The Clock in the Brain: Neurons, Glia, and Networks in Daily Rhythms

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Abstract The master coordinator of daily schedules in mammals, located in the ventral hypothalamus, is the suprachiasmatic nucleus (SCN). This relatively small population of neurons and glia generates circadian rhythms in physiology and behavior and synchronizes them to local time. Recent advances have begun to define the roles of specific cells and signals (e.g., peptides, amino acids, and purine derivatives) within this network that generate and synchronize daily rhythms. Here we focus on the best-studied signals between neurons and between glia in the mammalian circadian system with an emphasis on time-of-day pharmacology. Where possible, we highlight how commonly used drugs affect the circadian system.

Keywords SCN • VIP • GRP • AVP • Little SAAS • GABA • ATP

1 Neurons of the SCN

The nearly 20,000 neurons of the suprachiasmatic nucleus (SCN) have been identified as the alarm clock, or master circadian pacemaker, to the remaining 100,000,000,000 neurons in the human brain (Klein et al. 1991). Put succinctly, the SCN has been ascribed a single function—to synchronize the body's daily rhythms to local time. Although most of the evidence comes primarily from mice, rats, and hamsters, the SCN appears to be highly conserved in its anatomical and physiological organization. The SCN acts as a central timer in vivo and in vitro. In vivo, multiple brain regions exhibit circadian changes in electrical activity, with the SCN peaking during the day and the others at night (Inouye and Kawamura 1982;

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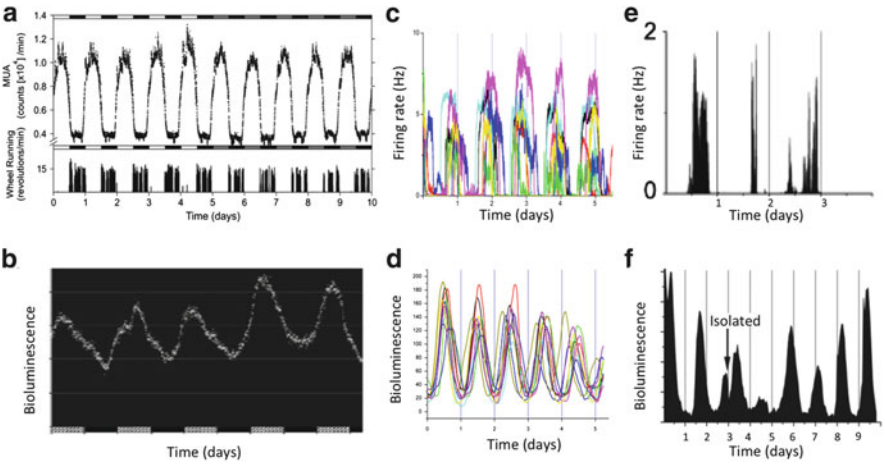

Fig. 1 Pharmacology of the circadian system must be considered in the context of daily changes in gene expression and membrane excitability. SCN neurons are capable circadian pacemakers in vivo, in vitro, and in isolation. (**a**) In vivo multiunit firing rhythms in the SCN. (**b**) In vivo Per1 transcription rhythms in the SCN. (**c**) In vitro firing rhythms recorded from 10 representative neurons with synchronized circadian periods. (**d**) In vitro Period1 rhythms from 10 representative cells with synchronized circadian periods. (**e**) Isolated SCN neuron shows rhythm in firing rate. (**f**) Isolated SCN neuron shows daily rhythms in Period2 protein expression

Yamazaki et al. 1998; Meijer et al. 1998) (Fig. 1). Ablation of the SCN abolishes many of these coordinated daily rhythms in the brain and behavior (Ralph et al. 1990; Moore and Eichler 1972; Stephan and Zucker 1972). Critically, SCN transplants restore behavioral circadian rhythms in SCN-lesioned animals with the period of the donor (Ralph et al. 1990; Sujino et al. 2003). When isolated in vitro, the SCN continues to express circadian rhythms in glucose metabolism, gene expression, neuropeptide secretion, and electrical activity similar to its rhythmicity in vivo (Green and Gillette 1982; Earnest and Sladek 1986; Shinohara et al. 1995; Herzog et al. 1997; Quintero et al. 2003; Yamazaki et al. 2000) (Fig. 1). Thus, the SCN acts as a pacemaker that generates and drives daily rhythms in the brain and body.

