijk is voor fractale regulatie van SCN elektrische activiteit. We concludeerde dat in dagdieren, zoals de mens, gedragsactiviteit een positief effect kan hebben op de amplitude van het ritme van de SCN klok.

In **Hoofdstuk 7** hebben we vervolgens getest in welke mate SCN elektrische activiteit betrokken in de regulatie van temporele gedragsactiviteitspatronen in dagdieren. Voor dit doel hebben we *in vitro* en *in vivo* SCN elektrische activiteit gemeten in de dag-actieve gras rat (*Arvicanthis ansorgei*) met twee verschillende gedragsfenotypen; ofwel actief gedurende de gehele licht periode (unimodaal gedrag) of actieve periodes rond de lichttransities (licht naar donker en vice versa, bimodaal gedrag). Daarnaast hebben we dag-actieve ratten blootgesteld aan een korte of lange fotoperiode waarna we SCN elektrische activiteit *in vitro* gemeten hebben. Interessant genoeg, in zowel de dag-actieve ratten met een unimodaal en bimodaal ritme volgde SCN elektrische activiteit een unimodaal patroon *in vitro*. En terwijl er fotoperiode-geïnduceerde verkorting of verlenging van de gedragsactiviteit fase werden waargenomen, was een fotoperiode geïnduceerde verandering van het vuurpatroon van de SCN afwezig *in vitro*. De resultaten tonen aan dat in dag-actieve ratten gedragsfenotypen (unimodal vs. bimodaal) noch fotoperiode-geïnduceerde veranderingen in gedrag overeenkomen met het endogene elektrische activiteit ritme van de SCN *in vitro*. Omdat de elektrische activiteit patronen van de SCN *in vivo* in alle gevallen wel overeenkomen met de gedragsactiviteit patronen verwachten we dat in dagdieren een samenspel van de SCN en de periferie van groot belang is voor het genereren van gedragsritmen in dagdieren.

Naast de rol van circadiane klok, hebben de SCN vanwege hun daglengte-afhankelijke output naar de pijnappelklier (epifyse) en andere delen van het centrale zenuwstelsel ook een functie al seizoen klok. De SCN coderen informatie over de externe fotoperiode door veranderingen van fase distributie van de activiteitspatronen van de individuele SCN neuronen. Fundamenteel voor de seizoen aanpassingen in fase distributie zijn veranderingen in expressie profielen van neurotransmitters en klokgenen. In **Hoofdstuk 8** presenteren we een literatuurstudie die inzicht verschaft in de unieke lichtrespons eigenschappen van de SCN die essentieel zijn voor het 'meten' van de externe fotoperiode. We bespreken op welke wijze de heterogene neuronale organisatie van belang is voor het opslaan van daglengte informatie, en hoe efferente routes betrokken zijn in het regisseren van seizoen aanpassingen in fysiologie.

Tot slot worden in **Hoofdstuk 9** alle resultaten van dit proefschrift bediscussieerd en worden mogelijke implicaties voor een verbeterde gezondheid en welzijn besproken. Samenvattend hebben we aangetoond dat de functie van het SCN neuronale netwerk afhankelijk is van een groter hersennetwerk dat onder andere bestaat uit het netvlies en hersengebieden betrokken in gedragsactiviteit. Op het geïntegreerde netwerk niveau kunnen de eigenschappen van het SCN systeem onvoorspelbaar zijn, wat de relevantie van complexiteitsaanpak in hersenonderzoek benadrukt.

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CURRICULUM VITAE

Ashna Ramkisoensing werd op 15 december 1987 geboren te 's-Gravenhage. In 2006 behaalde zij haar VWO diploma aan het Alfrink College te Zoetermeer. In dat zelfde jaar begon zij met de studie Biologie aan de Universiteit Leiden, waarvan zij in 2009 het Bachelor of Science diploma behaalde. Begin 2010 begon zij met de masteropleiding Biomedical Science, eveneens aan de Universiteit Leiden.

In het kader van haar masteropleiding heeft Ashna twee onderzoeksstages gevolgd. De eerste stage werd uitgevoerd bij de afdeling Moleculaire Cel Biologie. Onder begeleiding van Prof. dr. Meijer en dr. Houben werd veroudering van de biologische klok bestudeerd. De tweede stage werd uitgevoerd bij de afdeling Vasculaire Heelkunde onder begeleiding van dr. Lindeman. Hier was zij betrokken bij onderzoek naar voorspellende factoren voor abdominaal aorta aneurysma ruptuur.

Op 15 december 2011 behaalde zij aan de Universiteit Leiden het Master of Science diploma Biomedical Sciences. Van december 2011 tot december 2015 voerde zij als onderzoeker in opleiding onder supervisie van Prof. dr. Meijer het in dit proefschrift beschreven onderzoek uit bij de sectie Neurofysiologie van het Leids Universitair Medisch Centrum.

Begin 2016 verhuisde zij naar Oxford, Groot-Brittannië. Sinds januari 2016 is zij aangesteld als post-doctoraal onderzoeker aan de University of Oxford, waar zij onder begeleiding van prof. Foster en dr. Vyazovskiy onderzoek doet naar de invloed van licht op de werking van hersengebieden die betrokken zijn bij slaap- en waak ritmen.