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REVIEWS

Linking Together the Mammalian Circadian Clock

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Abstract

Mammalian circadian rhythms are thought to be driven by the suprachiasmatic nucleus (SCN), the master biological pacemaker. Each SCN neuron contains a set of "clock genes" that are transcribed, translated, and degraded every ~24 hours. Outputs from the molecular clock influence intrinsic ionic currents to generate cell-autonomous rhythms in action potential frequency. The firing pattern output of the SCN neural network ultimately synchronizes daily rhythms in behavior and physiology. This review presents our current understanding of how clock genes influence electrical activity, and how electrical activity influences circadian behavior.

Keywords

Circadian
Suprachiasmatic
Electrophysiology
Clock genes
Firing rate
Behavior

Introduction

The fact that we can wake and sleep on a regular schedule without the help of an alarm clock hints at the existence of some internal time-keeping mechanism that influences our daily behavior. Indeed, nearly every organism on the planet, from cyanobacteria and plants to mice and humans, has evolved a way to anticipate the daily light-dark cycle that results from the Earth's approximately 24-hour, or circadian, rotation about its axis. In mammals, the timekeeper is found in the SCN, a small, bilateral collection of ~20,000 neurons located above the optic chiasm in the hypothalamus[1]. The SCN is unique in that it not only generates self-sustaining oscillations, but also directly receives photic input from the retina. This input allows it to entrain to the external light cycle by synchronizing downstream cellular oscillators, ultimately leading to overt circadian behavioral and physiological rhythms[2]. Locomotor rhythms are lost when the SCN is lesioned, but can be restored by grafting SCN tissue into the arrhythmic animal[3]. The period of the restored rhythmicity is determined by the period of the donor SCN rather than that of the lesioned host, thus firmly establishing the SCN as the master circadian pacemaker[4-6].

While circadian outputs involve the emergent properties of the entire interconnected SCN neural network, individual SCN neurons are autonomous pacemakers with a period of approximately 24 hours. Each neuron exhibits both endogenous molecular rhythms and endogenous electrophysiological rhythms of high daytime and low nighttime spontaneous firing rate[1]. A key question in chronobiology is how these parts interact to make a coherent circadian pacemaker: how genes are linked to electrical rhythms, and how electrical rhythms are linked to behavior. A helpful analogy for this interconnection of circadian clock components is to think of the molecular oscillations as the "gears" of a clock, which move the clock's "hands" – its daily rhythms in electrical activity[7]. And just as looking at the hands on a clock can let a student know that he is late for class, the hands of the SCN clock seem to be able to dictate behavioral rhythms. Understanding the interconnectivity of the SCN is essential because the dysregulation of the core clock components plays a role in a variety of neuropsychiatric disorders including depression, sleep disorders, and autism[1,8,9]. Therefore this review will introduce the genetic, electrical, and behavioral components of the circadian clock and will examine the evidence linking together the gears and the hands, and the hands and behavior.

Gears

Molecular basis for rhythmicity. In each SCN neuron there exists an auto-regulatory transcription / translation negative feedback loop of core clock genes consisting of the *Period* genes *Per1* and *Per2*, the *Cryptochrome* genes *Cry1* and *Cry2*, *Clock* and *Bmal1*[1,10-12] (Figure 1). The positive arm of the core feedback loop begins when CLOCK and BMAL1 are translated and heterodimerize outside the nucleus. The complex then enters the nucleus and binds to the E-box sequence (CACGTG) of the *Per* and *Cry* promoter regions, activates their transcription, and begins the negative arm of the feedback loop. Various combinations of PER and CRY proteins then heterodimerize outside the nucleus where they are first phosphorylated by casein kinase 1 ε/δ (CK1ε/δ) and then enter the nucleus to inhibit the CLOCK/BMAL1 heterodimer, effectively inhibiting their own transcription.

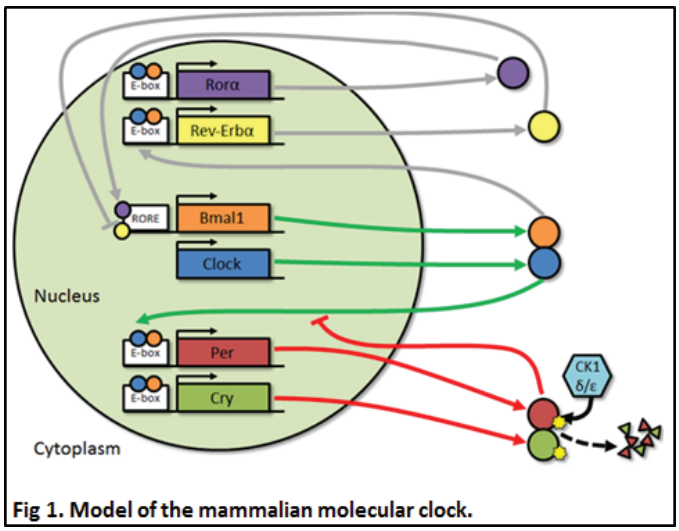

Fig 1. Model of the mammalian molecular clock.

