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Linking neural activity and molecular oscillations in the SCN

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Abstract

Neurons in the suprachiasmatic nucleus (SCN) function as part of a central timing circuit that drives daily changes in our behaviour and underlying physiology. A hallmark feature of SCN neuronal populations is that they are mostly electrically silent during the night, start to fire action potentials near dawn and then continue to generate action potentials with a slow and steady pace all day long. Sets of currents are responsible for this daily rhythm, with the strongest evidence for persistent Na^+ currents, L-type Ca^{2+} currents, hyperpolarization-activated currents (I_H), large-conductance Ca^{2+} activated K^+ (BK) currents and fast delayed rectifier (FDR) K^+ currents. These rhythms in electrical activity are crucial for the function of the circadian timing system, including the expression of clock genes, and decline with ageing and disease. This article reviews our current understanding of the ionic and molecular mechanisms that drive the rhythmic firing patterns in the SCN.

Our brains encode information through changes in the patterns and frequency of action potentials. These neural activity patterns change dramatically with the circadian cycle, so in a fundamental sense our brains behave differently as a function of the time of day. In addition, many cells within our body generate robust, synchronized rhythms in the transcription, translation and degradation of key ‘clock genes’ and their protein products through an autoregulatory loop. These rhythms have an endogenous periodicity of approximately 24 hours^{1,2}. Moreover, our bodies are made up of a network of oscillators, each of the major organ systems (heart, liver and pancreas) with its own clockwork to regulate the transcription of genes that are important to the specific target organ³. These circadian rhythms are synchronized by central pacemaker neurons located in a small subset of cells in the CNS — known in mammals as the suprachiasmatic nucleus (SCN). In all of the animal species that have been examined so far, these pacemaker neurons exhibit circadian rhythms in spontaneous neural activity. One of their striking features is that their spontaneous activity is highest during the day, regardless of whether the species is diurnal or nocturnal.

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Competing interests statement

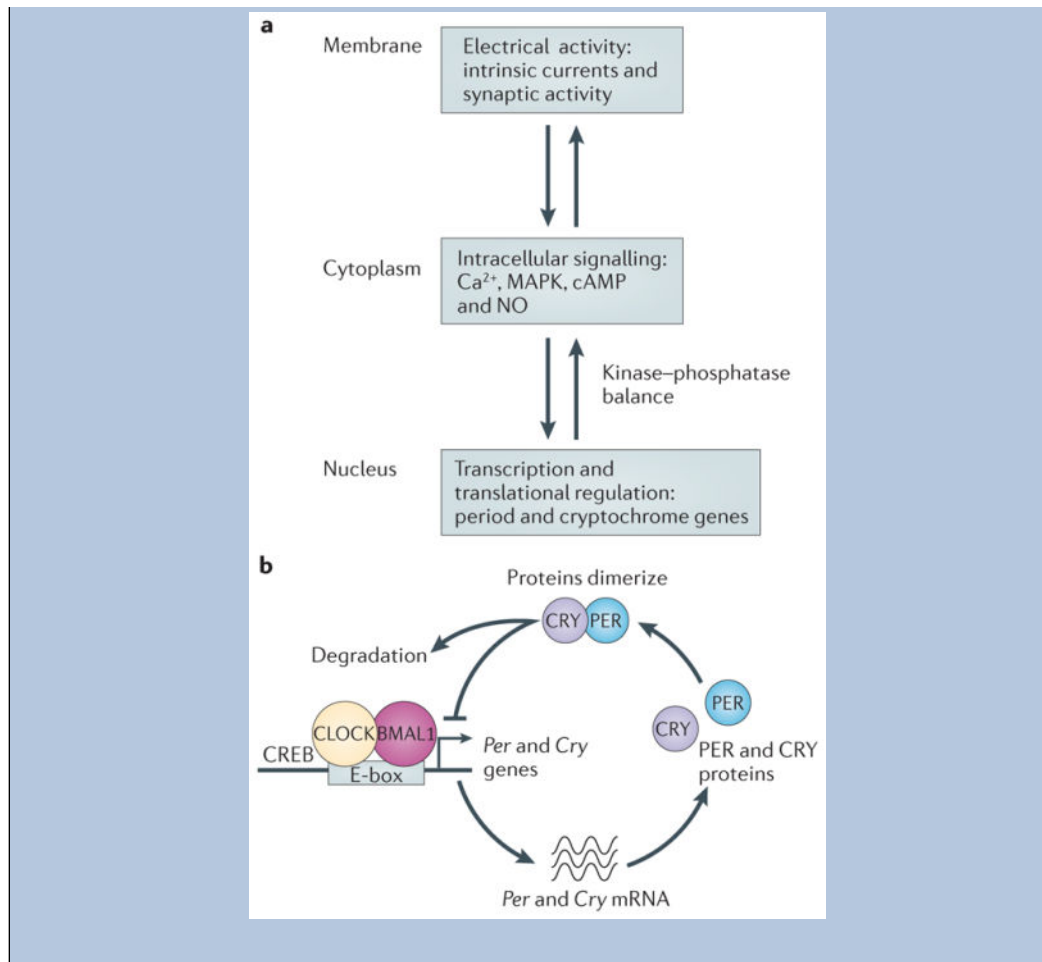
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We have a good conceptual understanding of the cell-autonomous molecular clockwork that regulates the generation of circadian rhythms in gene expression, but there is a lack of a mechanistic understanding of how this molecular feedback loop interacts with the membrane to produce physiological circadian rhythms (BOX 1). Clearly, the signals travelling to and from this molecular feedback loop must travel through the membrane, but not much is known about how the molecular feedback loop drives the rhythm in electrical membrane processes.

Box 1

Questions of coupling

One of the major problems in the field of circadian rhythms is to understand the inter-relationships between membrane events, intracellular signalling cascades and transcriptional and translational regulation. Suprachiasmatic nucleus (SCN) neurons generate rhythms of neural activity that peak in the day. Neural activity regulates Ca^{2+} as well as other signalling pathways through voltage-sensitive currents and the release of neurotransmitters (see the figure, part **a**). In the SCN, and perhaps other neurons, many of these signalling networks — including Ca^{2+} and cyclic AMP, nitric oxide (NO), casein kinases and RAS-dependent mitogen-activated protein kinases (MAPKs) — are strongly rhythmic in levels and activity. The balance between the activity of kinases and phosphatases at the end of these pathways regulates the transcription and translation of genes. Within many cells in the body, a transcriptional–translational negative feedback loop drives rhythms in gene expression (see the figure, part **b**). At the beginning of the cycle, CLOCK–BMAL1 protein complexes bind DNA at specific promoter regions (E-box) to activate the transcription of a family of genes including the period (*Per1*, *Per2* and *Per3*) genes and cryptochrome (*Cry1* and *Cry2*) genes. The levels of the transcripts for *Per* and *Cry* genes reach their peak during the period from midday to late in the day, whereas the PER and CRY proteins peak in the early night. The PERs, CRYs and other proteins form complexes that translocate back into the nucleus and turn off the transcriptional activity driven by CLOCK–BMAL1 with a delay (owing to the time required for transcription, translation, dimerization and nuclear entry). The proteins are degraded by ubiquitylation, allowing the cycle to begin again. Thus, in its simplest form, many cells contain this molecular feedback loop that regulates the rhythmic transcription of a number of genes. Other feedback loops within the cells contribute to the precision and robustness of the core oscillation. In the nervous system, many of the genes involved in control of excitability and secretion are rhythmically regulated by this molecular feedback loop. To produce a functional cellular oscillator within SCN neurons, there must be reciprocal signalling between membrane, cytosolic and nuclear processes. CREB, cyclic AMP-responsive element (CRE)-binding protein.



In this article, I will review our present understanding of the ionic mechanisms that underlie circadian rhythms in electrical activity in the SCN, how electrical activity may regulate clock gene expression and how clock gene expression may alter electrical activity in SCN neurons. I propose the idea that a decline in neural activity in the SCN may be a crucial mechanism by which ageing and disease may weaken the circadian output and contribute to a set of symptoms that impacts human health. Part of the goal of this Review is to identify gaps in our knowledge in the hope that this will stimulate further work in this area.

The SCN circuit

In mammals, the SCN of the hypothalamus contains the ‘master’ oscillatory network that is necessary for coordinating circadian rhythms throughout the body^{4–6} (FIG. 1). The SCN is a bilaterally paired nucleus made up of tightly compacted, small-diameter neurons that are located just lateral to the third ventricle, atop the optic chiasm⁷. Anatomical studies generally support the division of the SCN into at least two subdivisions: a ventral (core) region and a dorsal (shell) region^{8–10}. The core neurons are thought to act as an integrator of external input, receiving information through three major pathways: the retinohypothalamic tract (RHT), the geniculohypothalamic tract from the intergeniculate leaflet of the thalamus and projections from the raphe nuclei¹¹. Core neurons communicate this environmental

information to the rest of the SCN. These sensory processing ventral cells exhibit relatively low amplitude rhythms in clock gene expression. Low amplitude rhythms may be easier to reset to environmental perturbations — a concept that has been supported by mathematical modelling studies^{12,13}. Many of the neurons that receive retinal input within the core SCN express the neuropeptides vasoactive intestinal peptide (VIP) or gastrin-releasing peptide (GRP), as well as the neurotransmitter GABA. By contrast, neurons of the dorsal shell seem to generate robust circadian oscillations, at least at the level of gene expression^{14–16}. The neurons in the shell express arginine vasopressin (AVP) or prokineticin 2 (PK2), as well as GABA.

