

# Collective timekeeping among cells of the master circadian clock

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## Abstract

The suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the master circadian clock that coordinates daily rhythms in behavior and physiology in mammals. Like other hypothalamic nuclei, the SCN displays an impressive array of distinct cell types characterized by differences in neurotransmitter and neuropeptide expression. Individual SCN neurons and glia are able to display self-sustained circadian rhythms in cellular function that are regulated at the molecular level by a 24 h transcriptional–translational feedback loop. Remarkably, SCN cells are able to harmonize with one another to sustain coherent rhythms at the tissue level. Mechanisms of cellular communication in the SCN network are not completely understood, but recent progress has provided insight into the functional roles of several SCN signaling factors. This review discusses SCN organization, how intercellular communication is critical for maintaining network function, and the signaling mechanisms that play a role in this process. Despite recent progress, our understanding of SCN circuitry and coupling is far from complete. Further work is needed to map SCN circuitry fully and define the signaling mechanisms that allow for collective timekeeping in the SCN network.

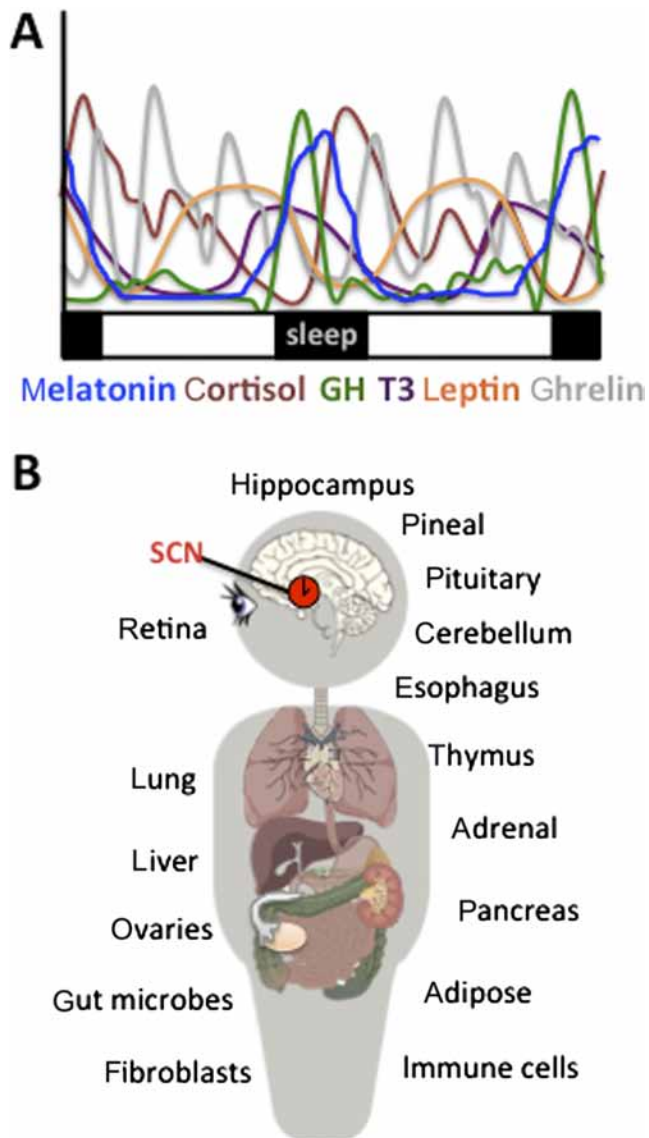
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## Introduction

Daily rhythms in metabolic and endocrine function serve to anticipate predictable changes in the environment. These rhythms are not driven externally, but instead arise from an intrinsic cellular process that tracks the hours of the day. In other words, cells are daily clocks. Because cellular clocks tick with a period close to but not exactly 24 h, they are referred to as ‘circadian’ (i.e. about a day). Biological timekeeping at the circadian timescale ensures that physiological and behavioral processes occur at the appropriate time of day. In this respect, it is an essential and ubiquitous feature of life on this planet. For example, glucocorticoid release surges just before waking to proactively marshal important energy resources (Fig. 1, Takahashi *et al.* 1968). Likewise, the secretion of growth hormone is highest during slow-wave sleep when its

ability to repair and strengthen bones is most effective (Fig. 1, Takahashi *et al.* 1968). In both cases, the timing of these rhythms is programmed by a biological clock and influenced by sleep (Czeisler & Klerman 1999). Over the last few decades, we have gained a deep understanding of the mechanisms that produce circadian timekeeping at the molecular and cellular levels. A key remaining question is how the numerous clock cells of our bodies form a coordinated system.

In mammals, the circadian system is a hierarchical collection of tissue clocks located throughout the brain and body (Mohawk *et al.* 2012). Many of these clocks are endocrine tissues that regulate hormone synthesis and release across the day (Fig. 1). Examples include the pituitary gland, the pineal gland, the adrenal gland,



**Figure 1**

Daily hormone rhythms are regulated by the circadian timekeeping system. (A) Hormone release fluctuates over the circadian cycle, with peak times occurring at specific times of day. Figure based on data from (Takahashi *et al.* 1968, Gavrilu *et al.* 2003, Koutkia *et al.* 2005, Natalucci *et al.* 2005, Russell *et al.* 2008). (B) The circadian timekeeping system is a hierarchical collection of tissue clocks located throughout the brain and body, many of which are endocrine tissues that regulate hormone synthesis and release across the day.

adipose tissue, and immune cells. These myriad body clocks are coordinated by the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Mohawk & Takahashi 2011). The SCN serves as a master clock that receives cues from the environment (e.g., light) and relays them to downstream tissues through a variety of outputs (e.g. synaptic connections, humoral cues, behavioral and

physiological control of sleep and body temperature). In this manner, the SCN harmonizes the various body clocks with one another and with the local time zone. Classic work demonstrates that the SCN is necessary for maintaining circadian rhythms in numerous processes, including sleep, feeding, drinking, melatonin production, and reproductive function (Klein *et al.* 1991, Weaver 1998). Given its extensive influence on endocrine function, understanding SCN circuitry is of prime importance.

The SCN is a neuronal network of cellular clocks that coordinate with one another to form a functional and cohesive population (Welsh *et al.* 2010). Like other hypothalamic nuclei, the SCN produces a diverse range of neurotransmitters and neuropeptides (van den Pol & Tsujimoto 1985, Lee *et al.* 2013), which have been used to define distinct neuronal subclasses (Antle & Silver 2005). In addition to relaying information to downstream clocks, SCN signaling molecules serve as local coupling factors that are important for maintaining network function. In particular, when SCN neurons are embedded in the network, they produce rhythms that are more precise, higher amplitude, and more robust than those displayed by isolated SCN neurons. Thus, intercellular communication within the SCN network is critically important for ensuring the fidelity of outputs to downstream tissues. Moreover, SCN neurons coordinate as a population to encode environmental conditions that are critical to survival (e.g. seasonal changes in day length). Despite its importance, the process by which SCN neurons influence one another is not completely understood. For instance, it is not known precisely how different SCN factors contribute to network function, how these signals are transmitted, and how they act to influence cellular rhythms. This review will describe recent progress that has advanced the understanding of SCN circuitry and highlight several issues that remain outstanding.

### Circadian timekeeping at the cellular level

As a tissue, the SCN displays daily rhythms in numerous cellular processes, including metabolism, electrical activity, gene/protein expression, peptide release, and response to photic stimulation (Klein *et al.* 1991, Weaver 1998). Most of these rhythms persist when the SCN is studied in isolation from the environment and the rest of the brain. Thus, autonomous rhythmicity is an intrinsic property of the SCN itself. A key question posed almost immediately after this discovery was whether SCN rhythms are a network- or cellular-driven phenomenon.