Progressive phosphorylation of PER and CRY by CK1ε/δ leads to their degradation, which releases their inhibition of CLOCK/BMAL1 and restarts the feedback loop. A second negative feedback loop consisting of REV-ERBα and RORα contributes to clock precision and robustness by regulating the transcription of *Bmal1*[1,10,11].

Linking the gears to the hands. A series of experiments involving animals with mutations in their molecular clockwork provided the first evidence linking the gears of the clock to its hands. Multielectrode array recordings of spontaneous neural activity from hamsters with the dominant-negative *tau* (R178C) mutation in CK1ε showed a drastic reduction in the normal 24-hour period of electrical activity with homozygous mutants exhibiting shorter periods than heterozygotes[13]. *tau* was later found to promote the degradation of PER protein, causing an acceleration of the molecular feedback loop that paralleled the reduction in the period of electrical activity[14]. Using the same recording technique, neurons from mice heterozygous for an exon-19 deletion in the core clock gene *Clock* were found to have a lengthened period of electrical activity. Neurons from homozygous mutants, depending on the study, exhibited either an even longer period or complete arrhythmicity[15,16]. Likewise, when again recorded with a multielectrode array, mice with a double knockout of the essential core clock genes *Cry1* and *Cry2* exhibited a complete lack of circadian oscillation in firing rate[17].

These results with mutant animals clearly indicate a necessary interaction between the molecular clock and electrical activity but raise the question of how clock genes interact in a wild-type mouse. To address this, brain slices from mice expressing a degradable form of green fluorescent protein (GFP) under the control of the *Per1* promoter were used as a real-time indicator of single-cell molecular rhythms in combination with patch-clamp electrophysiological recording[18,19]. It was initially found that after a nighttime light pulse, the degree of *Per1* induction as reported by the fluorescent reporter was positively correlated with the frequency of spontaneous firing in individual neurons[18]. Similar experiments showed that such a correlation between GFP fluorescence and firing rate could be obtained at midday[19]. These results seem to indicate that SCN neurons increase their firing rate in tandem with an increase in *Per1* promoter activity. A computational model of SCN neuron pacemaker activity also suggests that there is a positive correlation between *Per1* mRNA levels and firing rate[20]. Evidence from a more recent study seems to contradict these previous results by suggesting that SCN neurons expressing maximal levels of *Per1* during midday are in fact electrically silent. These *Per1*-positive neurons are so depolarized that they exhibit depolarization block and cannot fire action potentials. The cells exhibiting the previously reported high daily firing rate appear to be *Per1*-negative[21]. Future experiments are necessary to clarify the relationship between *Per1* and firing rate.

Hands

Ionic basis of electrical rhythms. SCN neurons are unique in that not only do they fire spontaneously, but they also rhythmically alter their firing rate with a high firing rate during the day and a low firing rate at night (Figure 2). In most neurons, firing rate is set by synaptic activity, which is required for a cell's resting membrane potential to reach spike threshold and elicit an action potential. SCN neurons, however, are intrinsically driven towards spike threshold by a depolarizing persistent, slowly-inactivating Na^+ current[22-24], which opens voltage-gated Na^+ and L-type Ca^{2+} channels and initiates the characteristic "spike" of an action potential[23-25]. The membrane subsequently hyperpolarizes due to the opening of fast-delayed rectifier (FDR) and A-type (IA) K^+ channels[26,27]. After-hyperpolarization and repolarization back to resting membrane potential are thought to be due to the closing of voltage-gated Na^+ and Ca^{2+} channels and the opening of Ca^{2+}-dependent K^+ channels, particularly the large conductance BK channel[28-30]. The cell is then ready to fire again – at a rate that is circadianly modulated.