The fact that many core projections terminate on shell neurons supports the idea that interplay between these two centres is responsible for the output of circadian information from the SCN¹⁷. The outputs of the SCN from both core and shell subpopulations travel mainly to other hypothalamic regions, including the subparaventricular zone¹⁸. These hypothalamic relay nuclei send projections throughout the nervous and endocrine systems, providing multiple pathways by which the SCN can convey temporal information to the brain and body^{3,19,20}.

SCN neurons as intrinsic pacemakers

A unique property of central clock neurons and one that is essential to the function of the circadian timing system is the ability to generate circadian rhythms in electrical activity^{21–23}. The SCN has been shown to generate neural activity rhythms *in vivo*^{24–26}, in brain slice preparations^{27–30} and in SCN tissue cultures^{31–33}. These findings are consistent with the idea that many SCN neurons are stable, self-sustained oscillators that have the intrinsic capacity to generate circadian rhythms in electrical activity. Regardless of whether an animal is diurnal or nocturnal in terms of behaviour, cells in the SCN are electrically active in the day and show circadian rhythms in firing action potentials, with peaks of around 6–10 Hz in the middle of the day^{30,34,35}. Even when isolated from the circuit, single SCN neurons can exhibit a rhythm in firing rate, with most estimates placing the number of neurons that exhibit such a rhythm at about 60–70% of the total SCN neuron population^{36,37}. Individual neurons do not seem to spend a full 12 hours firing action potentials; however, it is estimated that single neurons may be active for 4–6 hours^{38–40}. In this electrically active state, the neurons do not respond strongly to excitatory stimulation but do respond to synaptic input that reduces their firing. During the night, the SCN neuron populations are electrically inactive and are most responsive to excitatory or depolarizing stimulation. Clarifying the ionic mechanisms responsible for the generation of rhythms in electrical activity in SCN neurons is an important step towards understanding the generation and output of circadian oscillations in a wide range of species, including humans.

The ionic players

SCN neurons generate action potentials in the absence of synaptic drive, and can therefore be considered endogenously active neurons. To maintain spontaneous activity, a set of intrinsic currents must interact to depolarize the cell membrane to threshold, elicit an action potential and return the membrane to negative potentials from which the next spike can be initiated. This ‘spontaneous’ firing arises from specific combinations of intrinsic membrane

currents^{41,42}. Some progress has been made in identifying the ion channels that drive spontaneous activity in the SCN. Conceptually, it can be useful to divide the ionic mechanisms: first, into currents that are responsible for providing the excitatory drive required for all spontaneously active neurons; second, into currents that translate this excitatory drive into a regular pattern of action potentials; and third, into currents that drive the membrane hyperpolarization that underlies the nightly silencing of firing (TABLE 1).

Excitatory drive to SCN neurons

During the day, SCN neurons are much more depolarized than neurons that do not show spontaneous activity. They have a resting membrane potential between -50 mV and -55 mV, which places them close to the threshold for generating an action potential (-45 mV). A subset of SCN neurons may even move to such a depolarized state (-30 mV) during the day that they cannot generate action potentials⁴³. This relatively depolarized resting potential is the result of excitatory drive provided by multiple cation currents^{44–46}. In dissociated SCN neurons, in which this has been investigated in most detail, the spontaneous interspike depolarization was found to be primarily attributable to Na^+ currents that flow at between -60 mV and -40 mV⁴⁵. SCN tissue expresses mRNA for the tetrodotoxin (TTX)-sensitive voltage-gated sodium channels Nav1.1 (encoded by *SCN1A*) and Nav1B (encoded by *SCN1B*), as well as Nav1.5 (encoded by *SCN5A*) and Nav1.6 (encoded by *SCN8A*) (TABLE 1). Nav1.5 and Nav1.6 channels are likely to be responsible for the persistent Na^+ current described in SCN neurons, as both are expressed in the SCN^{47,48}. There is currently no evidence for a possible circadian regulation of persistent Na^+ currents. The persistent Na^+ currents in the SCN can be blocked by the neuroprotective agent riluzole⁴⁶. Chronic application of riluzole prevents the expression of daily rhythms in neural activity⁴⁹, indicating a role for persistent Na^+ currents in the excitatory drive. However, one caveat regarding these experiments is that riluzole can also activate the two-pore K^+ (K2P) channels that are expressed in the SCN (see below).

Another source of excitatory drive that is important in many pacemaking neurons is the opening of hyperpolarization-activated, cyclic nucleotide-gated (HCN) ion channels⁵⁰. When these channels open, Na^+ enters the neuron and K^+ goes out, with the net result being a slow excitation of the membrane. Almost all SCN neurons carry a very prominent hyperpolarization-activated conductance (I_H), which generates a depolarizing voltage sag in response to hyperpolarization^{51–54}. Probably as a result of this current, SCN neurons exhibit a robust rebound excitation in response to membrane hyperpolarization. There is some evidence for a modest circadian rhythm in the magnitude of this current — it peaks during the day⁵⁴ — although this rhythm was not detected in earlier studies⁵³. Acutely blocking I_H with ZD7288 did not greatly impact the firing rate of SCN neurons^{44,53}, but sustained pharmacological block of HCN channels greatly reduced the frequency of action potentials during the day⁵⁴ and curtailed circadian gene expression⁵⁵. Microarray analysis indicates that the mRNAs for both the HCN2 and HCN3 subunits are present in the SCN and suggests that their expression may be rhythmic⁴⁷. An immunohistochemical study found that HCN3 and HCN4 are the predominant subunits in the SCN⁵⁶.

Ca²⁺ channels can also contribute to spontaneous activity in neurons. The genes coding for T-type (Cav3.1, Cav3.2 and Cav3.3) and L-type (Cav1.2 and Cav1.3) Ca²⁺ channel subunits are strongly expressed in the SCN^{48,57}, with some evidence for rhythmic expression of transcripts^{47,57}. Both the low voltage-activated T-type Ca²⁺ channels and Cav1.3 L-type Ca²⁺ channels exhibit the low threshold, steep voltage-dependence and low inactivation kinetics that are important for oscillations in the membrane potential^{42,58}. Subthreshold membrane oscillations in SCN neurons have been characterized in both brain slices⁵⁹ and cell cultures⁴⁵. These oscillations are sensitive to the Ca²⁺ channel blocker nimodipine and seem to be mediated mostly by L-type Ca²⁺ currents. The magnitude of the L-type Ca²⁺ current amplitude exhibits a diurnal rhythm in the SCN, with subthreshold membrane oscillations found in the day but not the night⁵⁹; however, blocking these currents had a relatively minor effect on spontaneous neuronal activity. The T-type current is also prominent in SCN neurons^{60,61}, but present data indicate that this Ca²⁺ current probably mediates the response to glutamate⁶¹ rather than driving spontaneous firing during the day.

To summarize, a set of currents (persistent Na⁺, HCN, T- and L-type Ca²⁺ currents) provide the excitatory drive that is necessary for any neuron to be spontaneously active. Action potentials in the SCN are associated with a Ca²⁺ influx, which is presumably due to opening of T- and L-type Ca²⁺ channels^{45,62}. These Ca²⁺ channels are also likely to play a crucial part in the regulation of Ca²⁺ levels in the SCN and may explain the higher levels of Ca²⁺ in the day compared to in the night (see below). However, broadly speaking, the excitatory drive in SCN neurons seems to be relatively constant throughout the daily cycle.

The sodium–potassium pump (Na⁺–K⁺ ATPase) is crucial for maintaining the resting membrane potential in response to this excitatory drive. The pump transports three Na⁺ ions out and two K⁺ ions into the cells using ATP hydrolysis. The pump is more active in the day than in the night^{63,64} and its activity may be crucial to the daily rhythm in membrane potential. It is electrogenic, adding a virtual –1 charge for every cycle of three Na⁺ out and two K⁺ in. So, if the pumps are shut off, the neural membrane could become depolarized, depending on the ratio of the two leak currents and on the typical electrogenic pump output. The use of this pump is very energy expensive and is probably responsible for the robust daily rhythm in 2-deoxyglucose use that characterizes the SCN^{21,65}.

Translating excitatory drive into action potentials

In response to the excitatory drive, SCN neurons exhibit sustained discharge for 4–6 hours in the day without spike adaptation. Recent work suggests that the fast delayed rectifier (FDR) K⁺ current may allow for this type of discharge at least in other types of neurons^{66,67}. Within the SCN, the FDR current is of particular interest because the magnitude of this current exhibits a circadian rhythm and sustained pharmacological blockade of the FDR current reduces the magnitude of the daily rhythm in firing in a brain slice preparation⁶⁸. Two members of this family are expressed in the SCN: Kv3.1 (*KCNK1*) and Kv3.2 (*KCNK2*). Immunohistochemical analysis suggests a day–night difference in protein expression⁶⁸, whereas *in situ* hybridization showed a low amplitude day to night difference in mRNA expression⁶⁹. SCN neurons from mice lacking both *Kcnc1* and *Kcnc2* genes exhibit reduced spontaneous activity during the day and reduced NMDA-evoked excitatory

responses during the night⁶⁹. In addition, the width of the action potential in SCN neurons is almost double in the double knockout mice. Thus, the FDR K⁺ current is necessary for the circadian modulation of electrical activity in SCN neurons and represents an important part of the ionic basis for the generation of rhythmic output.

In other neurons, the subthreshold-operating A-type K⁺ current (I_A) is mainly involved in the regulation of neuronal excitability and the timing of action potential firing. Within the SCN, this current has been described and proposed as a likely candidate to regulate the spontaneous firing rate^{60,70–72}. The magnitude of the I_A current exhibits a diurnal rhythm that peaks during the day in the dorsal region of the mouse SCN⁷². This rhythm is driven by a subset of SCN neurons with a larger peak current and a longer decay constant⁷². Importantly, this rhythm in neurons in the dorsal SCN continues in animals placed in constant darkness, providing an important demonstration of the circadian regulation of an intrinsic voltage-gated current in mammalian cells. Both *in situ* hybridization and Western blots have detected expression of the Shal-related family members 1 and 2 (Kv4.1 (*Kcnd1*) and Kv4.2 (*Kcnd2*)) within the SCN⁷² but did not see evidence for rhythmic expression of these channels.