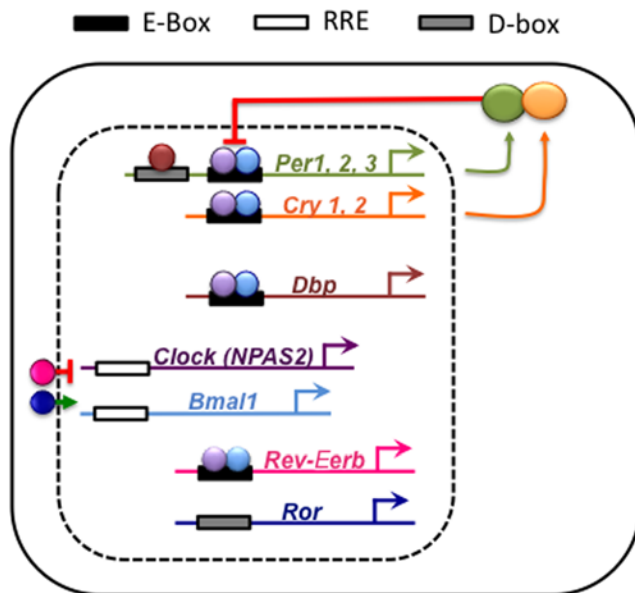
The simple answer: SCN neurons are intrinsic clock cells. Pioneering work demonstrated that individual SCN neurons are capable of sustaining cellular rhythms even when dispersed at low density (Welsh *et al.* 1995, Herzog *et al.* 1998, Honma *et al.* 1998). Based on these results, it is commonly stated that SCN neurons are autonomous, self-sustained clocks. But the reality of the situation is slightly more complex because SCN neurons communicate with one another in ways that strengthen cellular rhythms (see more in next section).

But how does a cellular clock keep circadian time? Research conducted over the last few decades has revealed an elegant molecular mechanism that operates in nearly every cell of the body (Buhr & Takahashi 2013). Briefly, cellular rhythms are generated by interlocking feedback loops controlling the daily transcription of 'clock genes' and 'clock-controlled genes' (Fig. 2). At its core, the molecular circadian clock is a delayed negative-feedback loop, with positive elements that drive transcription and negative elements that repress transcription on a daily basis. The positive elements are the transcription factors CLOCK and

BMAL1, whereas the negative elements are PERIOD and CRYPTOCHROME proteins. Daily transcription is initiated when CLOCK and BMAL1 form a dimer that activates the expression of a family of *Period* (*Per1*, *Per2*, *Per3*) and *Cryptochrome* genes (*Cry1*, *Cry2*). This increases the levels of PER and CRY proteins, which dimerize, translocate into the nucleus, and inhibit their own transcription by repressing CLOCK-BMAL1 function (Fig. 2). This transcriptional repression, along with ubiquitination and degradation, causes PER and CRY levels to decline. Falling levels of PER and CRY then allow for derepression of CLOCK-BMAL1, which reactivates the transcription of *Per* and *Cry*. And so, the cycle renews the following day. This type of negative-feedback mechanism forms the basis of circadian timekeeping in a wide range of organisms (bacteria, fungi, plants, insects, and mammals) although the genes involved differ across species (Mackey 2007).

It remains a key area of research to understand fully the molecular control of cellular rhythms (Zhang & Kay 2010). With each passing day, our understanding of the circadian network at this level grows. We now appreciate that there are additional gene networks that regulate the core loop described above. For example, one interconnected loop regulates *Bmal1* and *Clock* transcription via opposing actions of ROR and REV-ERB (Fig. 2). This ancillary loop regulates the precision, robustness, and amplitude of core clock gene expression. Also, there are numerous 'clock-controlled genes' that are influenced by the molecular clock either directly or indirectly. For example, the transcription factor D-binding protein (DBP) is directly controlled by CLOCK-BMAL1 (Fig. 2). By regulating the timing of DBP expression, the core molecular clock can influence myriad downstream targets. To further demonstrate the interlocked nature of these feedback loops, DBP can influence *Per* transcription by binding to an upstream promoter sequence. Moreover, clock genes may contain additional regulatory elements (e.g., CRE) through which their expression is regulated by cellular and environmental signals. Collectively, the circadian molecular clock operates in nearly every cell of the body to regulate at least half the genome (Zhang *et al.* 2014).

In SCN neurons, the molecular oscillator controls myriad cellular processes (Kuhlman 2007). Notably, SCN neurons typically display daily rhythms in membrane potential with high firing during the day and low firing during the night (Brown & Piggins 2007). The temporal patterning of SCN electrical activity has important implications for network signaling and outputs to downstream tissues, so this is a critical area of research. Yet this approach is often limited in the number of SCN



**Figure 2**

The molecular circadian clock comprises interlocking transcriptional-translational feedback loops. CLOCK and BMAL1 are transcription factors that bind to E-box elements within the promoter sequences of a variety of clock genes. The protein products of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes form repressors that inhibit their own transcription. Additional feedback loops involve other clock genes that interact with the elements of the core loop to amplify and stabilize molecular clock function. For example, the protein products of *Rev-Erb* and *Ror* genes compete for binding at ROR elements to influence *Bmal1* and *Clock* transcription. Further, the protein products of clock-controlled genes, such as *Dbp*, can influence both downstream molecular targets and core clock genes by binding at D-box elements.

neurons that can be recorded at the same time. Recent technological advances enable a more comprehensive view of SCN network function using real-time imaging of molecular activity. Over the years, researchers have engineered an impressive array of mouse models with genetically encoded optical reporters (e.g. *Per1*-GFP, *Per1*-luciferase, *PER2::LUCIFERASE*, *Bmal1*-luciferase, and *Cry1*-luciferase) that allow one to track molecular clock function in real time (Kuhlman *et al.* 2000, Yamaguchi *et al.* 2003, Yoo *et al.* 2004, Noguchi *et al.* 2010, Maywood *et al.* 2013). In addition, genetic mouse models and viral tools have been developed to monitor daily rhythms in Ca<sup>2+</sup> and other intracellular signals (Enoki *et al.* 2012, Brancaccio *et al.* 2013, Irwin & Allen 2013). Provided there is good penetrance and faithful recapitulation of native molecular function, real-time imaging approaches provide an unprecedented view of the dynamic clock because they can be used to monitor hundreds of SCN neurons simultaneously. Relative to standard electrophysiological approaches, they also allow for cellular recordings to be performed in a relatively noninvasive manner for many days in culture. Together with more traditional approaches, real-time imaging techniques provide excellent tools to investigate cellular clock function and network properties.

### The SCN network: greater than the sum of its parts

It is now well established that the SCN contains multiple cellular clocks that interact with one another to form a functional network. As described above, individual SCN cells can express self-sustained circadian rhythms (Welsh *et al.* 1995, Herzog *et al.* 1998, Honma *et al.* 1998). Nevertheless, it is clear that SCN neurons are weaker clocks when isolated from one another. For instance, ~60% of SCN neurons are competent oscillators when dispersed in low-density culture, but only ~30% of neurons are rhythmic under conditions of complete isolation (Webb *et al.* 2009). Strikingly, this is much lower than that observed when SCN neurons are connected in the network, with >90% of cells sustaining rhythms in a slice preparation. Further, coupled SCN neurons display rhythms that are more precise, higher amplitude, and more robust to perturbation and stochastic noise (Herzog *et al.* 2004, Abraham *et al.* 2010, Buhr *et al.* 2010). Remarkably, network interactions can even preserve cellular clock function when the molecular oscillator is crippled by genetic defects (Nakamura *et al.* 2002, Liu *et al.* 2007, Ko *et al.* 2010, Evans *et al.* 2012). This series

of observations indicates that SCN neurons can keep time by themselves, but are more capable clocks when they receive signals from other cells in the network. Of note, this is not true for all types of clock cells. Fibroblasts are strong circadian oscillators that appear to depend very little on one another for sustaining cellular rhythms (Nagoshi *et al.* 2004, Leise *et al.* 2012). While this may be viewed as evidence that SCN neurons are 'weaker' clocks than fibroblasts, this is not necessarily the case. Fibroblast clocks may oscillate well as individual cells, but they lack network-level properties that can buffer them from clock gene mutations (Liu *et al.* 2007). By contrast, SCN neurons have strength in numbers because they influence and bolster one another.