The first evidence for the ionic mechanism behind the circadian modulation of firing rate came from record-

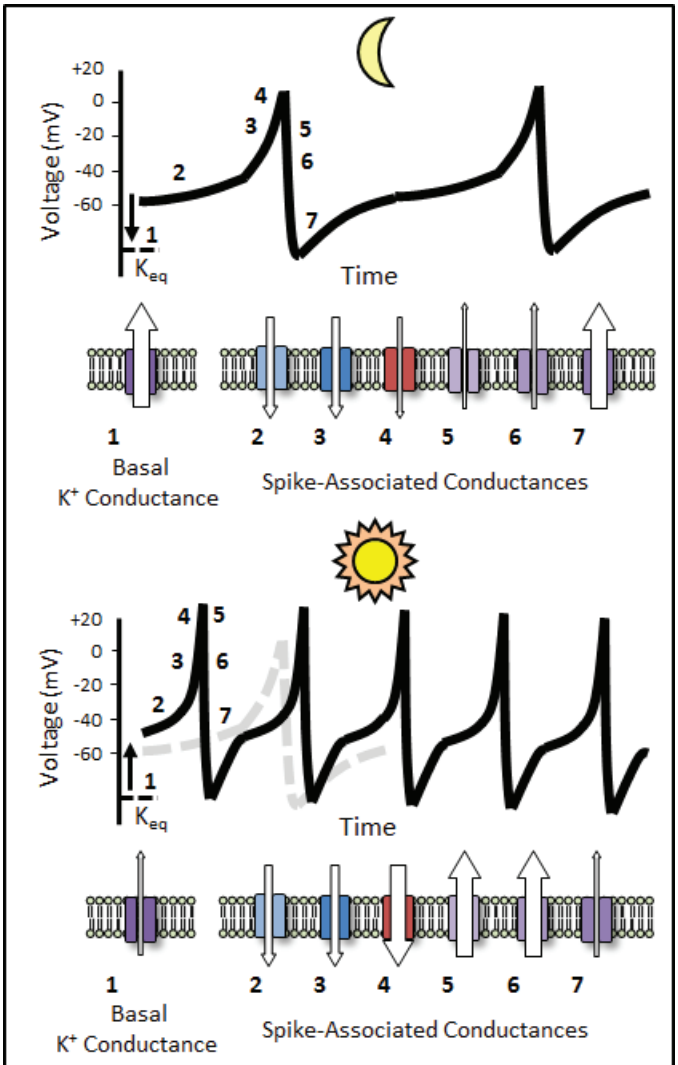

Fig 2. Ionic basis of SCN electrical rhythms. *(1)* Leak K^+ channel; *(2)* Persistent Na^+ channel; *(3)* Voltage-gated Na^+ channel; *(4)* L-type Ca^{2+} channel; *(5)* FDR K^+ channel; *(6)* A-type K^+ channel; *(7)* BK channel. Size of arrows represents relative size of current passing through channels.

ings taken from the basal retinal neurons of the sea slug *Bulla gouldiana*. Circadian rhythms in compound action potentials were shown to be driven by rhythms in resting membrane potential[31]. These daily membrane potential rhythms also exist in the neurons of the mammalian SCN, which, due to a daytime decrease in "leak" K^+ channel conductance, are depolarized and have a high input resistance (low conductance) during the day and are hyperpolarized and have a low input resistance at night[32,33]. A nighttime increase in K^+ conductance drives the neuron's resting membrane potential closer to the hyperpolarized equilibrium potential for K^+ and makes it harder for the cell to elicit a spike. Conversely, a daytime decrease in K^+ conductance brings the cell closer to spike threshold[34]. In addition to the rhythms in K^+ conductance, SCN neurons also show diurnal L-type Ca^{2+} channel-dependent current oscillations that may facilitate the high firing rate during the day[25]. Finally, circadian modulation of ionic conductance contributes to circadian rhythms in firing rate: the depolarizing L-type Ca2+ and the hyperpolarizing FDR and IA currents are higher during the day, which contribute to higher daytime spike rate[25-27,35]. The repolarizing BK current is higher during the night, which most likely contributes to a low nighttime spike rate by lengthening the refractory period required to initiate a new spike[28-30,35].

Linking the hands to behavior. Over two decades ago, it was shown that blocking electrical activity by transiently infusing the Na^+ channel blocker tetrodotoxin (TTX) into the SCN of freely behaving rats abolishes locomotor activity rhythms. However, once the drug was washed out, the animals resumed locomotor activity with an unaltered phase. These results strongly suggest that electrical activity is required for overt expression of circadian rhythms, but not for accurate molecular timekeeping[7]. More recent work either agrees with this[29,36] or instead implicates SCN electrical activity as being essential for robust molecular oscillations, as, for example, TTX or membrane hyperpolarization appears to dramatically but reversibly dampen clock gene rhythms[1,37,38]. Either way, it is now clear that electrical activity serves as the output of the intracellular clock. The calcium-activated BK channel, which normally suppresses spontaneous firing rate in the SCN at night, has been found to be necessary for circadian rhythms in locomotor activity. Mutant mice lacking the BK channel exhibit severely disrupted circadian behavioral rhythms and altered neuronal activity. Extracellular recordings taken during the daytime and nighttime from wild-type and knockout mice showed that BK-null mice had much higher firing rates at night while still having normal expression of clock genes such as *Bmal1*[29].