Lastly, Ca²⁺-activated K⁺ channels (BK channels) are a major contributor to repolarization of the membrane after an action potential in the SCN. A study in which BK currents were inhibited with iberiotoxin suggests that this current may contribute 40% of the afterhyperpolarization that occurs after an action potential in the SCN⁷³. The impact of this current on daytime firing of SCN neurons is still unclear. Iberiotoxin did not alter spike frequency during daytime⁷³ and mice lacking the gene that encodes the pore-forming subunit of the BK channel (*Kcnma1*) did not show a change in firing during the day⁷⁴. However, another study that focused on a subset of period 1 (*Per1*)-expressing SCN neurons found that blocking BK conductance with iberiotoxin can reduce daytime peak firing rate⁷⁵. The *Kcnma1* gene and the BK channel are rhythmically expressed in the SCN^{47,74,75}, and the BK current is involved in the nightly hyperpolarization of the membrane (see below).

Therefore, FDR, I_A and BK K⁺ currents all seem to have a role in the regulation of spontaneous action potential firing in SCN neurons during the day. The biophysical properties of these currents, measured in the SCN and other brain regions, suggest that these three currents are also critically involved in determining how SCN neurons respond to synaptic stimulation from other regions, particularly to photic information via the RHT.

Nightly silencing of neuronal firing

SCN neurons show a diurnal rhythm in membrane potential, such that cells are depolarized by about 6–10 mV during the day compared to the night^{43,76,77}. This day–night difference in resting membrane potential is mediated to a large extent by a hyperpolarizing K⁺-dependent conductance that is active at night at resting membrane potential and inactive during the day^{34,77}. Resting membrane potentials of neurons are mainly set by a class of two-pore domain K⁺ channels (K2P, TASK and TREK channels)^{78–80}. These K⁺ channels are active over the whole voltage range (unlike other K⁺ channels) and are referred to as providing ‘leak’ or background currents⁸⁰. K2P channels are encoded by the *KCNK* gene family. The transcripts for *Kcnk1* and *Kcnk2* are expressed in the SCN⁴⁸, with transcripts for *Kcnk1*

exhibiting a robust rhythm in the SCN⁴⁷. Unfortunately, specific pharmacological blockers of these channels are not available, but the rhythmic pattern of expression of K2P channels in the SCN means that they are promising candidates for driving the nightly hyperpolarization in membrane potential.

BK currents are also involved in the nightly silencing of SCN neurons. The expression of *Kcnma1*, the gene encoding the pore-forming subunit of the BK channel, peaks in the middle of the night⁷⁵, and the relative contribution of the BK current to the outward currents is larger in the night than in the day. In addition, deletion of *Kcnma1* increases night time firing in SCN neurons, although it does not completely abolish the day–night difference in firing rate^{74,81}.

Contribution of chloride pumps

The SCN expresses at least two members of a family of cation-chloride (Cl^-) cotransporters that regulate the chloride equilibrium potential (E_{Cl^-})^{82,83}. The $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter NKCC1 raises intracellular Cl^- levels and thus contributes to a more depolarized E_{Cl^-} . The effects of NKCC1 are balanced by the other cotransporter, KCC isoform 2 (KCC2), which normally lowers intracellular Cl^- levels. The balance between KCC2 and NKCC1 seems to be a critical regulator of the impact of GABA on SCN neurons⁸³. The upregulation of NKCC1 leads to a neuron showing a depolarizing membrane response to GABA. Data indicate that the continued expression of NKCC1 in the SCN is responsible for GABA-evoked excitation⁸³ and although this regulation varies from day to night, it is probably not important in setting the membrane potential of SCN neurons. There is some evidence that the transcript levels of the gene encoding NKCC1 (*Slc12a2*), but not the gene encoding KCC2 (*Slc12a5*), may be regulated by the circadian system⁴⁷.

How is the network involved?

Although SCN neurons are circadian pacemakers that can generate rhythms in neural activity in isolation from other neurons, *in vivo* they are part of a circuit^{5,6,84} (FIG. 2). For example, SCN neurons receive a stream of GABA-mediated synaptic input that peaks at the day–night transition⁸⁵. These neurons communicate through the release of peptides, and synaptic communication between cells in the SCN circuit seems to be crucial for rhythms in neural activity. For example, mice with a deletion of secretory vesicle proteins show substantial disruptions in the neural activity rhythms in the SCN⁸⁶. In addition, there is compelling evidence that the neuropeptide VIP and the vasoactive intestinal polypeptide receptor 2 (VIPR2, also known as VPAC2R) may have roles in the generation of rhythms in neural activity. Extracellular recordings from cultured SCN neurons indicate that in mice lacking VIP or VIPR2, fewer neurons express rhythms in neural activity³⁶. There is evidence that SCN neurons from *Vipr2*^{-/-} mice may be chronically hyperpolarized⁸⁷. The ionic mechanisms underlying this phenomenon are not yet known, and it is also not clear whether VIP acts as a transmitter or as a cofactor in the SCN. Nevertheless, the data suggest that although SCN neurons are cell-autonomous oscillators, the network plays an important part in generating the rhythms of firing and membrane potential that characterize SCN neurons.

In summary, our understanding of the ionic mechanisms responsible for the generation of rhythms in electrical activity in SCN neurons is increasing. It is probable that a set of intrinsic voltage-sensitive currents are responsible for the circadian variation in firing rate in SCN neurons (TABLE 1). One possibility is that these cells fluctuate between two subthreshold membrane potentials, a hyperpolarized down state and a depolarized up state. During the up state (day), most SCN neurons are spontaneously active and can fire action potentials. During the down state (night), most SCN neurons are inactive owing to a membrane hyperpolarization associated with an increase in total membrane conductance⁷⁷. From a circadian perspective, it is important not only to identify the currents that regulate spontaneous firing but also to understand how those currents change from day to night, to drive the rhythm in neural activity.

Activity regulates the molecular clock

For many years, the assumption was that the neural activity rhythms of the circadian system were driven — through some unknown mechanism — by the molecular clockwork that is present in single cells. However, in the past decade, a few key studies have suggested that neural activity is not only driven by the molecular clockwork but is itself required for the rhythmic expression of gene expression. An elegant study in *Drosophila* manipulated the expression of a non-voltage sensitive K^+ channel to assess the impact of chronically hyperpolarizing, pigment dispersed factor (PDF)-expressing circadian pacemaker neurons on circadian output⁸⁸. As expected, this construct electrically silenced the neurons and blocked behavioural rhythms in *Drosophila*. The unexpected finding was that it also blocked the rhythm in expression of the PER and timeless (TIM) proteins⁸⁸. These types of observations have changed the thinking in the field for the past decade.

Other findings also suggest that membrane excitability and/or synaptic transmission may be required for the generation of molecular oscillations in SCN neurons. One of the most direct lines of evidence comes from experiments using TTX to block fast inward Na^+ currents. Application of this toxin can block the expression of rhythms in behaviour²¹, glucose utilization⁸⁹, secretion⁹⁰ and, of course, electrical activity³¹. After TTX wash-out, the phase of each of these rhythms seems to re-emerge in phase with the prior oscillation. These results suggest that TTX does not stop the clock but blocks the expression of rhythmicity — that is, blocks circadian output — although this has not been explicitly tested and more analysis is needed to confirm that the phase is not altered by these treatments. Several groups have used optical reporters that allow real-time bioluminescence imaging to monitor oscillations at the level of single neurons. Real-time imaging of single SCN cells in a slice culture preparation from transgenic mice carrying the *mPer1* promoter-driven luciferase reporter gene showed that TTX dramatically reduced both the amplitude of the molecular circadian rhythms of PER1::LUC activity and the synchrony of the SCN population^{23,91}. Again, after wash-out of TTX, the molecular oscillation seems to re-emerge with a phase that is predicted by the bioluminescence rhythms before treatment. Interestingly, a detailed analysis of the impact of TTX on PER2::LUC rhythms recorded from cultured SCN neurons indicates that although most SCN neurons lost rhythmicity in the presence of TTX, a small minority (10 %) did not³⁷. So, in addition to blocking circadian output, TTX may also block

the expression of the core molecular clockwork within SCN neurons. But what is the underlying mechanism?

Physiological studies indicate that TTX application has three effects on SCN neuron activity. First, it stops the generation of action potentials by blocking most voltage-dependent sodium currents⁹². Second, through this mechanism, TTX application stops synaptic transmission, although the release of small packets of neurotransmitters still occurs. Third, in at least some SCN neurons, TTX application depolarizes the resting membrane potential through as yet unknown ionic mechanisms^{45,59}. It is difficult to parse out the different effects of TTX to determine which of these mechanisms underlies the effect of TTX on clock gene expression. An additional consideration for the interpretation of these experiments is that TTX is the most disruptive to clock gene expression in preparations of immature neurons. Neural activity may have an organizational role in the development of the SCN circuits, which could explain why clock gene expression in adult tissue seems to be less affected by the application of TTX⁹³.