Another key property of SCN neurons is that they are able to synchronize with one another. SCN neurons embedded within the network display well-coordinated rhythms both *in vivo* and *in vitro*. By contrast, SCN neurons that are dissociated 'run at different speeds' due to the expression of different period lengths (Welsh *et al.* 1995, Herzog *et al.* 1998, Honma *et al.* 1998). As a consequence, dissociated SCN neurons gradually lose synchrony with one another over time in culture. But SCN neurons remain synchronized when they are able to interact fully with one another. These results indicate that SCN neurons adopt a common period due to intercellular communication, which prevents loss of synchrony among SCN neurons. Studies of mutant SCN neurons likewise suggest that they interact with one another to determine overall period (Liu *et al.* 1997, Herzog *et al.* 1998, Low-Zeddies & Takahashi 2001). Due to their ability to synchronize, SCN neurons remain coordinated as a population, maintain tissue-level rhythms, and send strong outputs to downstream tissues. Importantly, the coherence of SCN rhythms is a key determinant of behavioral and physiological rhythmicity (Ciarleglio *et al.* 2009). This is a property that appears to be unique to the SCN because most other types of cells appear unable to synchronize with one another (Yamazaki *et al.* 2000, Nagoshi *et al.* 2004, Welsh *et al.* 2004). Cellular clocks that lack communication would be expected to rely on SCN-controlled signals to maintain tissue coherence (Farnell *et al.* 2011). For example, fibroblasts are strong cellular oscillators, but they desynchronize and lose population-level rhythms (Welsh *et al.* 2004). Interestingly, it may be their strongly autonomous cellular clock that prevents fibroblasts from synchronizing with one another (Locke *et al.* 2008).

Lastly, network interactions influence the relative timing of neuronal activity to regulate the waveform of their collective rhythm. When embedded in the network,

SCN neurons 'prefer' to adopt specific phase relationships with electrical and molecular rhythms that are slightly dispersed in time rather than occurring all at the same time (Quintero *et al.* 2003, Saeb-Parsy & Dyball 2003, Yamaguchi *et al.* 2003, Hamada *et al.* 2004, Rohling *et al.* 2006, Yan *et al.* 2007, Evans *et al.* 2011, Myung *et al.* 2012, Brancaccio *et al.* 2013). This is not a random event; rather, there are clear regional phase differences that are reproducible and stereotyped across animals. Phase mapping analyses of PERIOD2::LUCIFERASE (PER2::LUC) expression in the murine SCN reveal several consistent patterns (Evans *et al.* 2011). First, the caudal SCN typically assumes an earlier phase than the rostral SCN. Also, there are fairly complex gradients of expression, with the dorsal SCN phase leading more central and ventral regions. These spatiotemporal arrangements are intrinsically regulated by the network itself (Quintero *et al.* 2003, Yamaguchi *et al.* 2003, Evans *et al.* 2011), and yet can be modulated markedly by environmental lighting conditions (reviewed in Meijer *et al.* 2012, Evans & Gorman 2016).

In terms of environmental modulation of SCN temporal organization, one of the most studied contexts is seasonal changes in day length. Day length regulates the phase coherence of SCN neurons, with more clustered phases under short winter-like days than long summer-like days (Jagota *et al.* 2000, Hazlerigg *et al.* 2005, Inagaki *et al.* 2007, Naito *et al.* 2008, Evans *et al.* 2013, Myung *et al.* 2015). Temporal coherence of phase among SCN neurons influences the overall waveform of rhythms produced by the network, with longer duration of clock gene expression and electrical activity under long days. This photoperiodic encoding is determined largely by changes in SCN phase relationships rather than changes in cellular rhythms (Rohling *et al.* 2006, Brown & Piggins 2009). Effectively, photoperiodic encoding by the SCN network alters the patterning of outputs transmitted under summer vs winter conditions. Seasonal changes in SCN outputs provide time of year information to downstream tissues to produce seasonal changes in behavior and physiology (Schaap *et al.* 2003, Inagaki *et al.* 2007). Thus, the SCN network is both a daily clock and an annual calendar.

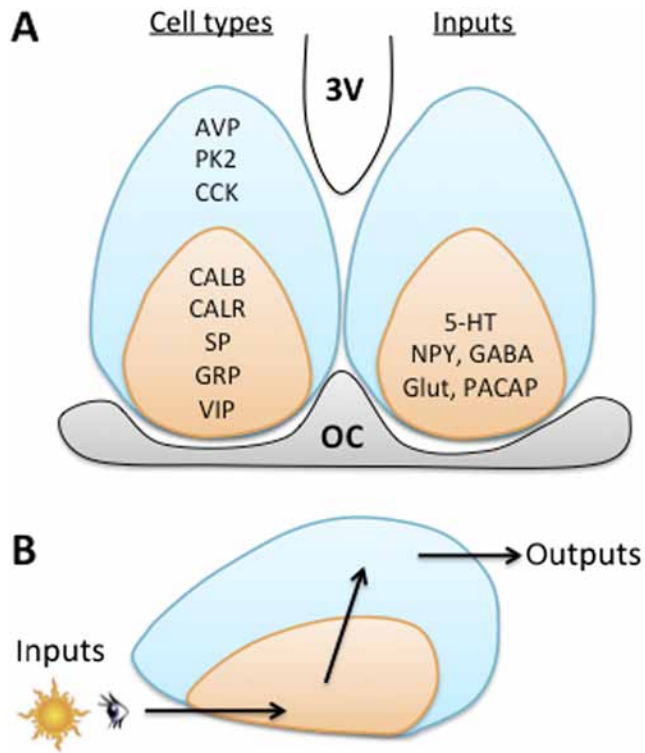
In addition to its ecological significance, plasticity in SCN phase relationships can be exploited to test mechanisms of intercellular communication (Evans *et al.* 2013, 2015). In this approach, SCN neurons are desynchronized by light *in vivo* and then allowed to resynchronize *in vitro*, so that the process of network coupling can be tracked in real time. Specifically, very long day lengths reorganize the SCN network, so that it adopts a highly polarized state

with two groups of SCN neurons cycling in antiphase. After release from these lighting conditions, SCN neurons within these two groups interact with one another and gradually resynchronize over the course of a week. Leveraging this form of network plasticity, the process of SCN coupling can be tracked in real time using an *ex vivo* slice preparation. By capturing the dynamic process of SCN communication, this analytical assay may provide a novel discovery tool for further defining the circuitry of the SCN network (Evans *et al.* 2013).

### SCN network organization: functional differences among neuronal subclasses

The SCN contains local projection neurons that communicate with one another and with other hypothalamic structures (Abrahamson & Moore 2001, Moore *et al.* 2002). The axons of many SCN neurons terminate within the nucleus itself, thus forming local circuit connections and/or collaterals from longer range projections. Nearly all SCN neurons produce  $\beta$ -aminobutyric acid (GABA), yet they can be categorized into distinct subgroups based on co-expression of different neuropeptides (Moore & Speh 1993, Abrahamson & Moore 2001). The SCN is typically subdivided into two spatially segregated compartments: the shell and the core (Moore & Silver 1998). These two compartments contain distinct subclasses of neurons that differ neurochemically (Fig. 3). The SCN shell contains a dense population of neurons that express arginine vasopressin (AVP), as well as other types of neurons. The SCN core contains a variety of subclasses, including neurons that express vasoactive intestinal polypeptide (VIP) or gastrin-releasing peptide (GRP). These subgroups have distinct developmental patterns (Antle *et al.* 2005b, Bedont & Blackshaw 2015) and are thought to represent distinct subclasses with minimal overlap in neuropeptide expression. However, as the index of SCN peptides grows, it is becoming apparent that SCN neurons can co-express different peptides that may contribute to their functional activity (e.g. Geoghegan & Carter 2008, Atkins *et al.* 2010, Drouyer *et al.* 2010, Hundahl *et al.* 2012, Lee *et al.* 2015). Although the shell–core scheme of the SCN network continues to be a convenient construct, it will likely morph to become more sophisticated as understanding of SCN circuitry increases (Morin 2007, 2012).