From this it seems probable that BK channels comprise at least part of the SCN output leading to behavior. Indeed, long-term multielectrode array recordings from BK-null mice demonstrate that the behavioral deficit appears to be due to a disruption of circadian rhythms in spontaneous firing rate[30]. Likewise, mice with a genetic knockout that attenuates the current carried by FDR K^+ channels, which greatly reduces the daytime spontaneous firing rate recorded from SCN neurons, were either behaviorally arrhythmic or exhibited severely attenuated rhythms. However, they did not show altered PER2 expression[36]. *In vivo* multiple-unit

recordings in freely-moving mice further clarified the link between electrical activity and behavior by determining that the onset and offset of behavioral activity corresponded with the half-maximal spontaneous firing rate[39].

Together, these studies indicate that firing rate is highly correlated with behavior. Intriguingly, however, a recent study found that the behavioral activity phase of nocturnal mice that were forced to work for food was shifted into the day. The behavior of these mice immediately reversed to the nocturnal phase when given *ad libitum* access to food, suggesting that the internal circadian pacemaker was maintained at its original phase[40]. A possible, though controversial, interpretation of this result is that the SCN is inherently rhythmic solely to inform the brain about the current light/dark cycle, and that an unexplored, malleable downstream brain region dictates circadian behavior from this input. Whether the SCN truly drives circadian behavior is yet to be determined.

Behavior

Rhythmic outputs of the SCN network. Individual SCN neurons are cell-autonomous circadian oscillators in both firing rate and clock gene expression[41], yet such an oscillation (in clock gene expression) has been observed in a variety of tissues outside the SCN[42]. What makes the SCN unique, however, is that it is a neuronal network of many coupled single-cell oscillators that together are able to produce robust behavioral and physiological rhythms. The SCN network can be organized into two broad regions based on their neuropeptide content: the vasoactive intestinal peptide (VIP)-expressing ventrolateral core, and the arginine vasopressin-expressing dorsomedial shell, which is densely innervated by the core[2]. VIP, the core's main neurotransmitter, is thought to mediate coupling in the SCN; VIP's effects may be modulated by the inhibitory neurotransmitter GABA, which is expressed in most, if not all, SCN neurons[1,43,44]. When coupled by VIP, the periods of the individual SCN neural oscillators are essentially identical; however, their phases are widely distributed[1,34]. Recent evidence suggests that an animal's behavioral phase and coherence are determined by the timing and degree of synchrony of individual rhythms, respectively, encoded by the average temporal population vector of the SCN network. The more synchronized the SCN, the more coherent the phases of its constituent neurons. Consequently, the average phase of the individual oscillators, represented as a time vector, determines the circadian time of activity onset[45]. Mutant animals lacking SCN synchrony, such as VIP knockouts, are behaviorally arrhythmic[45,46], and when behavioral arrhythmicity is induced in animals exposed to constant light, the synchrony within their SCN is disrupted[47].

The coordinated firing of the SCN network is sufficient to induce behavioral rhythmicity, but whether this output is synaptic or humoral in nature is unclear. Major axonal projections from the SCN synapse in other hypothalamic areas, including the subparaventricular zone and dorsomedial hypothalamus[48]. Some SCN neurons also secrete signaling molecules such as transforming growth factor alpha[49], prokineticin 2[50], and cardiotrophin-like cytokine[51]. Implanting encapsulated SCN tissue into SCN-lesioned animals, thus preventing neural outgrowth but allowing diffusion of secreted factors, is able to restore locomotor rhythms but not rhythms in melatonin or corticosterone secretion[52,53]. This suggests that both synaptic innervation and humoral secretion are necessary for complete circadian rhythmicity. Through downstream signaling, the SCN rhythmically regulates such diverse functions as corticosterone and melatonin release, orexin secretion, and cortical arousal[34,48]. Ultimately, these SCN outputs likely lead to overt circadian regulation of behavior.

Conclusions

The dynamic interaction between the molecular transcription/translation feedback loop, oscillations in electrical activity, and behavioral and physiological rhythms results in a functioning mammalian circadian clock, which provides an extremely useful model system to further understand the basic science question of how genes, through membrane events, can control behavior. The analogy for this interconnectivity presented in this review – the gears moving the hands, which in turn control behavior – is simplified. There is ample evidence that, unlike a physical clock, electrical activity can feed back onto the molecular clock[37,38], and that behavior can influence electrical activity[1,18]. However, to fully understand the mechanics of the biological clock, and to understand how its dysfunction can produce neuropsychiatric illness, it is necessary to understand the outputs of the circadian system: to link the gears to the hands, and to link the hands to behavior.

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