Other studies have used pharmacological treatments to block synaptic transmission in the SCN without affecting action potential generation or membrane depolarization, and have found that these treatments alter clock gene expression. For example, treating SCN slices with botulinum toxin A or using dynasore to block exocytosis or endocytosis, respectively, compromises circadian gene expression as measured by PER2::LUC expression⁹⁴. Similarly, the targeted deletion of secretory vesicle proteins receptor-type tyrosine-protein phosphatase-like N (IA2) and IA2 β alters the rhythms in a variety of circadian outputs, including SCN neural activity and clock gene expression⁸⁶. Most SCN neurons release GABA, and blocking synaptic transmission would be expected to block this release; however, some studies indicate that GABA is not crucial for coupling the circadian oscillations in gene expression between neurons⁹⁵. Instead, as mentioned above, most data suggest that SCN neurons are coupled by peptides including VIP and its receptor VIPR2 (REFS 96,97). Mice that are deficient in VIPR2 exhibit low amplitude PER2::LUC rhythms that resemble those seen with the application of TTX⁹⁸. Importantly, this loss of amplitude in the molecular clockwork in the VIPR2 knockout mice was 'rescued' by the addition of a medium containing high levels of K⁺ that broadly depolarizes the membrane. This suggests that one role of the VIPR2 may be to maintain membrane potential of SCN neurons within a range that is consistent with molecular timekeeping. In support of this model, the loss of VIPR2 results in the hyperpolarization of the membrane of SCN neurons⁸⁷. So, the effect of treatments that block synaptic transmission within the SCN has to be considered in the context that they are blocking peptide signalling within this structure.

In the SCN cell population, the rhythms in *Per1* and *Per2* mRNA peak between circadian time 4 and circadian time 6 (REFS 99,100), whereas electrical activity peaks around circadian time 7. So, the peak of CLOCK–BMAL1 (brain and muscle ARNT-like 1) -driven transcription occurs before the highest levels of action potentials, but neural activity and CLOCK–BMAL1-driven transcription rise together in the beginning of the daily cycle. The temporal dynamics of transcriptional activation of *Per1* can be optically monitored using green fluorescent protein (GFP) fluorescence in *Per1::GFP* mice. In these mice, there is a direct correlation between the frequency of action potentials and the level of *Per1*

expression in SCN neurons^{101,102}. Specifically, *Per1* mRNA expression (as measured by the GFP construct) was higher in neurons firing at 10 to 12 Hz than in cells that were electrically inactive. These experiments raise the possibility that there may be a fixed phase relationship between electrical activity and gene expression; however, there is also evidence that when the circadian system is transiently disrupted by rapid changes in the light–dark cycle, the peaks of neural activity and *Per1* expression assume very different phases¹⁰³. It is also not clear whether the firing rate itself or the underlying membrane depolarization has the greatest effect on *Per1* expression, as a recent study reported a high GFP signal in SCN neurons that were so depolarized as to be unable to generate action potentials⁴³. At a population level, membrane hyperpolarization that is caused by lowering the extracellular concentration of K^+ in SCN cultures reversibly abolishes the rhythmic expression of *Per1* and *PER2* (REF. 104). Together, these studies in mammals and experiments in *Drosophila*^{88,105,106} suggest that electrical activity of SCN cells drive the molecular clockwork, and that keeping SCN cells in an appropriate voltage range may be required for the generation of circadian rhythmicity of clock gene expression at the single-cell level. One key issue that needs to be resolved is the role of development in these effects. Neural activity is crucial in development, as patterns of activity have organizational effects on the developing circuits. In addition, it is not clear whether the impact of blocking rhythmic electrical activity on gene expression is due to the loss of action potentials or the chronic hyperpolarization that will reduce Ca^{2+} and perhaps cyclic AMP-driven transcription.

Activity-regulated signalling pathways

Regardless of whether TTX affects action potentials or synaptic events, the effect of electrical activity of SCN neurons on clock gene expression is probably mediated, at least in part, by Ca^{2+} . In neurons, there is a close relationship between electrical activity and Ca^{2+} levels^{107–109}. SCN neurons exhibit a circadian rhythm in resting Ca^{2+} that can be detected by the Ca^{2+} sensitive fura2 indicator dye. During the peak in firing at midday, SCN neurons show resting Ca^{2+} levels of around 150 nM. These levels drop to about 75 nM during times of inactivity¹¹⁰. This rhythm is reduced, though not quite eliminated, by TTX and L-type calcium blockers. The action potential itself is one important source of Ca^{2+} in the SCN⁴², regulating Ca^{2+} influx into the soma through voltage-dependent activation of L-type Ca^{2+} channels. This was shown most clearly by recent work in which Ca^{2+} levels and firing of SCN neurons were measured simultaneously⁶². The data from this study show that driving the frequency of action potentials in the SCN neuron to 5–10Hz (daytime levels) induces a rise in somatic Ca^{2+} levels, and this was attenuated by the application of the L-type Ca^{2+} channel blocker nimodipine.

Interestingly, SCN neurons also have a rhythmically regulated pool of Ca^{2+} that is not driven by membrane events. Experiments in which a Ca^{2+} -sensitive fluorescent protein cameleon was expressed in organotypic SCN cultures detected robust Ca^{2+} rhythms¹¹¹. These rhythms were sensitive to pharmacological manipulation of ryanodine-sensitive stores of intracellular Ca^{2+} but not to TTX or L-type Ca^{2+} channel blockers. However, the Ca^{2+} rhythm phase precedes the neural activity rhythm and previous studies showed that the ryanodine receptor can be a potent regulator of excitability of SCN neurons¹¹², suggesting that store-driven changes in Ca^{2+} are a critical part of the output pathway by which

intracellular processes drive rhythms in neural activity. Future studies should determine the subcellular location of these Ca^{2+} pools as well as the relationship between intracellular Ca^{2+} and fluorescence signals in SCN neurons as the cameleon reporters are not ideal for monitoring the relatively slow changes in firing rate observed in SCN neurons¹¹³.

Regardless of the relative contribution of Ca^{2+} influx and release from intracellular stores, it is clear that Ca^{2+} oscillations are crucial in driving a robust rhythm in gene expression. For example, blocking Ca^{2+} influx in SCN cultures by lowering the extracellular Ca^{2+} concentration reversibly abolishes the rhythmic expression of *Per1::luc* and *PER2::LUC*¹⁰⁴. Blockade of voltage-sensitive Ca^{2+} channels also abolishes the oscillatory patterns of *Per2* and *Bmal1* expression in a cell line (SCN2.2) derived from the SCN⁵⁷. In *Drosophila melanogaster*, buffering intracellular Ca^{2+} in pacemaker neurons results in dose-dependent slowing of free-running behavioural and molecular rhythms¹¹⁴. These results strongly suggest that a transmembrane Ca^{2+} flux is necessary for sustained molecular rhythmicity in the SCN. Indeed, rhythms in Ca^{2+} levels, with the peak occurring during the day, seem to be a general feature of circadian systems, as studies in molluscs^{115,116}, flies¹¹⁴ and even plants^{117–119} have shown.

Another crucial signalling pathway that is involved in the coupling between the membrane and clock gene expression is the cyclic AMP (cAMP) signalling pathway. Several studies have found that cAMP levels are rhythmic in the SCN^{55,120,121}. Part of the underlying mechanism may be the rhythmic expression of the gene encoding adenylyl cyclase 1 (*ADCY1*) in the SCN and retina¹²². Peak cAMP levels in SCN tissue occur during the day^{55,120} and precede the peak in the neural activity rhythm. The transcriptional activity of the cAMP-responsive element (CRE) is also strongly rhythmic in the SCN⁵⁵. Importantly, pharmacological blockade of adenylyl cyclase activity reduced cAMP levels, suppressed CRE expression and greatly reduced *PER2::LUC* expression⁵⁵. Inhibition of adenylyl cyclase can also lengthen the free-running period of the *Per2* rhythm, suggesting a regulatory role of this enzyme in the core clock mechanisms. Interestingly, the key cAMP-driven effector seems to be exchange protein activated by cAMP (EPAC) rather than protein kinase A (PKA). Indeed, it has been suggested that EPAC phosphorylation of CRE-binding protein (CREB) drives clock gene expression¹²³, but the roles of firing rate and synaptically mediated events in this cytosolic oscillation are not known. However, a number of Ca^{2+} sensitive adenylyl cyclases are expressed in the SCN. For example, *Adcy1* is expressed in the SCN^{48,122} and *ADCY1* activity is stimulated by Ca^{2+} concentrations of around 150 nM¹²⁴. So, the daytime peak in intracellular Ca^{2+} (REF. 110) would be sufficient to trigger adenylyl cyclase 1, and the crosstalk between the rhythmic electrical activity and cAMP may therefore be mediated by Ca^{2+} . In addition, many of the synaptically evoked neurotransmitters that are expressed in the SCN, including pituitary adenylyl cyclase-activating polypeptide (PACAP), VIP, GRP, AVP and serotonin are potent regulators of cAMP levels. Interestingly, a recent study showed that *CRY1* can inhibit cAMP in response to G protein-coupled receptor activation, possibly through a direct physical interaction with G proteins¹²⁵. Thus, electrical activity levels can alter the cAMP signalling network in the SCN through a number of mechanisms.

