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# Beyond the molecular clock

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The molecular basis of the internal circadian clocks that control daily rhythms in almost all organisms has been characterized in detail over the last 40+ years. These clocks are set by external signals from the environment, allowing organisms to anticipate daily events no matter the season. In order modulate behavior and physiology, molecular clocks then regulate cellular properties in order to communicate within networks of clock-containing cells to generate particular outputs at specific times. Here we review the current understanding of 1) how networks of molecular clocks maintain robust, synchronized daily oscillations; 2) how clock networks respond to changing environmental conditions; and 3) how circuits of clock neurons are organized to impose daily rhythms on behavior, focusing on the latest developments from *Drosophila* and mice.

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## Introduction

Having an accurate internal representation of time appears an essential feature of life. Innumerable studies have identified 'circadian' clocks in organisms from bacteria to fungi, plants and animals that run at 24 h even when isolated from daily light:dark cycles. Under normal conditions these endogenous clocks are set (entrained) by the environment, so the clock tells the correct time as seasons change, but remains robust enough to keep accurate time from day to day. The advantage of having an endogenous clock is thought to be to anticipate daily environmental changes, and prepare accordingly - reindeer in the arctic switch off their internal clocks and lose daily rhythms during the constant dark of winter or light of summer [1], when there is no longer dawn or dusk to anticipate.

Although circadian rhythms have been studied since at least the 18th century, the characterization of the molecular basis of the clock began when Ron Konopka and Seymour Benzer were searching for a complex behavior controlled by a single gene, and eventually identified the first three clock gene mutations, all in the *period (per)* gene of *Drosophila* [2]. The reasons that circadian rhythms were the first behavior shown to be controlled by a single gene also explain why the clock been extensively studied ever since: mutations of core clock genes produce easily quantifiable phenotypes (for a behavior), either changing the period of the clock from 24 h, or rendering animals arrhythmic. Additionally, *per* and many other clock components are non-essential genes, and produce few detrimental phenotypes outside the clock.

We now know most overt physiological rhythms across phyla depend on transcription-translation feedback loops (TTFL), where proteins repress their own RNA, establishing oscillations which are extended to 24 h through regulation of transcription, translation, phosphorylation, and degradation [3]. For the molecular clock to regulate behavior and physiology, it must modify the cell in which it oscillates, and communicate time of day information to other cells within an organism. The transcription factors at heart of TTFL can drive rhythmic transcription of 'clock-controlled genes', which alter cellular properties in a time of day-dependent manner – for instance, driving neurons to fire at specific times of day. Here we will explore how networks of these clock-containing cells impose circadian rhythms on an organism.

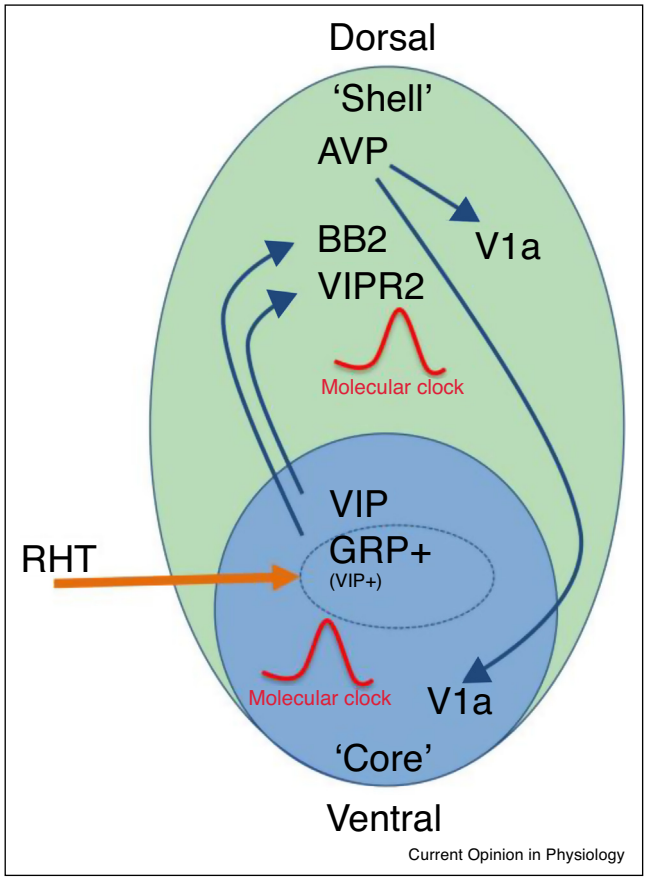
## Neural circuits and clocks

### Maintenance of phase relationships within the SCN

Unlike many other behaviors, circadian rhythms are predictable from day to day – the same neurons do the same thing every day if external conditions remain the same, an ideal system for studying neuronal communication. In mammals, the suprachiasmatic nucleus (SCN), 20 000 neurons at the base of the hypothalamus, is the 'master pacemaker', controlling daily rhythms of behavior and synchronizing clocks throughout the body. The SCN receives a direct signal from the visual system and therefore is rapidly reset by light, but it takes longer for signals released by the SCN to reset peripheral oscillators – hence some of the symptoms of jet lag result from desynchrony between the SCN that has entrained to a new time zone, and the rest of the body that has not. The light input pathway has recently been reviewed in [4]; in total, ~35 distinct brain regions directly project to the SCN, and a further 48 connect within 2 or more synapses, reviewed in Ref. [5].

The SCN is often divided into 'core' and 'shell' regions-based anatomy and the expression of the neuropeptides Vasoactive intestinal peptide (VIP, core) and Argenine vasopressin (AVP, shell) [6] (Figure 1). Once the SCN is entrained to a light cycle, it continues to oscillate in the entrained phase for several days *ex vivo*, facilitating the study of neural SCN networking using reporters of clock gene transcription, protein expression, $Ca^{2+}$ and cAMP. When communication between SCN neurons is blocked, through application of TTX [7], through neuronal dissociation [8], or in the absence of VIP signaling [9], individual SCN neurons continue to oscillate with multiple different periods, thus coupling is essential for accurate and robust daily rhythms. Within individual neurons, a daily rhythm in $Ca^{2+}$ peaks ~2.5 h in advance of clock-driven transcriptional rhythms of *Per1*, followed ~2 h later by PER1 protein [10]. $Ca^{2+}$ rhythms are also cell autonomous, driven by the clock and modulated by inputs from the SCN network [11].