Individual SCN neurons are competent circadian pacemakers. Just as single cyanobacteria and isolated retinal neurons from a marine snail show circadian oscillations (Mihalcescu et al. 2004; Michel et al. 1993), SCN neurons have recently been shown to cycle on their own (Fig. 1) (Webb et al. 2009). This is consistent with the standard model in which intracellular molecular events regulate daily rhythms in transcription and translation (Welsh et al. 2010). SCN cells retain many of their circadian properties when isolated from their network such as a genetically determined period near 24 h that changes little over a wide range of temperatures (Herzog and Huckfeldt 2003). Importantly, when isolated either physically or pharmacologically from their neighbors, SCN cells lose their daily precision and become relatively unstable oscillators (Webb et al. 2009; Liu et al.

2007; Abraham et al. 2010). The SCN thus comprises a multi-oscillator system that depends on intercellular signaling to synchronize the component oscillatory cells to each other and to environmental cycles.

The population of heterogeneous SCN neurons has spatial organization. Anatomically, the SCN has been divided into a dorsal shell and ventral core (Moore et al. 2002; Antle et al. 2003, 2007; Morin 2007). The retinal inputs are most dense in the ventral SCN where light first induces immediate genes (e.g., cFOS and Period1; Hattar et al. 2002; Abrahamson and Moore 2001). The dorsal SCN has been noted for its circadian rhythms in gene expression and as a recipient of projections from the ventral SCN (Leak and Moore 2001). Indeed, there are lighting conditions that can force the rhythms in the dorsal and ventral SCN apart, supporting a model where the ventral SCN lacks intrinsic oscillations and conveys photic information to the intrinsically rhythmic neurons of the dorsal SCN (Karatsoreos et al. 2004; LeSauter et al. 1999; Shigeyoshi et al. 1997). However, there is also strong evidence that cells in both the top and bottom of the SCN are intrinsically circadian (de la Iglesia et al. 2004; Cambras et al. 2007; Yamaguchi et al. 2003; Shinohara et al. 1995; Albus et al. 2005; Webb et al. 2009). It is not yet clear whether some, most, or all SCN cells are functional circadian pacemakers.

1.1 Neuron–Neuron Signaling in the SCN

Although intercellular communication within the SCN has been the focus of significant experimental effort, little is known about how SCN cells synchronize to each other to coordinate behavior. Most neurons within cultured explants of the SCN express synchronized circadian rhythms (Herzog et al. 1997; Quintero et al. 2003; Yamaguchi et al. 2003; Nakamura et al. 2001), while neurons dispersed at low density tend to oscillate with different periods (Welsh et al. 1995; Liu et al. 1997b; Herzog et al. 1998; Honma et al. 1998b; Nakamura et al. 2002). Dispersed SCN cells, when transplanted into SCN-lesioned animals, restore circadian behaviors (Silver et al. 1990) and, when plated at higher densities in vitro, secrete vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) (Murakami et al. 1991; Honma et al. 1998a) in a coordinated circadian pattern. This indicates that SCN cells release and receive signals that allow them to synchronize to each other.