Besides Ca^{2+} and cAMP, a number of rhythmically regulated signalling molecules, including casein kinases and RAS-dependent mitogen-activated protein kinases (MAPKs), have been described in the SCN. For example, recent work suggests that protein kinase C (PKC) and receptor of activated C kinase 1 (RACK1) are integral components of the circadian clock¹²⁶. These Ca^{2+} -sensitive signalling molecules are physically recruited into the nuclear BMAL complex. Overexpression or depletion of these proteins altered the molecular clockwork, suggesting a role for PKC in the basic circadian feedback loop. It may be useful to think of these signalling pathways collectively, as a 'cytosolic' oscillator¹²³. Although it is premature to throw out the molecular feedback loop concept, recent work in a variety of preparations, from cyanobacteria¹²⁷ to human red blood cells¹²⁸, suggests that rhythmic gene transcription is not essential for circadian timekeeping¹²⁹. In neurons, cytosolic networks are regulated by neural activity, both directly and through the controlled release of neurotransmitter, and they converge to regulate the transcription factor CREB, CREB-binding protein and p300 to drive the transcription and translation of clock genes such as *Per*. It seems likely that the cAMP- and Ca^{2+} -dependent activation of CREs is a necessary complement to activation of E-boxes by CLOCK::BMAL for normal circadian regulation of *Per* and the SCN clockwork¹³. Thus, dysregulated neural activity and synaptic transmission could weaken the basal Ca^{2+} -CRE activity to a level that is insufficient to drive *Per* and *Cry* expression.

Light-evoked changes

In addition to the changes in gene expression that are driven by the daily increase in spontaneous neural activity, exposing an animal to light during the night also drives a robust increase in neural activity in the SCN²⁵. The consequence of light exposure on the molecular clockwork varies with the phase of the daily cycle. A light stimulus during the night can induce a phase shift of the molecular oscillator, whereas it has no such effect when applied during the day. This is a central feature of entrainment, that is, the process by which circadian oscillators are synchronized to the environment. One way to consider how the membrane and the molecular clockwork are coupled is therefore by examining the signalling mechanisms through which light resets the circadian system^{9,10}.

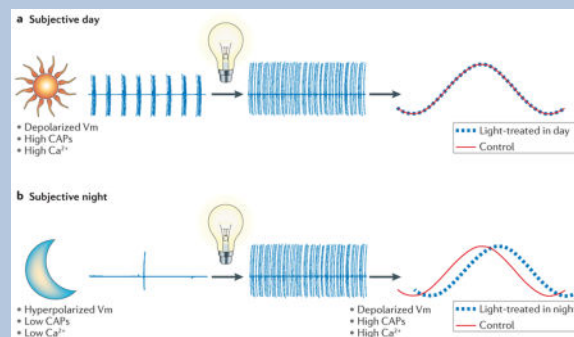
The cellular and molecular mechanisms by which light regulates the expression of *Per1* is the subject of much analysis (FIG. 2). The melanopsin-expressing retinal ganglion cells (mRGCs) are directly light-sensitive and receive information from rods and cones¹³⁰⁻¹³². These mRGCs thus encode ambient lighting¹³³ and generate action potentials that travel down the RHT and innervate the SCN. The RHT terminals release glutamate and, under certain conditions, the neuropeptide PACAP^{134,135}. The net result of RHT stimulation is an increase in firing rate of SCN neurons. These retinal-evoked excitatory postsynaptic responses in the SCN are mediated by AMPA¹³⁶⁻¹³⁹ and NMDA receptors^{140,141}. PACAP¹⁴² and the metabotropic glutamate receptors¹⁴³ seem to provide a mechanism to modulate the magnitude of these responses. In addition, there is a clear role for GABA regulation of the RHT input, with GABA type B (GABA_B) receptors acting presynaptically to regulate the release of glutamate^{144,145} and GABA type A (GABA_A) receptors acting postsynaptically to regulate the SCN neuron response to glutamate. Depending on the time

of day and the SCN region, these GABA_A receptor-mediated inputs can evoke hyperpolarizing or depolarizing responses in the SCN neuron^{83,146,147}.

During light exposure, the firing rate of SCN neurons is greatly increased, with larger increases during the night than during the day²⁵. Pioneering work by McMahon, Block and colleagues in molluscs has led to the development of a model that explains how the circadian system responds to photic stimulation in the night but not the day (BOX 2). Conceptually, elements of this model have begun to be applied to the SCN^{62,140}. The basic idea is that the circadian pacemaker neurons are already electrically active during the day and that additional excitatory input from the RHT does not induce many additional action potentials. By contrast, during the night, when the neurons are electrically silent, RHT stimulation results in a large change in electrical activity and the system responds to the change in action potential frequency with a phase shift.

Box 2

Diurnal gating of light input to the circadian system in the mollusc



One of the fundamental features of circadian oscillators is that their response to environmental stimulation varies depending on the phase of the daily cycle when the stimuli are applied. For example, the same light treatment can produce phase shifts of the oscillator when applied during the subjective night but have no effect when applied during the subjective day. This periodic sensitivity to photic stimulation is a central feature of entrainment, that is, the process by which circadian oscillators are synchronized to the environment. What are the cellular mechanisms that underlie this periodic sensitivity to environmental stimulation? Studies on the circadian oscillators in the eye of the marine molluscs *Aplysia* and *Bulla* have led to the development of a credible model to explain this daily variation in response to photic stimulation (see the figure). In these species, specialized retinal cells known as basal retinal neurons or secondary cells show a daily rhythm in membrane potential and spontaneous neural activity¹¹⁶. Central to the model is the hypothesis that this circadian rhythm in membrane potential drives a rhythm in Ca²⁺ influx through voltage-sensitive Ca²⁺ channels, and that light acts to cause a Ca²⁺ influx in these cells^{115,116,234}. During the day, the membrane potential (Vm) is relatively depolarized and Ca²⁺ enters the cell through voltage-sensitive Ca²⁺ channels. Light does not cause phase shifts because the intracellular Ca²⁺ concentration is already elevated (see the figure, part a). During the night, the membrane is relatively hyperpolarized and light-induced depolarization can cause a Ca²⁺ influx,

leading to a shift in the phase of the rhythm (see the figure, part **b**). Elements of this model are now being successfully applied to the mammalian SCN^{25,62,140,235}. CAPs, compound action potentials.

During the night, SCN neurons respond to photic stimulation transduced by mRGCs that generate action potentials of up to 20 Hz^{148–150}. These fast synaptic responses, which can be mediated by AMPA or GABA_A receptors, exhibit a short time course and move the SCN neuron into a voltage range that maximizes the contribution of the I_A, FDR, and BK currents that are described above. Indeed, mice lacking the FDR current show greatly attenuated NMDA-evoked responses in the SCN⁶⁹. It is likely that each of the K⁺ currents (I_A, FDR and BK) has a major role in determining how an SCN neuron responds to RHT input throughout the daily cycle, although this has not been tested yet. This light-induced increase in neural activity drives synaptic communication with the rest of the cells in the circuit through GABA, GRP and VIP. GRP produces long-term increases (lasting hours) in excitability in SCN neurons^{151,152}. Interestingly, the GRP-driven increases in firing rate are dependent upon the CREB pathway and activation of *Per1*. VIP acts presynaptically to alter GABA release¹⁵³ and postsynaptically to regulate voltage-gated currents in the SCN^{87,96}. These changes in electrical activity also trigger intracellular signalling cascades in SCN neurons.

The RHT stimulation during the night and the resulting increase in the frequency of action potentials produce a robust increase in Ca²⁺ in SCN neurons. In dendrites, the Ca²⁺ influx is probably mediated by NMDA receptors, with a major contribution from the NR2B subunit^{140,154}, whereas in the soma the L-type voltage sensitive Ca²⁺ channels are the main contributor⁶². There is also evidence indicating that T-type Ca²⁺ currents are required for glutamate-induced phase shifts in the SCN neural activity rhythm in rats⁶¹. The concentration of intracellular Ca²⁺ in neurons is tightly controlled by various channels, pumps and buffers. In addition, ryanodine receptors have a role in mediating the effects of light- and glutamate-induced phase delays of the circadian system¹⁵⁵ and in the regulation of the electrical activity of SCN neurons¹¹². Thus, RHT-evoked Ca²⁺ influx is likely to be a major transducer of light information to the circadian system.

The signal transduction events following the influx of Ca²⁺ that is induced by RHT stimulation during the night are beginning to be understood and include a number of signalling pathways^{156–158}. There is evidence for activation of MAPK–extracellular signal-regulated kinase (ERK)^{159–161}, calmodulin¹⁶², nitrogen oxide–cyclic GMP^{163,164}, PKC¹⁶⁵ as well as cAMP¹⁶⁶. The relative contributions of each pathway to regulating the molecular clockwork are not yet clear and there is evidence that the relative importance of each signalling cascade may vary between early and late night¹⁵⁷. There is no doubt that other signalling pathways are also involved, and the role of phosphatase cascades in counterbalancing these kinase cascades has not been adequately examined in the SCN. Moreover, light stimulation causes a robust and reliable increase in the proto-oncogene *c-Fos* and its phosphoprotein product FOS, and many research groups have made use of *Fos* induction to examine the impact of light on gene expression in the SCN^{167,168}. The kinase signalling pathways converge on CREB phosphorylation at Ser133 and Ser142 (REFS 155,169,170).

CREB became phosphorylated only at times during the circadian cycle when light induces *Fos* expression and behavioural phase shifts of circadian rhythms¹⁶⁹. These results suggest that circadian clock gating of light-regulated molecular responses in the SCN occurs upstream of CREB phosphorylation. Phosphorylated CREB is translocated into the nucleus, where it can bind to CREs in the promoter regions of *Per1* and *Per2* (REF. 171). The net result is an increase in *Per1* and *Per2* message that occurs over the next two hours¹⁷². This suggests that increasing the transcription of *Per* genes in the early night delays the molecular clockwork by postponing the normal decline in *Per* expression, whereas increasing the transcription of these genes in the late night speeds up the normal increase in *Per* expression at the beginning of the next cycle, although this has not been explicitly tested.