**Figure 1**




Structure of the suprachiasmatic nucleus (SCN).
The SCN is divided into distinct regions based upon the expression of VIP more ventrally, in the 'core' and AVP more dorsally, in the 'shell'. Core neurons innervate shell neurons, with the VIP receptor VIPR2 found in the shell, while the AVP receptor V1a is expressed across the SCN, allowing bi-directional communication. Both VIP and AVP SCN neurons are required for robust daily rhythms of behavior [69]. There is functional heterogeneity of neuronal activity even within core and shell neurons – for instance the neuropeptide Neuromedin S is expressed in ~20% of VIP and ~30% of AVP neurons, and is required for SCN coupling and normal circadian behavior [70]. A subset of VIPergic SCN neurons also express gastrin-releasing peptide (GRP) and receive light input via the retinohypothalamic tract (RHT), then transmit light information to the rest of the SCN.

### Networking establishes phase differences across the SCN

Despite having identical molecular clocks in different neurons, the phase of clock oscillations varies across the SCN, with peak expression levels reached up to 8 h earlier in more dorsal regions [7,12]. These phase differences are a network property, and depend on G-protein mediated regulation of cAMP [13], itself a core component of the mammalian circadian clock [14] that acts to induce expression through cAMP-response elements, as found in the *Per1* promoter [10]. VIP signals via Gs to increase intracellular cAMP, ultimately changing clock phase in neurons expressing VIPR2. As PER1 promotes SCN neuron firing [15], this also results in SCN neurons firing at different times of day. This is consistent with the long-standing observation that the SCN is most electrically active during the day [16], but individual SCN neurons differ in terms of time or duration of electrical activity [17,18]. SCN electrical activity correlates with sleep in mice; as silencing the SCN with TTX causes mice to become active [19], while activating VIP neurons inhibits behavioral activity [20••], SCN electrical activity may directly promote sleep.

### Neural circuits and clocks – SCN plasticity

As seasons change the clock needs to be set ('entrained') by day length, and this depends on photoperiod-induced SCN plasticity (reviewed in Refs. [18,21]), with a broader distribution of electrical phases of SCN neurons when days are long, and consolidation under short days [22]. It has long been known that exposure of young mice to days longer or shorter than 24 h (22 h or 26 h t cycles) induces 'aftereffects', changes in behavioral free running period that persist in constant conditions (usually darkness (DD)). After exposure to altered t-cycles, the SCN becomes polarized in terms of clock phase, with the period of the core showing a positive correlation with the period of the t-cycle (long or short) whereas the shell shows an inverse relationship [23]. This plasticity revealed by altered t-cycles is likely an exaggerated response that normally deals with seasonal changes, as entrainment to long days (>20 h light in a 24 h cycle) also polarizes the SCN, with core leading the shell by ~6 h [12]. SCN polarization is a network property as physically separating core and shell, adding TTX or the $GABA_A$ antagonist gabazine restore the normal phase relationship after t cycle exposure [23]. Recordings from SCN slices as long day-induced polarization resets over time *ex vivo*, suggest that $GABA_A$ and VIP promote coupling in a polarized SCN, but opposes coupling in a non-polarized

SCN [12], consistent with the previously reported role of GABA in promoting SCN desynchrony [24]. Although SCN GABA is predominantly inhibitory, some more dorsal SCN neurons are excited by GABA, and the proportion these neurons increases as the ratio of the $Cl^-$ co-transporters KCC2 and NKCC1 changes, switching GABA from inhibitory to excitatory as day length increases [25], which may explain differences in GABA function during polarization.

Aftereffects induced by t cycles depend on epigenetic modifications within the SCN: changing day length alters expression of genes associated with chromatin modification resulting in global changes in DNA methylation, and blocking DNA methlylation prevents aftereffects on free running period [26], restoring SCN phase relationships [23]. Presumably day length-dependent modifications of methylation fine-tune the SCN clock to the precise environmental conditions in which an organism finds itself. Once the SCN has entrained to a new LD cycle (a new season) it must 'remember' the season, rather than immediately return to a steady state. One possibility is that GABA/VIP signals ultimately alter methylation within the SCN, forming a semi-permanent memory of time of day, similar to observed changes in methylation under changing seasons in the hamster hypothalamus [27]. As behavioral aftereffects are only observed in young mice, it is likely the SCN is more plastic during development, allowing the SCN clock of a newborn mouse to be precisely set to the environment in which it is likely to spend the rest of its life.

### Beyond the neurons: the role of glia in circadian time keeping

The robust and self-sustained nature of pacemaker oscillations is often regarded as a consequence of coupling between SCN neurons. However, glia within the SCN also make a major contribution as changing the period or stopping the glial clock alters SCN rhythms and behavior [28,29••,30]. In contrast to SCN neurons, SCN glia are most metabolically active at night where they inhibit SCN neuronal activity via glutamate [29••], with a functional clock within SCN glia required for normal, rhythmic GABA uptake [28]. Similarly, *Drosophila* glia are required for normal activity of 'master clock' neurons (defined below) [31], and a blood brain barrier rhythm where permeability is higher at night [32]. As the structure and transport through the *Drosophila* blood brain barrier is similar to mammals, this may have implications for human health and treatment of disease where drugs that target the brain may be more effective at specific times of day [32].