The list of candidate intercellular signals within the SCN is extensive and virtually unexplored. We must consider factors that could be secreted by neurons or glia through vesicular and non-vesicular release mechanisms. For example, a screen for genes expressed in the SCN that encode secreted and membrane-bound proteins identified more than 100 peptides, including growth factors, cytokines, chemotrophins, neuropeptide precursors, and transmembrane proteins that signal after cleavage (Kramer et al. 2001). A recent effort to sequester and sequence peptides secreted from SCN explants identified more than 100 peptides derived from 27 precursor proteins (Lee et al. 2010). These lists will include at least some of the synaptic and extrasynaptic releasates but will miss signals carried through gap

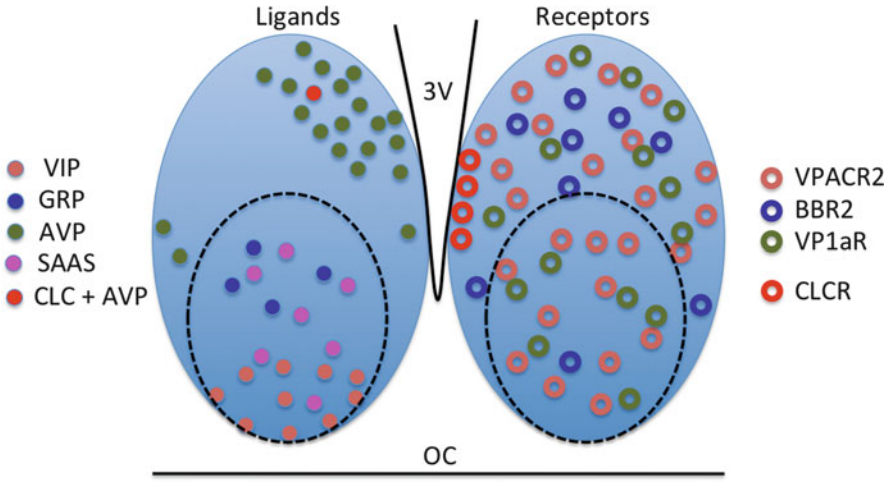

Fig. 2 Schematic localizing ligands and their cognate receptors in the SCN. For simplicity, the *left* SCN illustrates distributions of cells expressing identified ligands, and the *right* SCN shows somata expressing the relevant receptors. Based on neuropeptide expression, five distinct classes of cells account for approximately 50 % of the neurons in the SCN. These peptidergic cell classes are AVP, VIP, GRP, little SAAS, and CLC. Each *filled circle* represents the cell-body location of approximately 100 neurons. The broad distribution of the cognate receptors (*open circles*) in the SCN (largely based on mRNA expression) suggests extensive and convergent signaling from these distinct classes within the SCN. Here, CLCR-positive cells express the three genes believed to encode the heterotrimeric CLC receptor. The *dashed circles* delimit the area of densest retinal innervation often termed the SCN core. *3V* third ventricle, *OC* optic chiasm

and hemi-junctions. Here, we focus on the pharmacology of a short list of signals that have been most studied (Fig. 2). For each, we review the ligand and receptor, the time of day when most effective, the signaling cascade, and the potential role in SCN function.

1.1.1 VIP/VPAC2R

Produced by approximately 10–22 % of SCN neurons (Abrahamson and Moore 2001; Atkins et al. 2010; Moore et al. 2002), VIP is at the top of the hierarchy of influential signals in the SCN. Deletion of the *VIP* gene or the *Vipr2* gene for the VIP receptor, VPAC2R, results in the most severe circadian phenotype of any signaling molecule studied thus far: disrupted circadian behaviors and hormonal secretion, 8-h advance of the daily onset of activity in a light–dark cycle (i.e., phase angle of entrainment), and drastically reduced synchrony among circadian cells in the SCN (Maywood et al. 2011b). VIP neurons are primarily located in the ventral SCN where they receive dense innervation from the retina (Harmar et al. 2002; Hattar et al. 2002). VIP induces calcium influx (Irwin and Allen 2010) and changes in firing rate (Reed et al. 2001) and shifts the phase of the SCN through parallel

increases in adenylate cyclase and phospholipase C activities (An et al. 2011). Although *Vipr2* mRNA appears throughout the SCN (Usdin et al. 1994; Kalamatianos et al. 2004; Kallo et al. 2004), it is not yet clear if VIP acts directly on all or a subset of SCN cells.