Several lines of evidence indicate that the phosphorylation of CREB is a crucial mediator of the effect of light on the circadian system. For example, transgenic mice in which phosphorylation at Ser142 cannot take place¹⁷⁰ or that express a CREB repressor¹⁷³ show attenuated light-induced gene expression in the SCN and a reduced behavioural response to light exposure. Similarly, the use of a CRE decoy oligodeoxynucleotide in rats¹⁷⁴ reduced both light-induced behaviour *in vivo* and glutamate-induced increases in *Per1* mRNA and phase shifts *in vitro*. This evidence indicates that CRE-driven gene expression is part of the mechanism by which membrane events regulate clock gene expression in this system but it seems likely that other pathways are involved, including translational and post-translational regulation. Recent work suggests that light-evoked increases in firing also activate the mammalian target of rapamycin (mTOR) pathway and thereby regulate the translation of *Per* genes¹⁷⁵. Also, PKC seems to regulate photic resetting through post-translational mechanisms that impact the stability and intracellular distribution of PER2 (REF. 165). So, like the rest of neuroscience, the circadian field has to sort through an alphabet soup of intracellular signalling cascades to explain how a light-induced increase in the firing rate of SCN neurons drives chromatin remodelling, transcriptional, translational and post-translational processes to regulate the expression of clock genes in the SCN.

The activity-dependent transcription and release of brain-derived neurotrophic factor (BDNF) has various roles in the nervous system in linking neural activity with synaptic changes that underlie circuit development and functions^{176,177}. Previous work has raised the possibility that BDNF may be an important regulator of synaptic input into the SCN. The transcriptional repressor methyl-CpG-binding protein 2 (MECP2) is expressed in the SCN and this *Bdnf* regulator is phosphorylated in response to light¹⁷⁸. Both BDNF and its high-affinity BDNF/NT3 growth factors receptor (TRKB) are expressed in the SCN^{93,179,180}. Physiological data demonstrated that BDNF and neurotrophin receptors can enhance glutamatergic synaptic transmission within a subset of SCN neurons¹⁸¹, and can potentiate glutamate-induced phase shifts of the circadian rhythm of neural activity in the SCN¹⁸². Functionally, both BDNF-deficient and TRKB-deficient mice showed reduced behavioural phase shifts in response to light exposure during the subjective night^{183,184}. The evidence is consistent with the hypothesis that BDNF may gate light input to the circadian system — that is, BDNF secreted at night may be required for light-induced phase shifts.

The molecular clock regulates activity

The best evidence that the molecular clockwork in the SCN can drive the rhythms in electrical activity — and, by extension, in behaviour and physiology — comes from several studies that have explored the impact of mutations in the core clockwork on electrical activity rhythms recorded in the SCN. The *Tau* (casein kinase 1 ϵ) mutation in hamsters shortens the period of wheel-running activity and neural activity rhythms¹⁸⁵. Similarly, homozygote *Clock* mutant mice are behaviourally arrhythmic, whereas heterozygote animals show lengthened behavioural rhythms, findings that are paralleled by physiological recordings from the SCN^{32,186}. Furthermore, mutant mice lacking *Cry1* and *Cry2* show behavioural arrhythmicity and loss of rhythms in SCN neural activity²². These findings suggest that clock gene rhythms are translated into daily patterns of action potential activity in the SCN. Together, these studies provide clear evidence that the molecular clockwork can drive neural activity as an output.

However, very little is known about the mechanisms by which the molecular clockwork drives rhythms in neural activity (FIG. 3). The expression of the transcripts for the clock genes *Per1* and *Per2* peak at midday (circadian time 4–circadian time 6) in the SCN, a little before the peak in neural activity. So at dawn, when electrical activity is rising, the CLOCK–BMAL complex starts to drive the transcription of *Per* and *Cry* genes. An important issue, which has not been tested directly, is whether blocking the increase in *Per* or *Cry* transcripts alters the rise in neural activity. Application of the peptide GRP in the night produces a long-term increase in neural activity that is blocked by antisense oligodeoxynucleotides against *Per1* (REF. 151). In addition, a recent study in *D. melanogaster* suggests that CRY can alter membrane potential through redox-based regulation of a K⁺ channel conductance¹⁸⁷. Although it is too early to know whether a similar mechanism is involved in the SCN in mammals, the striking parallels between *Drosophila* and mammalian models certainly suggest that we should consider the possibility. In addition, the molecular clockwork is a potent regulator of transcription and there is evidence of rhythmic transcription of several ion channels, including L- and T-type Ca²⁺ channels, BK channels and K2P K⁺ channels. The genes that encode these channels could be regulated by the CLOCK–BMAL protein complex acting directly on E-box or other elements that are present in the regulatory sequences of these genes. In *Drosophila*, circadian rhythms in a mRNA that encodes a regulatory protein associated with BK channels have been described^{188,189}. It is difficult to obtain clear data on the half-life of the proteins coding for ion channels *in vivo*. In culture, Kv1.3 channel proteins have a half-life of 1–2 hours and this duration can be regulated by TRKB¹⁹⁰. The findings that channel-specific antisense oligodeoxynucleotides¹⁹¹ and RNA interference¹⁹² can produce physiological effects within 24 hours suggest that daily rhythms in transcripts for channel proteins may well be functionally important in generating daily rhythms in membrane potential.

Another possible mechanism by which the molecular clockwork drives rhythms in neural activity involves the circadian trafficking of proteins, and there are examples of channels that can be inserted or removed from the membrane to rapidly alter synaptic function. One of the core mechanisms that is thought to underlie long-term changes in the strength of excitatory synaptic transmission involves the rapid insertion and removal of AMPA-type

glutamate receptors into a postsynaptic membrane^{193–195}. These changes in AMPA receptor localization can occur within minutes, driven by neurotransmitter regulation of the cAMP–PKA signalling pathway. There is also evidence that this type of trafficking can occur with intrinsic voltage-gated channels. One of the most interesting examples comes from work on the Kv3.1 channels. In the auditory system, the distribution of Kv3.1b can become rapidly (within 30 min) and specifically modulated after brief periods of acoustic stimulation¹⁹⁶. This rapid modulation of the FDR current plays a part in maintaining appropriate levels of intrinsic neuronal excitability in different acoustic environments and may be the result of rapid translational regulation through the fragile X mental retardation protein 1 (FMR1)¹⁹⁷. The Kv3 channels seem to be directly bound to kinesin 1 heavy chain¹⁹⁸ and so can be directly targeted to the membrane to regulate excitability. We do not know if these types of rapid regulation of channel proteins occur in the SCN but it seems possible. The FDR current, Kv3.1 channels and *Kcnc1* transcripts do show evidence for circadian variation. The daily insertion of the Kv3 channels into the membrane of SCN neurons would be consistent with the experimental data. Interestingly, the loss of the gene encoding FMR1 causes substantial disruption of circadian rhythms in flies¹⁹⁹ and mice²⁰⁰. At present, there is no direct evidence for circadian clock regulation of the protein trafficking of ion channels.

Post-translational modifications of channels and associated auxiliary proteins are perhaps the most likely explanation for the circadian variation in the electrical activity of SCN neurons. Outside of the SCN, numerous studies have shown the importance of kinase and phosphatase activity in mediating short-term changes in current function that alter electrical excitability^{80,201,202}. In chick photoreceptors, circadian oscillations in the expression of cone cGMP-gated channels have been described²⁰³. Within the SCN, there is clear evidence for a daily rhythm in the levels of cAMP^{55,120,121} and Ca²⁺ (REFS 110,111). The daily rhythms in cAMP- and Ca²⁺-associated signalling pathways would be expected to drive daily rhythms in the activity of ion channels and membrane currents. In the nervous system, many ion channels contain auxiliary proteins that modulate pore forming subunits. These proteins are attractive targets for post-translational regulation that could mediate the daily rhythm in the Ca²⁺ and K⁺ currents observed in SCN neurons. Thus, circadian patterns of phosphorylation almost certainly drive circadian rhythms in the membrane properties of SCN neurons. Future studies are needed to explore the mechanistic links between the molecular circadian oscillator and channels (including pore forming subunits and auxiliary proteins) in the membrane of SCN neurons.

Effects of ageing and disease

Disruptions in sleep–wake cycles, including decreased amplitude of rhythmic behaviours and fragmentation of sleep episodes, are commonly associated with ageing in humans and other mammals^{204–206}. Although undoubtedly many factors contribute to these changes, a body of literature^{207–211} is emerging that is consistent with the hypothesis that an age-related decline in the output of the central circadian clock in the SCN may be key. *In vivo* multiunit recordings from the SCN of freely moving young and middle-aged (12–14-month-old) mice have shown that the day–night difference in neural activity was substantially reduced in the SCN of middle-aged mice²¹². Furthermore, the neural activity rhythms were clearly disrupted in the subparaventricular zone, one of the main neural outputs of the SCN.

Surprisingly, the molecular clockwork in the SCN, as measured by PER2 levels, was not disrupted in middle-aged mice, suggesting that the age-related disruption in the circadian output (measured at the level of neural activity rhythms in the SCN) occurs before any disruption of the molecular clockwork.

Patients suffering from neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and Huntington's disease commonly exhibit sleep disorders. These patients have difficulty in sleeping at night and staying awake during the day. This dysfunction in sleep timing may not be causal to their disorder, yet these symptoms have a major impact on the quality of life of the patients and on their caregivers. Although the pathology underlying these symptoms has not yet been identified, there has been some intriguing work using mouse models of neurodegenerative diseases^{213–216}. Most of these mouse models exhibit circadian disruption and there is at least some evidence that treatments that are designed to stabilize these rhythms can improve other, non-motor symptoms in these mice^{214,217}. In one of the mouse models of Huntington's disease (BACHD mice) in which the mutated human huntingtin (*HTT*) is expressed, the output of the circadian system, as measured by locomotor activity, heart rate and body temperature, was profoundly disrupted early in the life span²¹⁶. The neural activity rhythms in the SCN, but not rhythmic PER2 expression, were also reduced in the BACHD mice. Further studies are needed to identify the underlying mechanism, but it seems that the neural activity is disrupted while the molecular clockwork keeps ticking.