The expression and location of AVP and VIP neurons are consistent across mammalian species, but regional anatomy and chemoarchitecture can vary (Morin 2007, Cassone *et al.* 1988). For example, the SCN of rodents



**Figure 3**

The SCN network is a heterogeneous population of cellular clocks. (A) Organization of the mouse SCN, illustrating the compartmentalization of cell types and inputs in the coronal plane. AVP, arginine vasopressin; PK2, prokineticin 2; CCK, cholecystokinin; CALB, calbindin; CALR, calretinin; SP, substance P; GRP, gastrin-releasing peptide; VIP, vasoactive intestinal polypeptide; 5-HT, serotonin (from dorsal raphe); NPY, neuropeptide Y (from intergeniculate leaflet of the thalamus, which also releases GABA, neurotensin, and enkephalin), Glut, glutamate (from retina); PACAP, pituitary adenylate cyclase-activating polypeptide (from retina); 3V, third ventricle; OC, optic chiasm. (B) The shell–core model of the SCN network, representing how each compartment is thought to contribute to pacemaker function.

contains neurons that express calcium-binding proteins, but the location and specific proteins expressed differ among rodent species. In the Syrian hamster, calbindin-expressing SCN neurons form a spatially segregated subgroup densely packed into the central region of the core compartment. The mouse SCN contains both calbindin and calretinin neurons, with greatest expression within the core compartment. However, neither population forms a dense subnucleus. By contrast, calretinin neurons in the rat appear in the SCN shell rather than the SCN core. Another example of SCN variability across species is neuropeptide Y (NPY) expression. The rodent SCN does not produce NPY, but the human SCN does (Moore 1991). The consequence of species variation in SCN chemoarchitecture warrants further study given its potential influence on SCN function.

In addition to differences in neuropeptide expression, SCN neurons can be distinguished based on their response to afferent input. For instance, light exposure during the night typically increases SCN electrical activity and gene expression (i.e. *C-fos*, *Period*), but does so only in a subset of neurons (Meijer *et al.* 1986, Jiao *et al.* 1999, Aggelopoulos & Meissl 2000, Schwartz *et al.* 2000, Hamada *et al.* 2001, Nakamura *et al.* 2004, Brown *et al.* 2011). Of those SCN neurons affected directly by retinal stimulation, some respond with sustained increases in electrical activity, some respond with phasic responses, and others are inhibited. Further, photoreponsive SCN neurons may be distinguished based on the source of retinal input they receive (Brown *et al.* 2011). Tract tracing studies suggest that there is a denser retinal projection to the SCN core than the SCN shell (Abrahamson & Moore 2001, Lokshin *et al.* 2015), which maps onto functional differences in photic response. After light exposure, the SCN core displays changes in gene expression that precede those in the SCN shell (Silver *et al.* 1996, Dardente *et al.* 2002, Yan & Okamura 2002, Yan & Silver 2002, 2004, Kuhlman *et al.* 2003, Nagano *et al.* 2003). Regional differences in photic responsiveness have functional consequences that can influence how the network adjusts following changes in environmental lighting conditions. For instance, after simulated travel across time zones, the SCN core re-entrains faster than the SCN shell (Nagano *et al.* 2003, Albus *et al.* 2005, Nakamura *et al.* 2005, Davidson *et al.* 2009, Rohling *et al.* 2011, Sellix *et al.* 2012), and this form of light-driven desynchrony is thought to contribute to symptoms of jetlag. Moreover, regional patterning of afferent projections to the SCN is not unique to the retina, with inputs from the thalamus, pretectum, and median raphe also being densest in the SCN core (Fig. 3, for review, see Morin & Allen 2006). Furthermore, the SCN shell may receive input from a distinct set of structures, at least in the rat (Moga & Moore 1997, Leak & Moore 2001). Future studies should test whether this is consistent across mammalian species given the potential implications.

SCN neurons can also be distinguished based on efferent projections. The SCN communicates with downstream targets using both synaptic and humoral signals (Mohawk & Takahashi 2011). In the case of synaptic connections, the SCN innervates structures in the hypothalamus, thalamus, and forebrain (Watts 1991, Kalsbeek & Buijs 2002), and these projections may originate from specific SCN regions. With regard to outputs from SCN neuronal subclasses, those originating from AVP and VIP neurons have been most extensively studied. AVP and VIP neurons often project to the same

target, but the relative patterning of innervation can be target-specific (Watts & Swanson 1987, Abrahamson & Moore 2001, Kalsbeek & Buijs 2002). For instance, both AVP and VIP neurons in the murine SCN project to the paraventricular nucleus and the dorsomedial nucleus of the hypothalamus, but each target contains more AVP fibers than VIP fibers (Abrahamson & Moore 2001). The functional roles of projections from distinct SCN regions are far from fully understood. Thus far, research indicates that both SCN compartments influence rhythms in downstream targets, yet there are differences in the role of outputs from different SCN regions (Yan *et al.* 2005, Zhou & Cheng 2005, Lee *et al.* 2009, Schwartz *et al.* 2009, Kalsbeek *et al.* 2010, Butler *et al.* 2012, Smarr *et al.* 2012, Wotus *et al.* 2013, Evans *et al.* 2015). One emerging theme is that the SCN shell appears to set the phase of downstream tissues; however, both SCN shell and core neurons provide signals to downstream tissues that can influence their rhythms. The efferent projection patterns of distinct SCN subclasses should be revisited in future work given recent advances in cell-type-specific tract tracing (Callaway & Luo 2015). A deeper understanding of this issue may provide insight into the functional role of outputs from different SCN subclasses.

Lastly, there is a rich body of work describing regional differences in SCN function. As mentioned above, SCN neurons display regional differences in the phase of electrical and molecular activities, which can be detected even within a chemically defined subclass (Hamada *et al.* 2004, Kalsbeek *et al.* 2006, Evans *et al.* 2011, Yoshikawa *et al.* 2015). Although the majority of SCN neurons tend to exhibit clustered phases of electrical and molecular activities, antiphase rhythms are expressed by subgroups of SCN neurons whose chemical identity remains ill defined (Nakamura *et al.* 2001, King *et al.* 2003, Lee *et al.* 2003). In addition, SCN neurons from distinct regions or peptidergic subclasses can exhibit different period length (Shinohara *et al.* 1995, Nakamura *et al.* 2001, Noguchi & Watanabe 2008, Myung *et al.* 2012). Further, daily patterns of electrical activity can differ markedly among neuronal subgroups (Shibata *et al.* 1984, Jiao *et al.* 1999, Jobst & Allen 2002, Saeb-Parsy & Dyball 2003, Belle *et al.* 2009). This suggests that SCN neurons can oscillate with different properties, but the presence of cellular rhythms may not be universal among SCN neurons. Many studies examining the SCN network have found that photoreponsive neurons within the SCN core display a less rhythmic or arrhythmic phenotype (Jiao *et al.* 1999, Hamada *et al.* 2001, Jobst & Allen 2002). However, recent work indicates that cellular rhythmicity is stochastic, not

restricted to neurons of a particular peptidergic subgroup, and sustained by network interactions (Webb *et al.* 2009). This provides new insight, in that it suggests that there is no single subclass of 'pacemaker' neuron in the SCN network, and that cellular rhythmicity is strongly influenced by network connectivity rather than chemical phenotype.