VIP likely plays a role in synchronization between circadian cells and adjustments to the light–dark cycle. VIP is released in a circadian pattern from the cultured SCN (Shinohara et al. 1993, 1995), but there is conflicting evidence for circadian VIP release in vivo (Laemle et al. 1995; Francl et al. 2010). VIP release is stimulated also by light in vivo (Francl et al. 2010; Shinohara et al. 1998).

Importantly, the effects of VIP depend on the time of administration. During the subjective day and early subjective night, VIP dose-dependently delays circadian rhythms in the SCN with a maximal effect around subjective dusk (Reed et al. 2001; An et al. 2011). During the late subjective night and early morning, VIP modestly advances the SCN. When applied daily, VIP entrains the isolated SCN (An et al. 2011). These results are consistent with a model in which the 3,000 VIPergic neurons of the SCN synchronize their circadian rhythms to each other and coordinate circadian timing throughout the SCN.

To better understand whether VIP acts alone or in concert with other signals to synchronize SCN cells, Maywood and colleagues developed a novel coculture technique (Maywood et al. 2011b). They took advantage of the VIP-deficient SCN in which cells fail to synchronize their daily rhythms in gene expression. They found that a wild-type SCN could restore coordinated circadian cycling in a VIP-deficient SCN explant, confirming that VIP is both necessary and sufficient for sustained rhythms in the SCN and revealing that VIP can diffuse several millimeters to accomplish this task. However, when they discovered that wild-type SCN could slowly restore circadian rhythms to VPAC2R-deficient SCN, they concluded that other signals also must be capable of synchronizing SCN cells.

1.1.2 GRP/BBR2

Produced by approximately 4–10 % of SCN neurons (Abrahamson and Moore 2001; Antle et al. 2005; Atkins et al. 2010; Moore et al. 2002), gastrin-releasing peptide (GRP) in the SCN appears to have functions similar to and distinct from VIP. GRP-synthesizing neurons are found in the middle of the SCN and GRP receptor (BBR2). mRNA appears throughout the SCN, with more in the dorsal SCN (Aida et al. 2002; Karatsoreos et al. 2006). It is not yet clear if all or some SCN neurons respond directly to GRP.

Like VIP neurons, GRP neurons have been implicated in the SCN response to nighttime light exposure. Like VIP neurons, they receive retinal input and respond to nocturnal light with increased transcription of *cFOS* and the *Period* genes (Bryant et al. 2000; Karatsoreos et al. 2004). It is not yet clear if light induces the release of GRP. Like VIP, GRP signals through increases in cAMP (Gamble et al. 2007) and has been implicated also in synchronization between circadian SCN cells. GRP application in vivo and in vitro can shift SCN rhythms (Piggins et al.

1995; Antle et al. 2005; Kallingal and Mintz 2006; Gamble et al. 2007). Although blocking GRP receptors (BBR2) does not abolish SCN rhythms, it prevents SCN cocultures from restoring rhythms to VIP-deficient SCN (Maywood et al. 2011b). Finally, GRP application induces coordinated circadian rhythms in SCN deficient for VPAC2R (Brown et al. 2005; Maywood et al. 2006). Taken together, these results support the hypothesis that GRP is a weaker synchronizing agent than VIP but participates in entrainment of SCN circadian rhythms.

Anatomical data suggest an additional role for GRP neurons in communicating information from the dorsal to ventral SCN (Drouyer et al. 2010). In one model, synchrony among the circadian cells of the SCN requires this feedback onto retinorecipient SCN cells to gate their sensitivity to ambient light (Antle et al. 2007). Consistent with this hypothesis, mice lacking the GRP receptor show attenuated shifts to bright light (Aida et al. 2002). It will be exciting to see if animals deficient for GRP are slower to adjust to shifts in their light schedule and whether their dorsal and ventral SCN might fail to remain synchronized.