## Circadian neural circuits and behavior

### *Drosophila* clock circuits

Communication between neurons allows the SCN to contain multiple representations of time, respond to and remember changing seasonal conditions, and function as an accurate timekeeper, delivering coherent signals to downstream cells. However, there are 20 000 SCN neurons, making it difficult to precisely determine the function of individual neuronal subsets. In *Drosophila* there are just 150 clock neurons that display $Ca^{2+}$ oscillations in five distinct phases in DD, despite having similarly phased molecular clocks [33••]. Two of these populations of cells were previously identified as 'Morning' (M) and 'Evening' (E) cells based upon their roles in promoting activity anticipating dawn and dusk respectively [34,35], and the timing of M and E cell $Ca^{2+}$ is consistent with morning and evening activity; three other populations of cells peak at different times, a network property based upon neuropeptide signaling that allows a clock circuit to send different outputs at different times of day. Clock neuronal circuits must then signal to downstream targets in order to regulate behavior. In *Drosophila*, clock neurons send circadian signals via neuropeptides to impose rhythms on otherwise arrhythmic neurons, ultimately regulating activity, and promoting robust behavioral rhythms [36,37]. In mammals, where most neurons have molecular clocks, similar relays could provide a mechanism for promoting and spreading circadian synchrony across the brain, or to transmit clock information to motor control centers, regulating activity.

*Drosophila* M and E cells are required for seasonal adaptation, supporting a model originally proposed for nocturnal rodents [38]. Overall, there is great similarity between M cells and the SCN: both act as 'master pacemakers', required for 24 h rhythms of behavior and setting the phase of other neuronal clocks. M cells express the neuropeptide PDF, the SCN VIP; VIP and PDF receptors are both GPCRs that signal via Gs to induce cAMP, synchronize oscillations between clock neurons and promote rhythmicity [39]. At the cellular level, the morning firing of fly clock neurons depends on clock-driven expression of NCA localization factor-1, leading to depolarization in the morning via a sodium leak channel Narrow Abdomen (NA), and silencing by peak basal $K^+$ current in the evening; the mammalian NA homologue and daily anti-phase cycles of $Na^{2+}$ and $K^+$ currents also drive SCN firing rhythms [40]. Thus studying networks of *Drosophila* clock neurons can reveal universal mechanisms of circadian regulation, including daily sleep wake cycles.

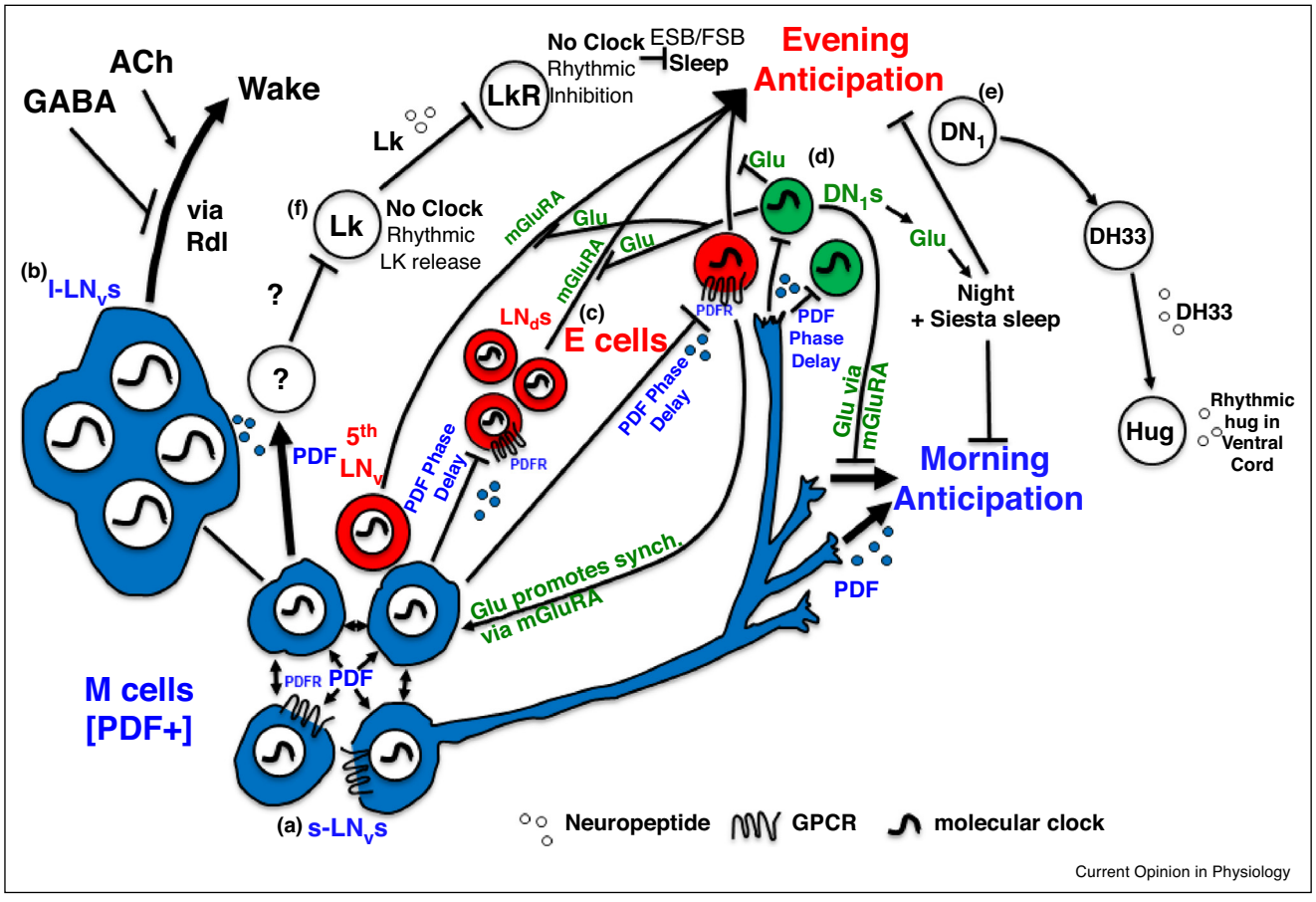
### Clocks and sleep

Normal sleep patterns depend on the clock and the sleep homeostat (sleep need), the two process model of sleep [41]. Clock mutations or SCN ablation both disrupt the rhythm of mammalian sleep and affect recovery after sleep deprivation [42,43], a measure of the homeostat. As enhanced $Ca^{2+}$-dependent hyperpolarization increases sleep duration, cell autonomous regulation of factors that change intracellular $Ca^{2+}$ throughout the brain by the clock could also change the probability of entering or

staying asleep in a time-dependent manner [44]. However, the mechanism by which the master clock and homeostat interact remains unknown.