In summary, SCN neurons in different regions of the network can be distinguished in many ways. The most common model of SCN organization highlights functional distinctions between its shell and core compartments. In this scheme, the SCN core contains first-order neurons that receive and process photic input. SCN core neurons display strong rhythms in light-induced responses, but electrical and molecular rhythms of these neurons may be low amplitude or nonexistent. Nevertheless, input transmitted from SCN core neurons is important for coordinating neurons in the rest of the network. In contrast, neurons in the SCN shell are strongly rhythmic and play a key role in transmitting daily outputs to downstream structures. Overall, there is utility in this linear model, but some key aspects of SCN circuitry remain unexplained. For example, if SCN core neurons are not different from SCN shell neurons when studied in isolation, what accounts for their differences when they are embedded in the network? If specific subclasses of SCN core neurons do indeed lack a strong molecular oscillator, how do they maintain circadian rhythms in photic responsiveness? Presumably, these observations reflect that molecular and photoreponsive rhythms of SCN core neurons are influenced by intercellular communication, but the relevant signals remain undefined. Additional work investigating SCN signaling is expected to expand the understanding of its circuitry.

## SCN coupling mechanisms

SCN neurons communicate through multiple mechanisms and signaling factors (van den Pol & Dudek 1993, Michel & Colwell 2001, Aton & Herzog 2005). An important role of synaptic signaling is based on observations that SCN neurons desynchronize when cultured with tetrodotoxin (TTX) to block Na<sup>+</sup>-dependent action potentials (Yamaguchi *et al.* 2003). However, there is also evidence that the SCN network can use other forms of coupling that do not depend on synaptic communication. For instance, SCN timekeeping can be maintained in the absence of Na<sup>+</sup>-dependent action potentials and Ca<sup>2+</sup>-dependent synaptic transmission (Schwartz *et al.* 1987, Earnest *et al.* 1991,

Schwartz 1991, Bouskila & Dudek 1993, Dudek *et al.* 1993, Shibata & Moore 1993). Further, circadian rhythms are maintained in some species *in vivo* under environmental conditions that severely compromise neuronal activity (Menaker 1961, Grahn *et al.* 1994). Further, the SCN displays metabolic rhythms at an embryonic age (Shibata & Moore 1987, Reppert 1992) that precedes the completion of synaptogenesis (Bedont & Blackshaw 2015). Collectively, this suggests that synaptic transmission is not the exclusive means by which SCN neurons can communicate with one another. Interestingly, functional studies indicate that SCN neurons can communicate through paracrine signaling (Maywood *et al.* 2011). In this work, co-cultured SCN slices were able to influence the rhythmic properties of one another even though they were unable to establish cross-slice synaptic connections. One potential mechanism driving this effect may be the nonsynaptic release of SCN neuropeptides from axons, dendrites, and somata (Castel *et al.* 1996). While it remains unclear which specific SCN neuropeptides are released at these sites, this form of communication may influence the function of neuronal networks (Ludwig & Leng 2006, van den Pol 2012). In the section below, recent insight into the roles of different SCN coupling factors has been reviewed. In particular, much has been learned about the ways in which VIP, GABA, and AVP signaling influence SCN function. Nevertheless, it remains a challenge to fully map the neurochemical, temporal, and spatial properties of SCN circuits.

### Vasoactive intestinal polypeptide (VIP)

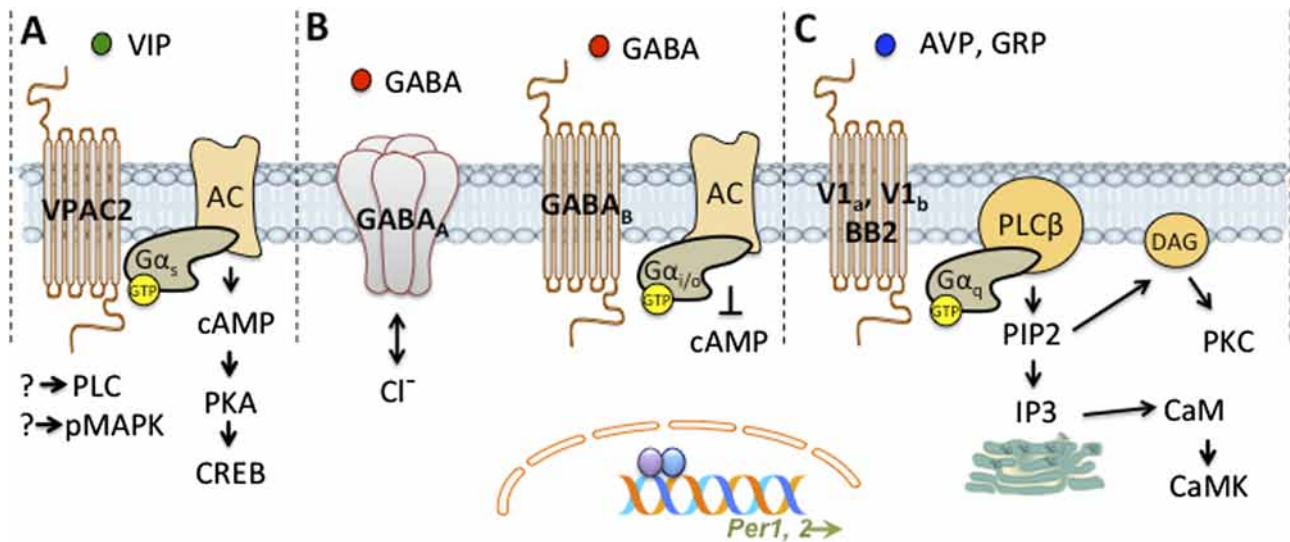
Over the past decade, clear evidence has emerged that indicates VIP is important for synchronizing SCN neurons (Vosko *et al.* 2007, Harmar 2003). As described above, VIP is produced by a subset of SCN neurons located within the ventral SCN core, with functional evidence suggesting that there are at least two subclasses of VIP neurons in the rat (Kawamoto *et al.* 2003). VIP is expected to have pervasive effects because VIP+ fibers innervate nearly all SCN regions (Card *et al.* 1981, Card & Moore 1984), and most SCN neurons express the VIP receptor, VPAC2 (Kalamatianos *et al.* 2004b, Kallo *et al.* 2004b, An *et al.* 2012). The influence of VIP signaling is expected to fluctuate daily since VIP and its receptor are expressed rhythmically in the SCN *in vivo* and *in vitro* (Takahashi *et al.* 1989, Glazer & Gozes 1994, Duncan *et al.* 1995, Shinohara *et al.* 1995, Cagampang *et al.* 1998, Dardente *et al.* 2004). The VPAC2 receptor is a G $\alpha_s$ -coupled receptor that activates adenylyl cyclase, cAMP, PKA, and

CRE-dependent transcription (Fig. 4A, Harmar *et al.* 1998, Couvineau & Laburthe 2011). Given that *Period* genes are activated by CREB, this suggests that VIP will influence cellular rhythms in SCN neurons. As expected, VIP signaling alters SCN electrical and molecular activities *in vivo* and *in vitro* (Piggins *et al.* 1995, Nielsen *et al.* 2002, Cutler *et al.* 2003, Itri & Colwell 2003, Pakhotin *et al.* 2006, Irwin & Allen 2010, Kudo *et al.* 2013). Moreover, it can phase-shift other SCN neuropeptide rhythms, such as AVP release (Watanabe *et al.* 2000). Consistent with VPAC2 being a G $\alpha_s$ -coupled receptor, the effects of VIP on SCN electrical and molecular rhythms depend on PKA signaling (Nielsen *et al.* 2002, Meyer-Spasche & Piggins 2004, An *et al.* 2011). However, VIP-induced effects also require activation of other intracellular cascades (i.e., mitogen-activated protein kinase, phospholipase C), which indicates that other signaling mechanisms may be involved (Fig. 4A).