Finally, infectious diseases may also be associated with disrupted neural activity rhythms within the SCN. For example, African sleeping sickness is characterized by disruption of the sleep–wake cycle²¹⁸. The underlying mechanisms have been studied in rats that are infected with *Trypanosoma* parasites. In this model, circadian behaviours and neural activity rhythms in the SCN are disrupted²¹⁹. Although there is some effect on clock gene expression in the SCN, there is a more profound impact on the gene expression rhythms in peripheral organs, such as the pituitary and the spleen²²⁰. Interestingly, these effects may be mediated by the proinflammatory cytokine interferon- γ (IFN γ), which has also been shown to alter neural activity within the SCN²²¹. As the release of this compound is part of the response of immune cells to infections, cytokine-induced changes in SCN neural activity could be part of the mechanism underlying the loss of a coherent sleep–wake rhythm that is so characteristic of our body's response to infectious disease.

Based on these findings, I propose that the neural activity rhythms in the SCN may be the 'weak link' of the circadian system. With ageing, and in models of neurodegeneration, there is a weakening of the neural output despite fairly normal functioning of the molecular clock. Importantly, the maintenance of neural activity is extremely metabolically demanding. 2-deoxyglucose studies have revealed robust rhythms in energy metabolism in the SCN, with peaks during daytime^{21,65}. Perhaps owing to this demand, neural activity may be a critically sensitive output of the SCN. It is possible that any health condition that compromises metabolism will alter SCN output through this mechanism. The disruption of circadian neural activity rhythms is likely to have profound consequences on patient health^{222,223}. It is becoming increasingly clear that robust daily sleep–wake rhythms are essential to good health. A wide range of studies have demonstrated that disruption of the circadian system

leads to a cluster of symptoms, including metabolic deficits^{208,224,225}, cardiovascular problems^{226,227}, difficulty in sleeping^{228,229} and cognitive deficits^{230–232}. Many of these same symptoms are seen in ageing and neurodegenerative diseases. This suggests that we should put a greater emphasis on the development of pharmacological tools and behavioural interventions that can boost neural activity rhythms in the SCN in situations in which the molecular clock may still be working.

Conclusions and future directions

The neural activity patterns of SCN neurons are dynamically regulated across the circadian cycle. Even in the absence of external stimulation, these neurons exhibit a robust daily rhythm in neural activity that is high in the day and low in the night. A set of intrinsic voltage-sensitive currents is responsible for the circadian variation in firing rate of SCN neurons; these include currents that are responsible for providing the excitatory drive required for all spontaneously active neurons, currents that translate this excitatory drive into a regular pattern of action potentials and currents that, through hyperpolarizing the membrane, are responsible for the nightly silencing of firing. The role of specific currents that regulate spontaneous firing and how those currents change from day to night, to drive the rhythm in neural activity, is beginning to be understood. Unfortunately, there are a number of important gaps in our knowledge. For example, the K^+ current that is turned on at night to silence firing has not yet been identified (although there are some candidate channels).

The daily increase in firing rate that starts near dawn has been assumed to be an output of the circadian system, that is, it has been assumed that the neural activity rhythms are directly driven by the molecular clockwork that occurs in single cells. There is now evidence to suggest that neural activity and synaptic activity are not just driven by the molecular clockwork but are also required for the generation of robust rhythms in gene expression. The possible signalling cascades that may be involved in this link are slowly being revealed and have led to the hypothesis that dysregulated neural activity and synaptic transmission weakens basal Ca^{2+} -CRE activity to a level that is insufficient to drive *PER* and *CRY* expression. One key issue that needs to be resolved is the role of development in these effects. During development, patterns of neural activity have organizational effects on developing neural circuits²³³. Genetic manipulations that alter neural activity in the SCN throughout development need to be interpreted in consideration of the possibility of organizational effects. Another issue that needs clarification in future experiments is whether the impact of a number of experimental manipulations of neural activity on gene expression is due to a loss of action potentials or to a chronic hyperpolarization, both of which reduce Ca^{2+} - and cAMP-driven transcription. Furthermore, many of the pharmacological and genetic manipulations that have been used to block action potentials also block synaptic transmission of crucial neuropeptides, and careful studies are required to tease apart these two mechanisms.

Our understanding of the mechanisms by which the molecular clockwork drives changes in the membrane is particularly incomplete. They may involve rhythmic transcription and/or translation of ion channels, rhythmic regulation of ion channel trafficking and distribution as

well as rhythmic regulation of the channels' open state by phosphorylation. This is an important area for future work.

Present evidence suggests that ageing and neurodegenerative diseases may have a particularly strong impact on neural activity rhythms in the SCN, although molecular rhythmicity in the SCN seems to remain relatively stable. This degradation of circadian output at the level of the SCN is likely to have profound consequences on the individual's health^{222,223}. If neural activity rhythms in the SCN are the 'weak link' of the circadian system in ageing and neurodegenerative diseases, we should be looking for interventions that boost the neural output of the circadian system for healthy ageing and management of neurodegenerative disease.

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Glossary

Riluzole	Riluzole preferentially blocks tetrodotoxin-sensitive sodium channels but has been suggested to have other effects, including activating glutamate uptake and increasing potassium currents
BMAL1	A protein that dimerizes with CLOCK to form a complex. Inside the nucleus, the protein complex binds to a site in the DNA known as the E-box, to drive transcription. The period and cryptochrome genes contain E-boxes and thus are transcriptionally activated by the complex formed of a brain and muscle ARNT-like (BMAL) protein and CLOCK, in the beginning of the daily cycle
Period	The time that it takes for the biological oscillator to complete one cycle. In the case of circadian oscillators, the period (τ) is close to, but not equal to, 24 hours
Phase shift	A shift in the phase of the biological oscillation. Phase (Φ) is one of the most important parameters that describe any oscillation, as it refers to the time points within the cycle. To measure the phase of the rhythm, a reliable reference point must be chosen. In the case of circadian oscillations, the onset of activity or the peak expression of a biochemical parameter are commonly used
Entrainment	In the context of the circadian system, entrainment refers to the process by which a biological oscillator with a free-running period that is close to 24 hours is adjusted to the exact 24-hour period of the environment. When entrained, the period of the

**CRE decoy
oligodeoxynucleotide**

biological rhythm equals the period of the entraining stimuli and the two oscillations exhibit a stable phase relationship

A synthetic DNA molecule that contains a cyclic AMP-responsive element (CRE). When expressed in a cell, the CRE decoys compete with native CRE sites in gene promoters for binding of phosphorylated CRE-binding protein (CREB). Thus, these oligodeoxynucleotides can function as competitive inhibitors of CREB binding

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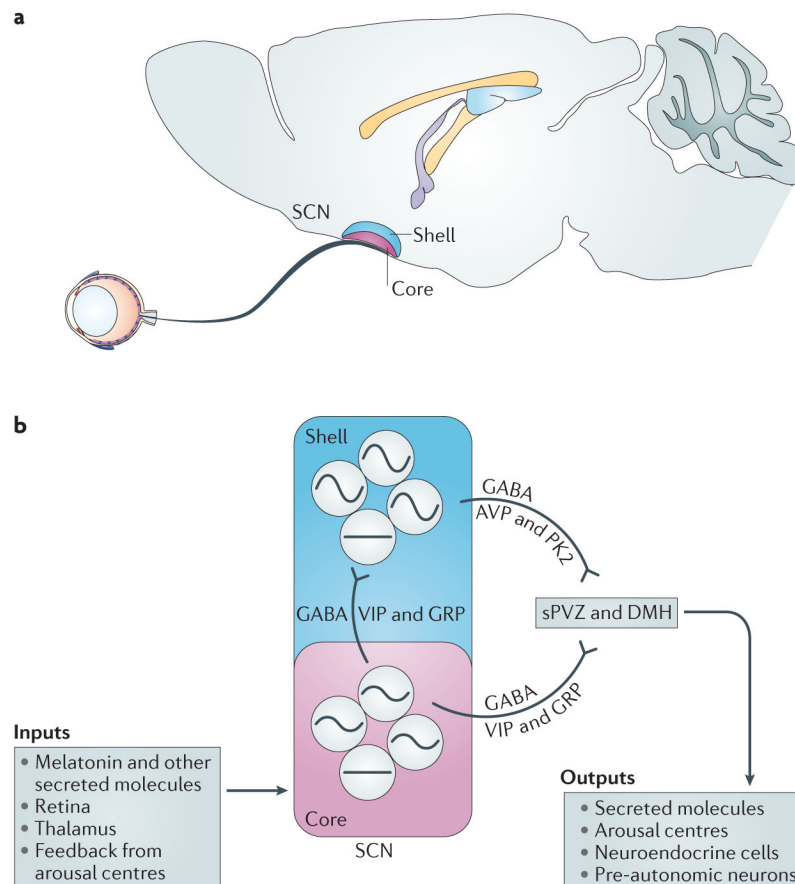


Figure 1. The suprachiasmatic nucleus circuit

a | The suprachiasmatic nucleus (SCN) in the hypothalamus is often divided into two anatomical and functional subdivisions: a ventrolateral ‘core’ and a dorsomedial ‘shell’. **b** | SCN core neurons are thought to integrate external input, including light information from the retinohypothalamic tract (RHT) and information from the thalamus and from midbrain structures such as the raphe nucleus. Core neurons relay this information to the rest of the SCN using GABA and vasoactive intestinal peptide (VIP) or gastrin-releasing peptide (GRP). Shell neurons use GABA and arginine vasopressin (AVP) or prokineticin 2 (PK2) to communicate with other cell populations, and at least some of the SCN shell neurons are neurosecretory cells that rhythmically release signalling molecules, including AVP, into the third ventricle. The amplitudes of the rhythms in gene expression and neural activity in core and shell neurons are relatively low and high, respectively. The outputs of core and shell SCN neurons travel mainly to other hypothalamic regions, including the subparaventricular zone (sPVZ) and the dorsal medial hypothalamus (DMH). These hypothalamic relay nuclei send projections throughout the CNS and endocrine system. Major centres in the brain that control arousal, such as the raphe nucleus, locus coeruleus, hypocretin or orexin neurons, and pars tuberalis, are rhythmically regulated through projections from the SCN.