*Drosophila* also display periods of quiescence that respond to stimulatory drugs, and sleep deprivation results in sleep rebound, characteristics shared with mammalian sleep [45]. Significant progress has been made in understanding circadian sleep regulation in flies, where M and E cells promote activity at dawn and dusk respectively, inhibiting sleep; a population of circadian $DN_1$ neurons then release glutamate to inhibit M and E cells and promote sleep at night and during a daily siesta (Figure 2) [34,35,46•,47]. Wake promoting M cells are then inhibited by GABA released from sleep promoting neurons outside the clock circuit [48,49].

**Figure 2**




The *Drosophila* clock circuit.
**(a)** s-$LN_v$s, or 'M cells' express PDF, and are required for the morning activity anticipating dawn. PDF sets the phase of other clock circuit neurons, and synchronizes oscillations of s-$LN_v$s, via the PDF receptor, PDFR. PDF released by M cells, in conjunction with light inputs in LD cycles, delays $Ca^{2+}$ activation in E cells by 12 h, allowing anticipation of dusk, while sNPF released from both M and E cells is required for the normal $Ca^{2+}$ oscillation in a population of night-active clock neurons (the $DN_1$s), with M cell signaling via sNPF determining phase [71].
**(b)** l-$LN_v$s express PDF and promote wakefulness. GABA, released by sleep promoting neurons inhibits the l-$LN_v$s via the $GABA_A$ receptor Rdl. The clock controls Rdl activity via Fbxl4, ensuring l-$LN_v$s are inhibited at the appropriate time of day [48,49].
**(c)** Evening cells (Red, 3 $LN_d$s, the 5th PDF- $LN_v$ and some $DN_1$s) are required for the evening peak of activity anticipating dusk. E cells express PDFR, and PDF delays the phase of E cell activity.
**(d)** $DN_1$ neurons have several functions. A subset (Red) promote evening activity. $DN_1$s also release glutamate to 1) synchronize s-$LN_v$ oscillations and 2) promote night and siesta sleep by inhibiting M and E cell outputs. These effects are mediated by the metabotropic glutamate receptor mGluRA [46•,47].
**(e)** $DN_1$s act within a relay circuit transmitting time of day information from $LN_v$s via DH33 expressing neurons to the ventral cord, where they impose rhythms in expression of the neuropeptide hugin (hug), controlling wake/activity [37].
**(f)** M cells signal via PDF to regulate an unknown population of neurons that in turn inhibit the activity of leucokinin (Lk) neurons, driving rhythmic release of Lk. Lk then imposes rhythmicity on neurons within the lateral horn that express the Lk receptor and project to the ellipsoid and fan shaped bodies (EB and FSB, see below), inhibiting sleep and promoting robust behavioral rhythms [36].

Multiple brain structures have implicated to play a role in sleep homeostasis in the fly [50,51,52,53••]. Of these, the Fan Shaped body (FSB) is a prime candidate for convergence of the clock and homeostat, as the FSB receives projections from Lk receptor neurons that relay clock signals [36] and also lies downstream of the EB, where ring two neurons change firing from spiking to bursts in response to sleep loss, acting as a bona fide sleep homeostat [53••], promoting sleep via ExF12 neurons that project to the FSB (Figure 2) [54].

The circadian contribution to sleep therefore appears to depend on the balance between neuronal excitation and inhibition by different populations of clock neurons with similarly phased molecular clocks signaling at different times of day, and on signals from other neurons that regulate clock neuronal activity, a pattern that could also apply to mammals.

## Mammalian clock circuits

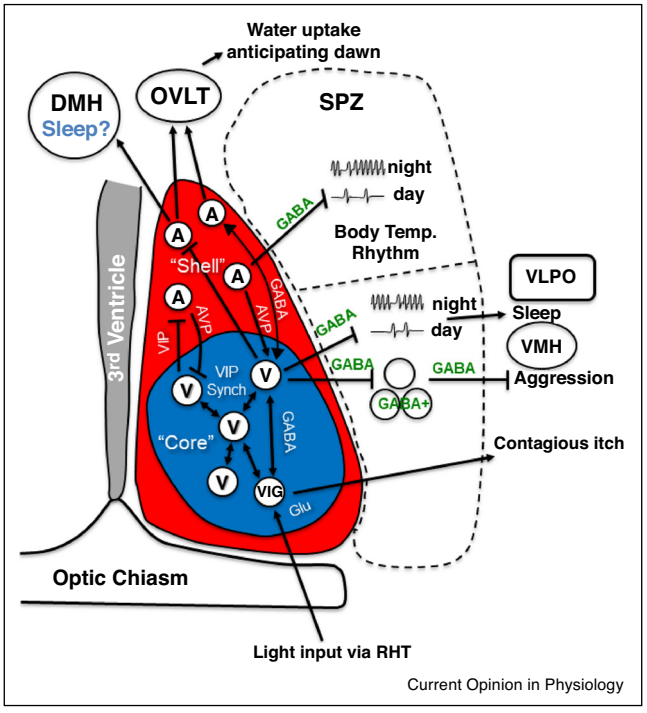
The SCN is essential for circadian behavior as SCN ablation renders rodents arrhythmic, while optogenetic activation of the SCN is sufficient to entrain behavior [55]. However, despite extensive study of SCN neurons (Section 'Neural circuits and clocks' above), it is unclear how SCN activity is translated into behavior. The SCN regulates many daily events, including when we eat, sleep, wake up, and are most mentally alert. Thus phase differences in clock phase, neurotransmitter expression and activity across the SCN may allow different SCN neurons to signal to different brain regions regulating different behaviors at different times of day. Recent experiments targeting-specific subsets of neurons have begun to reveal how subsets of SCN neurons modulate behavior. Optogenetics has been used to deliver physiologically relevant stimuli to VIPergic SCN neurons, revealing that rapid burst firing by VIP neurons is sufficient to entrain behavior and inhibit running wheel activity in a time-specific manner [20••]. AVPergic SCN neurons are electrically active during the late night (~ZT21.5–23.5), when they promote water intake in advance of daytime sleep onset through excitation of OVLT thirst neurons, preventing dehydration at the end of day sleep [56••]. Most surprisingly, GRPergic SCN neurons are required for contagious itch behavior, becoming activated and promoting scratching when a mouse observes another mouse scratching [57•]. While there is no evidence that this behavior is circadian, it is possible that modulation of light sensitivity of GRP neurons [that receive light input from the RHT] within the SCN over the course of the day affects this visual behavior.