1.1.3 AVP/V1aR

Produced by approximately 20–37 % of SCN neurons (Abrahamson and Moore 2001; Moore et al. 2002), arginine vasopressin (AVP) was the first neuropeptide discovered in the SCN, although years after it was found in the magnocellular neurosecretory hypothalamic (supraoptic and paraventricular) nuclei where it is produced in even greater abundance (Swaab et al. 1975; Vandesande et al. 1974; Burlet and Marchetti 1975). AVP-synthesizing neurons are found in the dorsal-medial SCN and, in mouse, also in a small group of magnocellular neurons in the lateral SCN. In the SCN, AVP signals primarily through V1a receptors which appear to be broadly expressed (Li et al. 2009). Although most SCN neurons increase their firing in response to AVP, it is not yet clear if the response is direct or through network interactions (Ingram et al. 1996).

The regulation of circadian AVP release occurs at the levels of transcription (Jin et al. 1999), translation, and neuronal excitability. AVP levels in the cerebrospinal fluid vary with time of day, depending on the SCN, with a morning peak about five times higher than in the evening (Abrahamson and Moore 2001; Moore et al. 2002). This rhythm is intrinsic to the isolated SCN (Swaab et al. 1975; Vandesande et al. 1974; Burlet and Marchetti 1975) and regulated, at least in part, through circadian transcription (Li et al. 2009) and polyadenylation (and subsequent translation) of the transcript (Ingram et al. 1996). Interestingly, the rhythm in AVP transcription depends on neuronal firing, VIP, cAMP, and Ca^{2+} signaling (Reppert et al. 1981; Jansen et al. 2007; Sodersten et al. 1985; Tominaga et al. 1992). This provides a nice example of how intercellular signaling and intracellular transduction cascades in the SCN are critical for the daily rhythms from gene expression to neuropeptide secretion.

AVP has been primarily implicated in regulating the amplitude of circadian rhythms in the SCN and paraventricular nucleus of the hypothalamus (Tousson and

Meissl 2004) and in hormone and behavior rhythms (Gerkema et al. 1999; Jansen et al. 2007). In contrast to animals deficient for VIP, animals deficient for AVP or the V1a receptor display circadian rhythms with normal periodicity but with attenuated amplitudes (Li et al. 2009). AVP-deficient Brattleboro rats display low-amplitude daily rhythms in sleep-wake, body temperature, plasma melatonin, and SCN firing rates. Similarly, AVP levels and periodicity in the SCN of common voles correlate with the amplitude of their locomotor rhythmicity. Mice lacking V1aR show diminished rhythms in locomotion and in expression of at least one gene, *prokineticin 2*, in the SCN (Robinson et al. 1988). Because AVP is likely released during the day when firing rates are high in the SCN and excites most SCN neurons, it is possible that AVP regulates the gain of the SCN and drives rhythms in downstream targets (Rusnak et al. 2007).

Coculture experiments have recently suggested an additional role for AVP in the SCN. Much like GRP, blocking AVP receptors does not abolish SCN rhythms in vitro, but does prevent SCN cocultures from restoring rhythms to VIP-deficient SCN (Maywood et al. 2011a). It is possible that AVP normally amplifies SCN rhythms and, when VIP signaling has been compromised, AVP, alone or through GRP, can act as a weak synchronizing agent to coordinate the rhythms among the many circadian cells of the SCN.

1.1.4 Little SAAS

The recent reports on little SAAS exemplify novel approaches to discovering new molecules involved in circadian communication. Historically, SCN signals were identified when a good antibody existed. Little SAAS emerged from a relatively unbiased screen for secreted molecules in the SCN (Hatcher et al. 2008). In this approach, spontaneous and electrically evoked releasates were concentrated from explanted SCN and characterized by mass spectrometry. Further improvements with a 12 Tesla LTQ-FT mass spectrometer and ProSightPC 2.0 software led to the identification of 102 endogenous peptides released from the SCN including 33 novel peptides and 12 with posttranslational modifications including amidation, phosphorylation, pyroglutamylation, or acetylation. These methods allow for simultaneous identification of many signals from identified tissues or even areas within the SCN under a variety of stimulation conditions.