Interest in the role of VIP signaling was especially piqued when it was discovered that the loss of VIP signaling is able to compromise circadian rhythmicity. When studied under constant dark conditions, the majority of mice lacking the gene for VIP or its receptor display either arrhythmic locomotor patterns or low-amplitude rhythms with altered period (Harmar *et al.* 2002, Colwell *et al.* 2003, Aton *et al.* 2005, Ciarleglio *et al.* 2009). Arrhythmic *Vip*-deficient mice also display loss of rhythms in SCN electrical activity and gene expression due to the lack of network synchrony and a decrease in the number of SCN neurons able to maintain viable cellular rhythms (Aton *et al.* 2005, Maywood *et al.* 2006, Brown *et al.* 2007, Hughes *et al.* 2008, Ciarleglio *et al.* 2009). Importantly, SCN cellular rhythms and synchrony can be rescued in *Vip* knockout slices by daily application of a VPAC2 agonist (Aton *et al.* 2005). Loss of cellular rhythms and synchrony likewise occurs in wildtype SCN slices exposed to a VPAC2 antagonist (Brown *et al.* 2007, Evans *et al.* 2013), which suggests that the effects of *VIP/VPAC2* deletion are not due to developmental abnormalities. Further, VIP signaling has important consequences for spatiotemporal arrangements of the SCN network, which involves intercellular communication triggered by G $\alpha_q$  signaling in VIP neurons (Brancaccio *et al.* 2013). Collectively, this work indicates that VIP is a local coupling factor that amplifies and entrains SCN neurons that are weak intrinsic oscillators. These effects are very important for maintaining cellular rhythms and synchronized network activity within the SCN network.

In addition to serving as a local coupling factor, VIP signaling also influences other processes. In particular, VIP





**Figure 4**

Intercellular signaling mechanisms that alter cellular activity and clock gene expression within SCN neurons. (A) SCN neurons express the VPAC2 receptor for VIP, which is a G-protein-coupled receptor with seven transmembrane domains. Upon VIP binding to VPAC2,  $G\alpha_s$  activates adenylyl cyclase, cAMP, PKA, and CRE-dependent transcription. (B) Within the SCN, both  $GABA_A$  receptors and  $GABA_B$  receptors are expressed. GABA binding to the ionotropic  $GABA_A$  receptor allows for influx or efflux of  $Cl^-$ , depending on  $[Cl^-]_i$ . By contrast, the metabotropic  $GABA_B$  receptor is coupled to  $G\alpha_{\gamma/o}$ , which inhibits adenylyl cyclase. (C) The receptors for both AVP and GRP are  $G\alpha_q$ -coupled receptors that stimulate phospholipase C (PLC $\beta$ ), which in turn activates phosphatidylinositol 4,5-bisphosphate (PIP2) to cause diacyl glycerol (DAG)-mediated activation of protein kinase C (PKC) and inositol triphosphate (IP3)-induced release of intracellular calcium stores. Intracellular release of calcium activates calmodulin (CaM), which stimulates  $Ca^{2+}$ /calmodulin-dependent protein kinase (CaMK). Due to the expression of CRE-elements in *Period* genes, changes in the intracellular signaling cascades illustrated in A–C can alter the function of the molecular clock.

is involved in photic signaling (Shen *et al.* 2000, Colwell *et al.* 2003, Kuhlman *et al.* 2003, Hughes *et al.* 2004, Dragich *et al.* 2010, Lucassen *et al.* 2012, An *et al.* 2013), and light influences VIP expression in the SCN (Duncan *et al.* 1995, Shinohara & Inouye 1995, Isobe & Nishino 1998, Smith & Canal 2009, Francl *et al.* 2010). Deficits in VIP signaling cause abnormal responses to light, with one result being an unusual responsiveness to light during the day. Furthermore, loss of VIP causes disruptions in a wide variety of physiological processes, including cardiovascular function, metabolism, and reproduction (Shimizu *et al.* 1996, Bechtold *et al.* 2008, Sheward *et al.* 2010, Hannibal *et al.* 2011, Schroeder *et al.* 2011, Loh *et al.* 2014). This indicates that VIP signaling is important for maintaining endocrine function, which could be due to its role in the SCN network itself and/or its role as an output signal to downstream tissues (van der Beek *et al.* 1993, Kalsbeek *et al.* 1993, Gerhold *et al.* 2001, Egli *et al.* 2004, Kallo *et al.* 2004b, Gerhold & Wise 2006, Loh *et al.* 2011, Fahrenkrug *et al.* 2012). Also, age- and sex-related differences in VIP expression may influence changes in the strength and robustness of circadian function (Zhou *et al.* 1995, Kawakami *et al.* 1997, Krajnak *et al.* 1998a,b, Kallo *et al.* 2004a, Mahoney *et al.* 2009). Thus,

VIP signaling is an important modulator whose absence has widespread consequences for behavior and physiology. There continues to be a need to define the properties and mechanisms of VIP signaling to better understand how it regulates clock function at the cellular, network, and systems levels. For instance, recent work demonstrates that VIP can desynchronize the phase of SCN neurons if given at the wrong time or at high doses (An *et al.* 2013, Ananthasubramaniam *et al.* 2014). This suggests that VIP expression is tightly regulated within the SCN network. Of interest, 4E-BP1 has been identified recently as a molecular repressor of *Vip* mRNA translation (Cao *et al.* 2013). Future studies are expected to provide additional insight into the temporal and spatial features of VIP signaling, as well as modulatory mechanisms.

#### $\gamma$ -aminobutyric acid (GABA)

Since it was first reported that nearly all SCN neurons express GABA, it has been suspected that this neurotransmitter regulates SCN communication. The SCN displays a high density of GABA terminals and cells (van den Pol & Tsujimoto 1985, Okamura *et al.* 1989, Decavel & Van den Pol 1990, Moore & Spoh 1993,

Huhman *et al.* 1996, Castel & Morris 2000, Abrahamson & Moore 2001). Receptors for GABA include both ionotropic GABA<sub>A</sub> receptors and metabotropic GABA<sub>B</sub> receptors coupled to Gα<sub>i/o</sub> (Fig. 4B, Bormann 2000), with both being expressed in the SCN (Gao *et al.* 1995, O'Hara *et al.* 1995, Naum *et al.* 2001, Belenky *et al.* 2003, 2008). As with other SCN factors, daily rhythms in GABA signaling are evident in the SCN, and these rhythms can be modulated by light exposure (Aguilar-Roblero *et al.* 1993, Huhman *et al.* 1996, Naum *et al.* 2001, Itri *et al.* 2004). Consistent with this anatomical work, SCN neurons are sensitive to GABA signaling (Liou *et al.* 1990, Mason *et al.* 1991, Gillespie *et al.* 1997, 1999, Jiang *et al.* 1997b, Strecker *et al.* 1997, Biggs & Prosser 1998, Cardinali & Golombek 1998, Mintz *et al.* 2002, Ehlen *et al.* 2008). Collectively, these studies indicate that GABA regulates SCN neuronal activity, modulates photic signaling, and serves as an output signal to downstream tissues (Wang *et al.* 2003). For modulation of photic signaling, an important role has been demonstrated for both GABA<sub>A</sub> and GABA<sub>B</sub> receptors (Gillespie *et al.* 1997, Biggs & Prosser 1998, Belenky *et al.* 2003, Novak *et al.* 2004, Ehlen & Paul 2009). By contrast, GABA<sub>A</sub> receptor signaling is thought to be the strongest local regulator of SCN network activity (Kim & Dudek 1992, Shimura *et al.* 1996, Strecker *et al.* 1997, Albus *et al.* 2005, Fan *et al.* 2015). Over the last two decades, work has revealed surprising ways in which GABA can modulate SCN function.