## Downstream of the SCN

Understanding circadian modulation of behavior ultimately requires identification of brain regions targeted by the SCN, and their function. The SCN directly projects to ~15 distinct brain regions [5], with the subparaventricular zone (SPZ) immediately adjacent to the SCN receiving most projections. The SPZ is inhibited by GABA released by the SCN, so is most electrically active during the night, when mice are active [58]. The SPZ likely has several roles in relaying circadian information around the brain (Figure 3). First, the SPZ is likely upstream of the sleep promoting VLPO [59]; daytime inhibition of the SPZ may be the first step in a relay pathway promoting circadian regulation of sleep. GABAergic neurons of the SPZ also receive GABA inputs from VIPergic SCN neurons, and release GABA to inhibit neurons within the VMH that inhibit aggression, resulting in a rhythm of aggression in mice peaking around dawn and dusk [60].

The DMH, which receives projections from SCN AVPergic neurons [61] signals to orexin neurons to promote wakefulness [62]; thus AVP neurons may both regulate sleep timing and prepare an animal for sleep by driving them to drink water at the end of the active phase

**Figure 3**




Outputs from the SCN.
V = VIPergic A = AVPergic G = GRPergic.
Most SCN neuronal projections go to the SPZ. GABA released by the SCN inhibits SPZ activity, causing it to fire in antiphase to the SCN. The dorsal SPZ regulates body temperature, and the ventral SPZ is required for sleep and locomotor activity rhythms. The VLPO likely lies downstream of the SPZ, and promotes sleep. GABA signals from VIP neurons also inhibit GABAergic SPZ neurons, imposing a rhythm on the VMH and aggression.
AVPergic SCN neurons signal to the DMH to regulate sleep, and to the OVLT, promoting water uptake before sleep. GRP signaling within the GRP neurons that receive light inputs via the RHT is required for contagious itch behavior.

[56••]. Like the SCN, the lateral habenula (LHb) receives direct connections from the visual system and has intrinsic daily oscillations in electrical activity, which are delayed in phase compared to the SCN. The LHb likely also receives inputs from the SCN either directly via prokinectin 2 and/or AVP-expressing SCN neurons, or indirectly via the DMH. Suggested functions of the LHb raise the possibility that the LHb and SCN work together to regulate cognition, addiction, depression and sleep [63]. Targets of the SCN with roles in energy metabolism rather than behavior, and the role of AVP as a circadian messenger molecule are reviewed in detail in [64].

## Conclusions

In order for a 'clock gene' to generate daily circadian rhythms in physiology, it must first change the properties of the cell in which it finds itself. In the case of a neuron, the molecular clock must regulate electrical activity, driving it to fire at the correct time of day. This neuron must then acts within a network of neurons (and astrocytes) to generate unified time for an organism. Approaching the clock at the cellular and circuit level should lead to an understanding of aspects of circadian timing that remain a mystery, including the interaction between the homeostat and circadian clock. Eventually we may even understand time memory (which is independent of the SCN [65,66]), daily rhythms of food anticipation (which do not require the circadian clock [67]), or the dopaminergic oscillator, which generates ultradian ~4 h rhythms [68].

## Conflict of interest statement

Nothing declared.

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## References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Lu W, Meng QJ, Tyler NJ, Stokkan KA, Loudon AS: **A circadian clock is not required in an arctic mammal**. *Curr Biol* 2010, **20**:533-537.

2. Konopka RJ, Benzer S: **Clock mutants of *Drosophila melanogaster***. *Proc Natl Acad Sci U S A* 1971, **68**:2112-2116.

3. Takahashi JS: **Transcriptional architecture of the mammalian circadian clock**. *Nat Rev Genet* 2017, **18**:164-179.

4. Hughes S, Jagannath A, Hankins MW, Foster RG, Peirson SN: **Photic regulation of clock systems**. *Methods Enzymol* 2015, **552**:125-143.

5. Morin LP: **Neuroanatomy of the extended circadian rhythm system**. *Exp Neurol* 2013, **243**:4-20.

6. Silver R, Schwartz WJ: **The suprachiasmatic nucleus is a functionally heterogeneous timekeeping organ**. *Methods Enzymol* 2005, **393**:451-465.

7. Yamaguchi S, Isejima H, Matsuo T, Okura R, Yagita K, Kobayashi M, Okamura H: **Synchronization of cellular clocks in the suprachiasmatic nucleus**. *Science* 2003, **302**:1408-1412.

8. Welsh DK, Logothetis DE, Meister M, Reppert SM: **Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms**. *Neuron* 1995, **14**:697-706.

9. Maywood ES, Reddy AB, Wong GK, O'Neill JS, O'Brien JA, McMahon DG, Harmar AJ, Okamura H, Hastings MH: **Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling**. *Curr Biol* 2006, **16**:599-605.