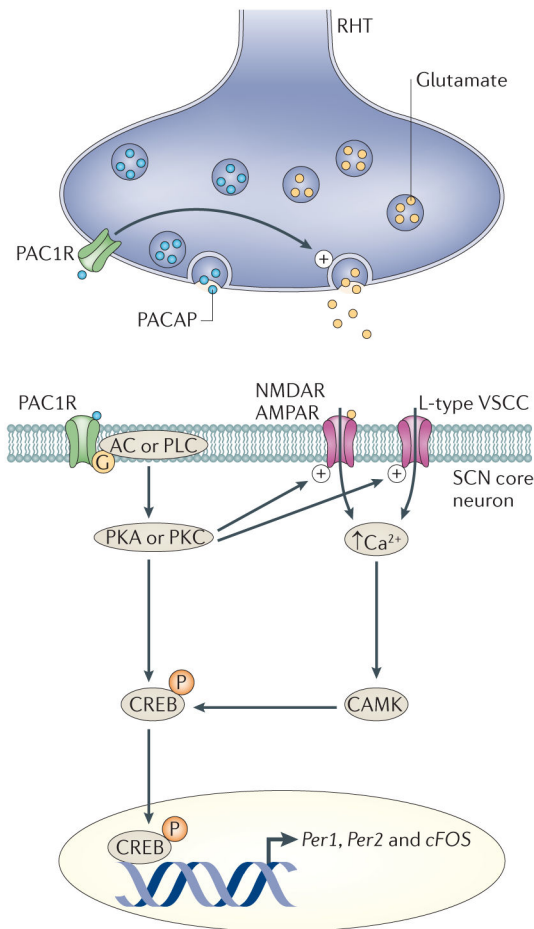


Figure 2. How light regulates the molecular clockwork in SCN neurons

The best understood pathways by which neural activity regulates clock gene expression comes from studies that have explored how light turns on the transcription of period 1 (*Per1*) during the night. Melanopsin-expressing retinal ganglion cells encode ambient light and generate action potentials that travel down the retinohypothalamic tract (RHT) and innervate the suprachiasmatic nucleus (SCN). The RHT terminals release glutamate and, under certain conditions, the neuropeptide pituitary adenylate cyclase activating peptide (PACAP). The net result of RHT stimulation is an increase in firing rate and Ca^{2+} increase in SCN neurons through both activation of glutamate receptors (AMPA and/or NMDA receptors) and voltage-sensitive calcium currents (VSCCs). So far, all of the available evidence indicates that the PACAP type I receptor (PAC1R; also known as PACAPR1) is responsible for mediating the effects of PACAP on SCN neurons. Functionally, PACAP presynaptically enhances the release of glutamate onto SCN neurons and postsynaptically enhances the magnitude of NMDA and AMPA currents within the SCN. The increase in Ca^{2+} activates a number of signalling pathways that converge to alter transcriptional and/or translational regulators, including cyclic AMP-responsive element (CRE)-binding protein (CREB). Phosphorylated CREB is translocated into the nucleus where it can bind to CREs in the promoter regions of *c-Fos*, period 1 (*Per1*) and *Per2*, and drives transcription of these genes over the course of hours. Within the SCN, the photic regulation of *c-Fos* and *Per1* is

rapid, whereas the regulation of *Per2* exhibits a slower time course. AC, adenylyl cyclase; CAMK, calcium/calmodulin-dependent protein kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C.

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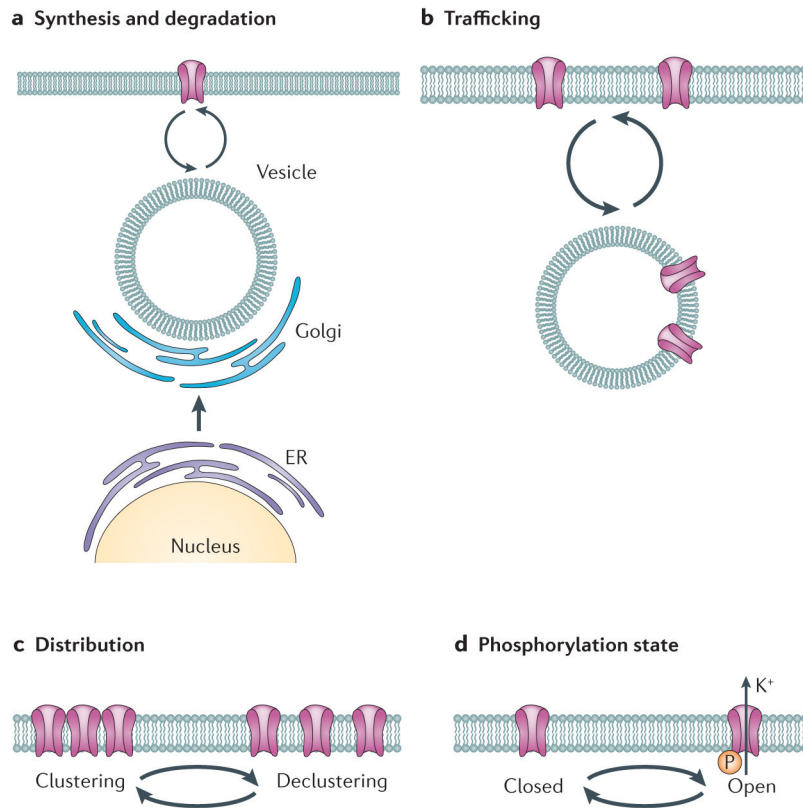


Figure 3. Possible mechanisms by which the molecular clock can regulate spontaneous neural activity in SCN neurons

a | Ion channels and other membrane proteins are made and then transported to the membrane through the rough endoplasmic reticulum (ER) to the Golgi. The membrane proteins are transported to the membrane and removed for degradation through vesicles. There is evidence of the rhythmic transcription of several ion channels, including L- and T-type Ca²⁺ channels, large-conductance Ca²⁺ activated K⁺ (BK) channels and two-pore K⁺ (K2P) channels. Although we do not know the half-life of these channels in the SCN, direct transcription and translational regulation of membrane proteins is an obvious candidate mechanism for driving the observed rhythms in spontaneous neural activity. **b** | AMPA receptors and potassium channels have been shown to be rapidly inserted and removed from the membrane in response to physiological stimulation. Therefore, the daily trafficking of ion channels and associated proteins is another possible mechanism that may be responsible for the firing rate rhythms. **c** | The distribution of ion channels within the plasma membrane can change from day to night, and this may also contribute to changes in firing rate rhythms between day and night. **d** | Robust circadian rhythms in signalling pathways, including pathways involving Ca²⁺, cyclic AMP, phospholipase C (PLC), casein kinases and RAS-dependent mitogen-activated protein kinases (MAPKs), have been described. These pathways are robust regulators (through phosphorylation) of ion channels and associated proteins. Circadian regulation in the phosphorylation state of ion channels through the balance between kinase and phosphatase activities is a likely mechanism that underlies the rhythm in spontaneous neural activity in SCN neurons. In summary, a diverse set of

mechanisms, including changes in transcription and translation, direct phosphorylation, trafficking and distribution of ion channels (and associated proteins (not shown)) could underlie the rhythms in membrane events that characterize SCN neurons.

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Table 1

Ion channels and functions in the suprachiasmatic nucleus

Function	Current	Is current rhythmic?	Channels and their genes	Rhythmic expression?	Refs
Excitatory drive	Persistent Na ⁺	No	Nav1.8 (<i>SCN8A</i>) and Nav1.9 (<i>SCN9A</i>)	No	45-49
	L-type Ca ²⁺	Yes	Cav1.3 (<i>CACNA1C</i>) and <i>CACNA1S</i>	Yes	45,47,48, 57,59
	T-type Ca ²⁺	?	Cav3 (<i>CACNA1G</i>)	Yes	47,48,51,57,60,61
Modulation of firing and action potential width	Fast delayed rectifier (FDR)	Yes	Kv3.1 (<i>KCNK3</i>) and Kv3.2 (<i>KCNK2</i>)	?	48,68,69
	A-type K ⁺	Yes	Kv4.1 (<i>KCND1</i>) and Kv4.2 (<i>KCND2</i>)	No	60,70-72
Nightly silencing	Large-conductance Ca ²⁺ -activated K ⁺ (BK)	Yes	BK (<i>KCNMA1</i>)	Yes	47,48,73-75,81
	Two-pore K ⁺ (K2P)	?	TASK1 (<i>KCNK1</i>) and TASK2 (<i>KCNK2</i>)	Yes	47,48
Others	Hyperpolarization-activated, cyclic nucleotide gated	No	HCN1 (<i>HCN1</i>) and HCN2 (<i>HCN2</i>)	No	47,52-54, 56
	Na ⁺ -K ⁺ ATPase	Yes	?	?	63,64
	Chloride pump	Yes	NKCC1 (<i>SLC12A2</i>)	Yes	47,82,8