Because it was in relatively high abundance and putatively involved in prohormone processing, little SAAS rose to the top of the list of peptides to be further characterized by the labs of Martha Gillette and Jonathan Sweedler. Produced by approximately 16 % of SCN neurons (Maywood et al. 2011b), little SAAS signaling in the SCN appears to have functions similar to and distinct from VIP and GRP. Little SAAS-synthesizing neurons are found primarily in the middle of the SCN. Approximately 33 % of them do not express either GRP or VIP, but the 67 % remaining represent 80 % of GRP- and 10 % of VIP-positive neurons (Hatcher et al. 2008). This suggests that little SAAS may be co-released, at least under some conditions, with other neuropeptides.

Like VIP and GRP, little SAAS has been implicated in the SCN response to nighttime light exposure. Neurons positive for little SAAS receive retinal input and respond to nocturnal light with increased cFOS (Lee et al. 2010). It is not yet clear if light induces the release of little SAAS, but electrical stimulation of the optic nerve increases little SAAS release (Fricker et al. 2000). Remarkably, an antibody to little SAAS can block glutamate-induced delays of the in vitro SCN (Atkins et al. 2010). In addition, little SAAS application in vitro can shift SCN rhythms (Hatcher et al. 2008) independent of VIP or GRP signaling (Atkins et al. 2010). Taken together, these results support the hypothesis that little SAAS signals in parallel to or independent of GRP and VIP in response to light.

This may indicate a high level of redundant functions for different neuropeptides in photic entrainment. Alternatively, we may need more sophisticated assays to distinguish their roles with higher spatial and temporal resolution and under diverse conditions. This is exemplified in central pattern generators where circuit properties depend on which neuropeptides are released (Dickinson 2006; Wallén et al. 1989). It will be exciting to see, for example, if animals deficient for little SAAS (Atkins et al. 2010) fail to entrain to specific light cycles or initiate their daily activity at abnormal times.

1.1.5 GABA

Most, if not all, of the diverse peptidergic neurons of the SCN share one important function—they synthesize γ-aminobutyric acid (GABA) (Belenky et al. 2007). Both the ionotropic, $GABA_A$, and metabotropic, $GABA_B$, receptors are expressed widely in the SCN and on the terminals of projections to the SCN (Gao et al. 1995; Belenky et al. 2003, 2007; Francois-Bellan et al. 1989). Thus, GABA is postulated to act directly on all SCN neurons and on inputs to the SCN.

Although it is rare in the adult nervous system, there is good evidence that GABA can excite neurons of the SCN. GABA was first reported in 1997 as excitatory during the day and inhibitory at night, thus amplifying the daily rhythm in firing rate (Wagner et al. 1997). Unfortunately, the time-of-day effect has not been reproducible with different labs reporting excitation by GABA: during the night (Pennartz et al. 2002), during the night in only the dorsal SCN at all times but in a fraction of the dorsal and ventral SCN neurons (Choi et al. 2008; Irwin and Allen 2009), or never (Gribkoff et al. 2003; Liu and Reppert 2000; Aton et al. 2006). Some of this confusion may be explained by difficulties in defining responses as excitatory when they reflect post-inhibitory rebound. It is, however, reasonable to conclude that GABA likely excites a subset of SCN neurons that have elevated chloride reversal potentials due to the activity of a chloride transporter, NKCC1. Future studies will clarify which neurons are excited, at what times of day, and to what functional end.

Because chronic blockade of endogenous GABA signaling in the SCN raises the daytime peak in firing with little effect on the already-low nighttime firing in most neurons, it has been postulated that GABA plays an important role in governing

peak firing rates and enhancing sensitivity to depolarizing inputs (Aton et al. 2006). This is consistent with the evidence that GABA and its receptor agonists can modulate light-induced phase shifts in vivo and optic nerve input to the SCN in vitro (Gannon et al. 1995; Ehlen et al. 2008). Notably, daily GABA application can synchronize cultured SCN neurons (Liu and Reppert 2000). However, endogenous GABA signaling is not required for SCN neurons to synchronize to each other (Aton et al. 2006). Instead, GABA signaling from the ventral SCN acutely excites neurons in the dorsal SCN and from the dorsal SCN acutely inhibits neurons in the ventral SCN (Albus et al. 2005). This reciprocal, long-range, rapid synaptic communication may play a role in coordinating rhythms between the top and bottom of the SCN. We will benefit from further studies on the necessity of GABA signaling for SCN entrainment to environmental cues.