10. Brancaccio M, Maywood ES, Chesham JE, Loudon AS, Hastings MH: **A Gq-Ca$^{2+}$ axis controls circuit-level encoding of circadian time in the suprachiasmatic nucleus**. *Neuron* 2013, **78**:714-728.

11. Noguchi T, Leise TL, Kingsbury NJ, Diemer T, Wang LL, Henson MA, Welsh DK: **Calcium circadian rhythmicity in the suprachiasmatic nucleus: cell autonomy and network modulation**. *eNeuro* 2017, **4**.

12. Evans JA, Leise TL, Castanon-Cervantes O, Davidson AJ: **Dynamic interactions mediated by nonredundant signaling mechanisms couple circadian clock neurons**. *Neuron* 2013, **80**:973-983.

13. Doi M, Ishida A, Miyake A, Sato M, Komatsu R, Yamazaki F, Kimura I, Tsuchiya S, Kori H, Seo K, Yamaguchi Y *et al.*: **Circadian regulation of intracellular g-protein signalling mediates intercellular synchrony and rhythmicity in the suprachiasmatic nucleus**. *Nat Commun* 2011, **2**:327.

14. O'Neill JS, Maywood ES, Chesham JE, Takahashi JS, Hastings MH: **cAMP-dependent signaling as a core component of the mammalian circadian pacemaker**. *Science* 2008, **320**:949-953.

15. Kudo T, Block GD, Colwell CS: **The circadian clock gene *Period1* connects the molecular clock to neural activity in the suprachiasmatic nucleus**. *ASN Neuro* 2015, **7**.

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

17. Brown TM, Piggins HD: **Spatiotemporal heterogeneity in the electrical activity of suprachiasmatic nuclei neurons and their response to photoperiod**. *J Biol Rhythms* 2009, **24**:44-54.

18. Meijer JH, Michel S, Vanderleest HT, Rohling JH: **Daily and seasonal adaptation of the circadian clock requires plasticity of the SCN neuronal network**. *Eur J Neurosci* 2010, **32**:2143-2151.

19. Houben T, Coomans CP, Meijer JH: **Regulation of circadian and acute activity levels by the murine suprachiasmatic nuclei**. *PLoS One* 2014, **9** e110172.

20. •• Mazuski C, Abel JH, Chen SP, Hermanstyne TO, Jones JR, Simon T, Doyle FJ 3rd, Herzog ED: **Entrainment of circadian rhythms depends on firing rates and neuropeptide release of VIP SCN neurons**. *Neuron* 2018, **99**:555-563 e555.
Mazuski*et al.* recorded electrical activity from SCN neurons cultured on a multi electrode array, and characterized VIP expressing neurons with 24 h rhythms of electrical activity. These neurons either fire tonically or with an irregular pattern (bursts of firing separated by longer, irregular intervals). They then used optogenetics to drive tonic or irregular firing from VIPergic SCN neurons, and found *in vitro* only irregular firing of VIPergic SCN neurons shifted the SCN molecular clock. *in vivo*, irregular activation of VIPergic SCN neurons entrained behavior more rapidly than tonic activation. VIP stimulation only induced phase delays and entrained behavior if delivered between ~CT8-18, suggesting a time of day function for VIPergic SCN signaling.

21. Porcu A, Riddle M, Dulcis D, Welsh DK: **Photoperiod-induced neuroplasticity in the circadian system**. *Neural Plast* 2018, **2018** 5147585.

22. VanderLeest HT, Houben T, Michel S, Deboer T, Albus H, Vansteensel MJ, Block GD, Meijer JH: **Seasonal encoding by the circadian pacemaker of the SCN**. *Curr Biol* 2007, **17**:468-473.

23. Azzi A, Evans JA, Leise T, Myung J, Takumi T, Davidson AJ, Brown SA: **Network dynamics mediate circadian clock plasticity**. *Neuron* 2017, **93**:441-450.

24. Freeman GM Jr, Krock RM, Aton SJ, Thaben P, Herzog ED: **GABA networks destabilize genetic oscillations in the circadian pacemaker**. *Neuron* 2013, **78**:799-806.

25. Olde Engberink AHO, Meijer JH, Michel S: **Chloride cotransporter KCC2 is essential for gabaergic inhibition in the SCN**. *Neuropharmacology* 2018, **138**:80-86.

26. Azzi A, Dallmann R, Casserly A, Rehrauer H, Patrignani A, Maier B, Kramer A, Brown SA: **Circadian behavior is light-reprogrammed by plastic DNA methylation**. *Nat Neurosci* 2014, **17**:377-382.

27. Stevenson TJ, Prendergast BJ: **Reversible DNA methylation regulates seasonal photoperiodic time measurement**. *Proc Natl Acad Sci U S A* 2013, **110**:16651-16656.

28. Barca-Mayo O, Pons-Espinal M, Follert P, Armirotti A, Berdondini L, De Pietri Tonelli D: **Astrocyte deletion of Bmal1 alters daily locomotor activity and cognitive functions via GABA signalling**. *Nat Commun* 2017, **8**:14336.

29. •• Brancaccio M, Patton AP, Chesham JE, Maywood ES, Hastings MH: **Astrocytes control circadian timekeeping in the suprachiasmatic nucleus via glutamatergic signaling**. *Neuron* 2017, **93**:1420-1435 e1425.

Robust circadian oscillations in the SCN are essential for circadian time keeping and usually regarded as a function of connectivity between SCN neurons. Here Brancaccio*et al.* show that while SCN neurons metabolically active during the day, SCN astrocytes are active during the night, when they suppress activity of SCN neurons through regulation of extracellular glutamate. Most surprisingly, changing the circadian clock within astrocytes alters circadian behavioral rhythms, adding to the emergent picture of a key role for molecular clocks within glia as well as neurons in circadian behavior [e.g. Refs. 17,19,20••].

30. Tso CF, Simon T, Greenlaw AC, Puri T, Mieda M, Herzog ED: **Astrocytes regulate daily rhythms in the suprachiasmatic nucleus and behavior**. *Curr Biol* 2017, **27**:1055-1061.