One of the first surprises discovered about GABA signaling in the SCN concerns the polarity of responses to this neurotransmitter. GABA is typically defined as an inhibitory neurotransmitter; however, GABA can depolarize and increase intracellular calcium concentration in SCN neurons (Wagner *et al.* 1997, Choi *et al.* 2008, Irwin & Allen 2009). Although excitatory GABAergic responses are not always observed (Bos & Mirmiran 1993, Dudek *et al.* 1993, Gribkoff *et al.* 1999), this may stem at least in part from spatial or temporal differences in recordings performed in different laboratories (De Jeu & Pennartz 2002, Albus *et al.* 2005, Choi *et al.* 2008, Ikeda *et al.* 2013, Alamilla *et al.* 2014, DeWoskin *et al.* 2015). Indeed, the SCN displays regional differences in the expression of the chloride co-transporters (Belenky *et al.* 2008, 2010, Choi *et al.* 2008) that determine chloride reversal potential (Vogt 2015). Adding to this complexity, it has been demonstrated recently that the polarity of GABA responses in the SCN is influenced by the duration of daily light exposure (Farajnia *et al.* 2014, Myung *et al.* 2015). This may have great relevance for the ability of the

SCN to serve as an annual calendar, and firmly places the SCN on the growing list of mature neural networks that exhibit plasticity in the polarity of GABA responses (Marty & Llano 2005).

Given that the nature of SCN responses to GABA remain unclear, it should come as little surprise that the precise role of GABA in SCN coupling has proven difficult to define. Early work demonstrated that dissociated SCN neurons exposed to GABA will synchronize their electrical rhythms via GABA<sub>A</sub> signaling (Liu & Reppert 2000, Shirakawa *et al.* 2000). Additional studies further indicated that GABA<sub>A</sub> signaling is involved in the transfer of resetting information from the SCN core to the SCN shell (Albus *et al.* 2005, Han *et al.* 2012). However, GABA receptor antagonists do not desynchronize SCN neurons *in vitro* (Aton *et al.* 2006). These results came as a surprise because they run counter to the hypothesis that GABA acts as a coupling factor. Recent work has provided some insight into these conflicting findings. GABA<sub>A</sub> signaling does influence SCN coupling, but surprisingly it destabilizes phase relationships of SCN neurons (Evans *et al.* 2013, Freeman *et al.* 2013, DeWoskin *et al.* 2015, Myung *et al.* 2015). Normally, this effect of GABA<sub>A</sub> signaling is hard to detect because VIP signaling is a potent synchronizing agent. But in the absence of VIP, SCN neurons desynchronize due to GABA<sub>A</sub> signaling (Evans *et al.* 2013, Freeman *et al.* 2013). In fact, antagonism of GABA<sub>A</sub> signaling will 'fix' a *Vip* KO slice and prevent desynchrony from occurring. This indicates that the loss of synchrony that emerges during VIP deficiency is not due to 'passive' desynchrony caused by cellular period differences but rather 'active' desynchronizing responses elicited by GABA signaling. This provides us with a new view into SCN circuitry because it indicates that some SCN signaling mechanisms promote synchronization, while others may cause desynchronization. This also reveals that SCN coupling factors may be arranged into pairs that oppose one another's effects. Building on this theme of interactive coupling mechanisms, GABA<sub>A</sub> signaling can interact with VIP signaling in either an antagonistic or cooperative manner depending on the state of the network (Evans *et al.* 2013), which may account for earlier work demonstrating synchronizing effects (Liu & Reppert 2000, Shirakawa *et al.* 2000, Albus *et al.* 2005, Han *et al.* 2012). Together with previous work, this strongly suggests that the functional role of a coupling mechanism can change based on the experience, history, or age of the animal (Evans *et al.* 2013, Bedont *et al.* 2014, Wang *et al.* 2014).

### Arginine vasopressin (AVP)

Although AVP has been traditionally viewed more as an SCN output signal, a more direct role in network synchronization has been suggested recently. AVP is rhythmically expressed both *in vivo* and *in vitro* (Cassone *et al.* 1988, Sukhov *et al.* 1993, Shinohara *et al.* 1998, Dardente *et al.* 2004, Van der Veen *et al.* 2005, Miller *et al.* 2006, Noguchi & Watanabe 2008, Mahoney *et al.* 2009, Yoshikawa *et al.* 2015). Of the three known AVP receptors,  $V_{1a}$  (V1) and  $V_{1b}$  (V3) are expressed within the SCN (Kalamatianos *et al.* 2004a, Li *et al.* 2009), which are both  $G\alpha_q$ -coupled receptors that stimulate phospholipase C to cause DAG-mediated activation of PKC and IP3-induced mobilization of intracellular calcium (Fig. 4C, Maybauer *et al.* 2008). Although AVP was one of the first SCN neuropeptides to be discovered, it was deemed not necessary for circadian rhythms based on work in rats with a spontaneous loss-of-function mutation (Grobowski *et al.* 1981, Boer *et al.* 1998). Further work, however, revealed that these rats display lower amplitude rhythms of sleep, melatonin release, and corticosterone (Schroder *et al.* 1988, Brown & Nunez 1989, Wideman *et al.* 2000), and this led to the suggestion that AVP was mostly an important output signal (Jin *et al.* 1999, Tousson & Meissl 2004). In support of a network role, however, AVP neurons project locally within the SCN itself (Castel *et al.* 1990, Romijn *et al.* 1997) and exogenous AVP is able to regulate the cellular activity of SCN neurons (Liou & Albers 1989, Mihai *et al.* 1994, Ingram *et al.* 1998). However, it was unclear whether these responses actually produced functional consequences because AVP application *in vitro* or *in vivo* does not phase-shift SCN rhythms. Nevertheless, recent work has revealed that AVP is able to influence the function of the SCN network.  $V_{1a}$  and  $V_{1b}$  receptor knockout mice display a pronounced circadian phenotype, in that they are resistant to jetlag and re-entrain almost instantly following a shift in the light:dark cycle (Yamaguchi *et al.* 2013). Wildtype mice receiving AVP receptor antagonists directed to the SCN likewise shift quickly, thus discounting a developmental basis of this effect. Rapid recovery following simulated jetlag is also observed in mice lacking *Bmal1* in AVP neurons, which can be reversed by SCN-specific rescue of molecular clock function (Mieda *et al.* 2015). Collectively, this work suggests that AVP signaling is involved in setting the pace of re-entrainment. It has been proposed that the loss of AVP signaling causes a change in SCN coupling that allows the entire network to shift rapidly to the new time zone. Consistent with this, application of AVP can

synchronize SCN neurons collected from mice deficient in VIP signaling (Maywood *et al.* 2011). Further work testing the specific role of AVP signaling in SCN coupling is warranted.

### Gastrin-releasing peptide (GRP)

The potential role of GRP in SCN coupling remains to be determined, although there is clear evidence that GRP signaling is involved in photic processing. GRP is rhythmically expressed in the SCN and modulated by light (Shinohara *et al.* 1993, Dardente *et al.* 2004, Lee *et al.* 2013). The GRP receptor, also known as the bombesin 2 (BB2) receptor, is likewise rhythmically expressed within the SCN in a manner influenced by light exposure (Karatsoreos *et al.* 2006). Like the AVP receptor, BB2 is a  $G\alpha_q$ -coupled receptor (Fig. 4C, Jensen *et al.* 2008). GRP neurons within the SCN receive retinal input that induces cellular responses (Dardente *et al.* 2002, Lesauter *et al.* 2011). Further, GRP can phase-shift SCN rhythms *in vitro* and locomotor rhythms *in vivo* in a pattern similar to those induced by light and VIP (Piggins *et al.* 1995, Gillespie *et al.* 1997, McArthur *et al.* 2000, Aida *et al.* 2002, Piggins *et al.* 2005, Antle *et al.* 2005a, Gamble *et al.* 2007, Biello 2009). These effects of GRP are dependent on BB2 receptor signaling, CREB-dependent transcription, clock gene activation, and changes in fast delayed rectifier potassium currents (Aida *et al.* 2002, Piggins *et al.* 2005, Gamble *et al.* 2007, 2011). However, the role of GRP may not be limited to photic signaling because GRP can enhance cellular rhythms in SCN slices collected from mice deficient in VIP signaling (Brown *et al.* 2005, Maywood *et al.* 2006, 2011). Further, cellular rhythmicity is attenuated by a BB2 receptor antagonist, but only when applied in the absence of VIP signaling (Brown *et al.* 2005). Together with the work on GABA described above, this suggests that VIP is a very strong modulator of SCN cellular rhythms that may mask the effects of other signaling mechanisms.