31. Ng FS, Tangredi MM, Jackson FR: **Glial cells physiologically modulate clock neurons and circadian behavior in a calcium-dependent manner**. *Curr Biol* 2011, **21**:625-634.

32. Zhang SL, Yue Z, Arnold DM, Artiushin G, Sehgal A: **A circadian clock in the blood-brain barrier regulates xenobiotic efflux**. *Cell* 2018, **173**:130-139 e110.

33. •• Liang X, Holy TE, Taghert PH: **Synchronous drosophila circadian pacemakers display nonsynchronous $Ca^{2+}$ rhythms *in vivo***. *Science* 2016, **351**:976-981.

Since the discovery of M and E oscillators in*Drosophila* [34,35] it has seemed likely that different neurons within the *Drosophila* clock circuit signal at different times of day despite having similarly phased molecular clocks. Liang *et al.* use objective-coupled planar illumination (OCPI) microscopy and the $Ca^{2+}$ sensor GcAMP6s to map the active phases of different groups of clock neurons across 24 h in live flies, identifying a wide range of phases of neuronal activity [including M cells active in the morning and E cells in the evening]. This diversity of activity would allow the clock to drive different outputs at multiple times of day.

34. Stoleru D, Peng Y, Agosto J, Rosbash M: **Coupled oscillators control morning and evening locomotor behaviour of *Drosophila***. *Nature* 2004, **431**:862-868.

35. Grima B, Chelot E, Xia R, Rouyer F: **Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain**. *Nature* 2004, **431**:869-873.

36. Cavey M, Collins B, Bertet C, Blau J: **Circadian rhythms in neuronal activity propagate through output circuits**. *Nat Neurosci* 2016, **19**:587-595.

37. King AN, Barber AF, Smith AE, Dreyer AP, Sitaraman D, Nitabach MN, Cavanaugh DJ, Sehgal A: **A peptidergic circuit links the circadian clock to locomotor activity**. *Curr Biol* 2017, **27**:1915-1927 e1915.

38. Pittendrigh C, Daan S: **A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons**. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 1976, **106**:333-355.

39. Collins B, Kaplan HS, Cavey M, Lelito KR, Bahle AH, Zhu Z, Macara AM, Roman G, Shafer OT, Blau J: **Differentially timed extracellular signals synchronize pacemaker neuron clocks**. *PLoS Biol* 2014, **12** e1001959.

40. Flourakis M, Kula-Eversole E, Hutchison AL, Han TH, Aranda K, Moose DL, White KP, Dinner AR, Lear BC, Ren D, Diekman CO *et al.*: **A conserved bicycle model for circadian clock control of membrane excitability**. *Cell* 2015, **162**:836-848.

41. Borbely AA: **A two process model of sleep regulation**. *Hum Neurobiol* 1982, **1**:195-204.

42. Laposky A, Easton A, Dugovic C, Walisser J, Bradfield C, Turek F: **Deletion of the mammalian circadian clock gene *Bmal1/Mop3* alters baseline sleep architecture and the response to sleep deprivation**. *Sleep* 2005, **28**:395-409.

43. Easton A, Meerlo P, Bergmann B, Turek FW: **The suprachiasmatic nucleus regulates sleep timing and amount in mice**. *Sleep* 2004, **27**:1307-1318.

44. Tatsuki F, Sunagawa GA, Shi S, Susaki EA, Yukinaga H, Perrin D, Sumiyama K, Ukai-Tadenuma M, Fujishima H, Ohno R *et al.*: **Involvement of $Ca^{2+}$-dependent hyperpolarization in sleep duration in mammals**. *Neuron* 2016, **90**:70-85.

45. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G: **Correlates of sleep and waking in *Drosophila melanogaster***. *Science* 2000, **287**:1834-1837.

46. • Guo F, Yu J, Jung HJ, Abruzzi KC, Luo W, Griffith LC, Rosbash M: **Circadian neuron feedback controls the drosophila sleep—activity profile**. *Nature* 2016, **536**:292-297.

Although the circadian clock and sleep homeostat are both required for normal sleep wake cycles, this is one of the first studies to identify a role for clock neurons in promoting sleep. By using cell-specific optogenetic manipulations of neuronal activity, Guo*et al.* extend the discovery that dorsal *Drosophila* clock neurons signal via Glutamate to inhibit locomotor activity [47], identifying the subset of dorsal clock neurons that regulate sleep.

47. Collins B, Kane EA, Reeves DC, Akabas MH, Blau J: **Balance of activity between $LN_v$s and glutamatergic dorsal clock neurons promotes robust circadian rhythms in *Drosophila***. *Neuron* 2012, **74**:706-718.

48. Li Q, Li Y, Wang X, Qi J, Jin X, Tong H, Zhou Z, Zhang ZC, Han J: **Fbxl4 serves as a clock output molecule that regulates sleep through promotion of rhythmic degradation of the $GABA_A$ receptor**. *Curr Biol* 2017, **27**:3616-3625 e3615.

49. Parisky KM, Agosto J, Pulver SR, Shang Y, Kuklin E, Hodge JJ, Kang K, Liu X, Garrity PA, Rosbash M, Griffith LC: **Pdf cells are a gaba-responsive wake-promoting component of the *Drosophila* sleep circuit**. *Neuron* 2008, **60**:672-682.

50. Pitman JL, McGill JJ, Keegan KP, Allada R: **A dynamic role for the mushroom bodies in promoting sleep in *Drosophila***. *Nature* 2006, **441**:753-756.

51. Joiner WJ, Crocker A, White BH, Sehgal A: **Sleep in *Drosophila* is regulated by adult mushroom bodies**. *Nature* 2006, **441**: 757-760.

52. Crocker A, Shahidullah M, Levitan IB, Sehgal A: **Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior**. *Neuron* 2010, **65**:670-681.

53. •• Liu S, Liu Q, Tabuchi M, Wu MN: **Sleep drive is encoded by neural plastic changes in a dedicated circuit**. *Cell* 2016, **165**:1347-1360.

Liu*et al.* use *Drosophila* to address one of the most intriguing questions in neurobiology - how does an organism measure sleep need? From a neural circuit screen, they identify a population of elipsoid body (EB) neurons that, when activated, induce sleep even if the fly is well rested, whilst blocking signaling from these EB neurons prevents rebound after sleep deprivation. EB neurons measure sleep need by increasing intracellular $Ca^{2+}$, NMDA receptor expression, and firing frequency in proportion to the time a fly has gone without sleep. These EB neurons lie upstream of, and signal to the ExFl2 neurons identified in Ref. [54].

54. Donlea JM, Pimentel D, Miesenbock G: **Neuronal machinery of sleep homeostasis in *Drosophila***. *Neuron* 2014, **81**:1442.

55. Jones JR, Tackenberg MC, McMahon DG: **Manipulating circadian clock neuron firing rate resets molecular circadian rhythms and behavior**. *Nat Neurosci* 2015, **18**:373-375.

56. •• Gizowski C, Zaelzer C, Bourque CW: **Clock-driven vasopressin neurotransmission mediates anticipatory thirst prior to sleep**. *Nature* 2016, **537**:685-688.

The role of the SCN in regulating circadian behavior has been extensively studied, usually by measuring the effects on rodent running wheel activity. Here Gizowski*et al.* use electrophysiological recordings and optogenetic manipulations to show that AVPergic SCN neurons are active towards the end of the night and release AVP to excite thirst neurons within the OVLT, causing mice to drink water independent of actual thirst - e.g. the clock prepares the animal for sleep; blocking this late-night AVP signal causes mice to become dehydrated before the end of light phase sleep.

57. • Yu YQ Barry DM, Hao Y, Liu XT, Chen ZF: **Molecular and neural basis of contagious itch behavior in mice**. *Science* 2017, **355**:1072-1076.

Watching someone else scratch an itch can lead oneself to experience itchiness. Remarkably, this is also true of mice, who experience contagious itch behavior upon exposure to video footage of other mice itching. This leads to an increase in activity within GRPergic SCN neurons, and blocking GRP signaling prevents contagious itch in mice, whilst optogenetic activation of GRPergic SCN neurons induces itching. Exactly how these neurons transform a visual input to a physical response remains unclear, but may depend more on the role of GRPergic SCN neurons receiving and transmitting information from the visual system, perhaps modulating the response in a circadian fashion.

58. Nakamura W, Yamazaki S, Nakamura TJ, Shirakawa T, Block GD, Takumi T: ***In vivo* monitoring of circadian timing in freely moving mice**. *Curr Biol* 2008, **18**:381-385.

59. Lu J, Zhang YH, Chou TC, Gaus SE, Elmquist JK, Shiromani P, Saper CB: **Contrasting effects of ibotenate lesions of the paraventricular nucleus and subparaventricular zone on sleep-wake cycle and temperature regulation**. *J Neurosci* 2001, **21**:4864-4874.

60. Todd WD, Fenselau H, Wang JL, Zhang R, Machado NL, Venner A, Broadhurst RY, Kaur S, Lynagh T, Olson DP, Lowell BB *et al.*: **A hypothalamic circuit for the circadian control of aggression**. *Nat Neurosci* 2018, **21**:717-724.

61. Abrahamson EE, Moore RY: **Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections**. *Brain Res* 2001, **916**:172-191.

62. Inutsuka A, Yamanaka A: **The physiological role of orexin/hypocretin neurons in the regulation of sleep/wakefulness and neuroendocrine functions**. *Front Endocrinol* 2013, **4**:18.

63. Bano-Otalora B, Piggins HD: **Contributions of the lateral habenula to circadian time keeping**. *Pharmacol Biochem Behav* 2017, **162**:46-54.

64. Kalsbeek A, Scheer FA, Perreau-Lenz S, La Fleur SE, Yi CX, Fliers E, Buijs RM: **Circadian disruption and SCN control of energy metabolism**. *FEBS Lett* 2011, **585**:1412-1426.

65. Ralph MR, Sam K, Rawashdeh OA, Cain SW, Ko CH: **Memory for time of day (time memory) is encoded by a circadian oscillator and is distinct from other context memories**. *Chronobiol Int* 2013, **30**:540-547.

66. Cain SW, Yoon J, Shrestha TC, Ralph MR: **Retention of a 24-hour time memory in syrian hamsters carrying the 20-hour short circadian period mutation in casein kinase-1epsilon (ck1epsilontau/tau)**. *Neurobiol Learn Memory* 2014, **114**:171-177.

67. Storch KF, Weitz CJ: **Daily rhythms of food-anticipatory behavioral activity do not require the known circadian clock**. *Proc Natl Acad Sci U S A* 2009, **106**:6808-6813.

68. Blum ID, Zhu L, Moquin L, Kokoeva MV, Gratton A, Giros B, Storch KF: **A highly tunable dopaminergic oscillator generates ultradian rhythms of behavioral arousal**. *eLife* 2014, **3**.

69. Li JD, Burton KJ, Zhang C, Hu SB, Zhou QY: **Vasopressin receptor V1a regulates circadian rhythms of locomotor activity and expression of clock-controlled genes in the suprachiasmatic nuclei**. *Am J Physiol Regul Integr Comp Physiol* 2009, **296**:R824-830.

70. Lee IT, Chang AS, Manandhar M, Shan Y, Fan J, Izumo M, Ikeda Y, Motoike T, Dixon S, Seinfeld JE, Takahashi JS *et al.*: **Neuromedin S-producing neurons act as essential pacemakers in the suprachiasmatic nucleus to couple clock neurons and dictate circadian rhythms**. *Neuron* 2015, **85**:1086-1102.

71. Liang X, Holy TE, Taghert PH: **A series of suppressive signals within the *Drosophila* circadian neural circuit generates sequential daily outputs**. *Neuron* 2017, **94**:1173-1189 e1174.