### Other neuroactive substances

In addition to these well-studied subgroups, there are likely novel subclasses of SCN neurons that play a role in network coupling. The SCN produces dozens of signaling factors, and the number of recognized SCN peptides continues to increase (van den Pol & Tsujimoto 1985, Lee *et al.* 2013, 2015). A recent study demonstrated that SCN network function is regulated by neuromedin S, which is an SCN peptide produced by both AVP and VIP neurons

(Lee *et al.* 2015). Also, a forward peptidomics screen identified little SAS as a novel peptide produced in the SCN core that relays photic signals independent of VIP- or GRP-dependent signaling (Atkins *et al.* 2010). Another interesting development is that intra-SCN glutamatergic signaling may play a role in coupling the left and right SCN (Michel *et al.* 2013), and yet glutamate is not expressed by SCN neurons (Strecker *et al.* 1997). Communication between the left and right SCN likely differs from that coupling neurons within each SCN (Bouskila & Dudek 1993), and additional studies may shed new light on mechanisms mediating this coupling. Overall, this work highlights that the SCN remains a complex structure, and it is likely that additional coupling signals will be identified in the future.

### Nonsynaptic interactions

One early hypothesis posited a role of local electric field effects produced by changes in the membrane potential of adjacent cells. Ephaptic interactions produced by electrical field potentials in dendrites can synchronize electrical activity in the mammalian neocortex, but it is unlikely that this mechanism couples oscillators within the SCN given its nonlaminar organization (Van den Pol 1980, van Esseveldt *et al.* 2000). Nevertheless, functional and anatomical evidence suggests that SCN neurons can communicate through nonsynaptic release of neuropeptides (Castel *et al.* 1996, Maywood *et al.* 2011). Moreover, electrotonic communication through low-resistance gap junctions may influence SCN coupling (Jiang *et al.* 1997a, Colwell 2000, Jobst *et al.* 2004, Long *et al.* 2005). Gap junctions are channels that allow the exchange of small molecules between neurons and/or glia in close apposition (Bennett *et al.* 1991, Rash *et al.* 2000, 2001, Connors & Long 2004). Gap junctions can be found in both SCN neurons and glia, with the diffusion of labeled molecules (i.e. dye coupling) occurring mostly between homotypic cells located in the same SCN compartment (Jiang *et al.* 1997a, Colwell 2000). Studies using paired intracellular recordings and dye coupling suggest that electrical coupling varies as a function of circadian phase (Colwell 2000, Long *et al.* 2005), which suggests that communication through gap junctions is not a passive process but one that is actively regulated in a dynamic fashion. Further evidence that gap junctions likely influence SCN function stems from pharmacological experiments demonstrating that SCN electrical rhythms become broader, arrhythmic, or bimodal after octanol

or halothane application (Prosser *et al.* 1994, Shinohara *et al.* 2000a,b, Shirakawa *et al.* 2001). These specific changes in waveform are thought to reflect changes in SCN phase relationships caused by altered coupling, although this has yet to be tested directly. Further, the cellular location of these gap junctions remains unclear since these pharmacological inhibitors would be expected to affect both neurons and glia, as well as producing off-target effects. A specific role for neuronal gap junctions is indicated by work demonstrating that SCN electrical coupling is dependent on the expression of the neuron-specific gap junction protein connexin36 (Long *et al.* 2005). Signaling through these gap junctions is thought to influence circadian behavior because mice lacking connexin36 display reduced amplitude of locomotor rhythms under constant darkness (Long *et al.* 2005). Another factor that may modulate circadian behavior via gap junction signaling is the expression of the polysialylated form of neural cell adhesion molecule (Lee *et al.* 1995, Glass *et al.* 2003), although this protein is also involved in synaptic transmission. This work suggests that gap junctions influence SCN function, but additional studies are needed to delineate their precise role.

### Astrocytes

While most circadian research has focused on the functional properties of SCN neurons, the SCN also contains glial cells (Guldner 1983). SCN astrocytes can be detected by labeling glial fibrillary acidic protein (GFAP), which displays a daily rhythm that is modulated by light (Lavialle & Serviere 1993, Moriya *et al.* 2000, Gerics *et al.* 2006, Becquet *et al.* 2008, Lindley *et al.* 2008, Canal *et al.* 2009). Like other cell types, astrocytes are intrinsic oscillators that display daily rhythms in metabolic function (Schwartz & Gainer 1977, van den Pol *et al.* 1992, Lavialle & Serviere 1993, Womac *et al.* 2009, Burkeen *et al.* 2011) and clock gene/protein expression (van den Pol *et al.* 1992, Prolo *et al.* 2005, Cheng *et al.* 2009, Yagita *et al.* 2010, Duhart *et al.* 2013). The available evidence points to several ways in which SCN glia and neurons interact (Jackson 2011); however, the precise role of glia in the SCN network is not well characterized. In general, astrocytes provide physical support, release pro-survival factors, influence synaptic clearance, and produce gliotransmitters that interact with presynaptic and postsynaptic receptors (Faissner *et al.* 2010). Anatomical and functional evidence suggests that SCN astrocytes may influence light-induced resetting by

regulating glutamate release from retinal terminals (van den Pol *et al.* 1992, Lavaille & Serviere 1995, Tamada *et al.* 1998, Moriya *et al.* 2000, Lavaille *et al.* 2001, Girardet *et al.* 2010). Interestingly, VIP and AVP neurons in the SCN core and shell compartments display differences in daily rhythms of glial coverage of dendrites, with higher coverage of VIP and AVP neurons during the night and day, respectively (Becquet *et al.* 2008). This suggests that SCN glia may differentially regulate signaling to these two subpopulations across the circadian cycle. Consistent with a glial role in SCN photic responses and/or interneuronal coupling, mice that have a mutation in GFAP have altered locomotor activity rhythms in LL (Moriya *et al.* 2000). More direct evidence for glial involvement in SCN coupling is provided by work demonstrating that SCN electrical rhythms become bimodal in the presence of the glial metabolism antagonist, fluorocitrate (Prosser *et al.* 1994). These changes in the waveform of SCN electrical rhythms are thought to be due to altered communication among SCN neuronal subpopulations (Wang *et al.* 2014), which could be tested further with real-time imaging techniques. Communication between SCN neurons and astrocytes is likely bidirectional since damped rhythms in astrocytes are enhanced by co-culture with SCN explants, but not cortical explants (Prolo *et al.* 2005). Further, VIP influences the phase and amplitude of astrocyte rhythms in a dose-dependent manner (Marpagan *et al.* 2009). Collectively, this work suggests that SCN astrocytes and neurons interact with one another, and future studies are expected to clarify the functional consequences of this relationship.

## Conclusions

In many ways, mapping the circuitry of the SCN remains a challenge. Defining the specific role of any given signaling mechanism can be complicated given that multiple processes interact and modulate one another (Shinohara *et al.* 2000b, Itri *et al.* 2004, Haas *et al.* 2011, Wang *et al.* 2014). In addition, it remains a challenge to separate the role of a given neuromodulator in mediating intranetwork coupling from its role in processing input and transmitting output. Despite the difficulty in addressing these issues, it remains critical to address how SCN cells integrate the various signals provided by other cell types in the network. The combinatorial effects of SCN coupling factors have yet to be systemically investigated, although some work has addressed this issue in the context of photic signaling (Albers *et al.* 1991, Piggins *et al.* 1995). It is likely

that future technological advances will prove critical for achieving a deeper understanding of SCN circuitry. Going forward, it will be important to define the precise cellular location of receptors for putative coupling factors. Further, whether these receptors are co-expressed spatially and temporally on specific subclasses of SCN neurons should be addressed in future work. Given the potential for rhythms in each component of SCN signaling (e.g. signal release, receptor expression, physical connectivity, and astrocyte function), advanced techniques for imaging cellular connections and responses will continue to be essential for making progress in this area.

### Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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