1.1.6 Other Signals Within the SCN

A number of other small molecules, primarily neuropeptides and cytokines, have been studied as intercellular signals in the SCN including prokineticin 2, neuromedin S and neuromedin U (Mori et al. 2005; Graham et al. 2005), met-enkephalin, angiotensin II (Brown et al. 2008), somatostatin (Ishikawa et al. 1997), and substance P (Kim et al. 2001). Cardiotrophin-like cytokine (CLC) falls into this category of signals of interest. Based on one report, approximately 1 % of SCN neurons synthesize CLC, and the genes encoding its receptor subunits (*Cntf*, *Gp130* and *Lifr*) are expressed along the third ventricle (Fig. 2) (Kraves and Weitz 2006). In vivo administration of CLC decreased running wheel activity in mice, whereas inhibition of GP130 increased locomotion without affecting the phase or period of circadian rhythms (Kraves and Weitz 2006). Similar results have been reported for Prokineticin 2 (Zhou and Cheng 2005), implicating them as humoral factors secreted by the SCN to regulate motor activity. In each case, these signals are made by a subset of dorsal SCN neurons, and their function within the SCN has yet to be elucidated.

2 Glia of the Circadian System

Although it is clear that much of daily rhythms in physiology and behavior arise from the activity of clock neurons (Nitabach and Taghert 2008; Hastings 1997), recent advances have revealed that the “other cells in the brain,” glia, also show circadian rhythms in vivo and in vitro. In 1993, Lavialle and Serviere discovered high-amplitude daily rhythms in the distribution of glial fibrillary acidic protein (GFAP) in astrocytes of the suprachiasmatic nucleus (SCN) (Lavialle and Serviere 1993). This rhythm persists in constant darkness in the SCN of hamsters, rats, and mice (Lavialle and Serviere 1993; Moriya et al. 2000), suggesting that this rhythm is intrinsic and independent of external light cues. The role of daily oscillations in

GFAP immunoreactivity on glial cells is unknown. Leone et al. suggest that oscillations in GFAP reflect a response of astrocytes in the SCN to inputs from the immune system. Moriya et al. speculate that GFAP plays a role in circadian rhythms in constant light conditions. Regardless, the conservation of the daily rhythms in GFAP distribution in the SCN among three mammalian species suggests it has some function.

Astrocytes communicate with nearby glia and neurons by releasing transmitter through a process known as gliotransmission (Perea et al. 2009; Fields and Burnstock 2006; Haydon 2001). The best known transmitters produced and released by astroglia are ATP, D-serine, and glutamate (Parpura and Zorec 2010). The mechanism of gliotransmission is thought to be dependent upon fluctuations in cytosolic calcium levels and vesicular release of transmitters.

The first (and only) direct demonstration that glial cells can modulate circadian physiology and behavior came from flies. In flies, the protein and mRNA levels of ebony, a glia-specific enzyme, are enriched around clock neurons and vary with time of day (Suh and Jackson 2007). Ebony is an *N*-beta-alanyl-biogenic amine synthetase capable of conjugating a beta-alanine to histamine, as well as other amine neurotransmitters (e.g., dopamine and serotonin). Mutants carrying any one of five *ebony* alleles show dramatic changes in the circadian period of their locomotor rhythms (Suh and Jackson 2007). This phenotype is rescued by glia-specific overexpression of *ebony* (Ng et al. 2011). These results led researchers to test whether glial signaling is required for circadian behaviors. They found that transgenic manipulation of the membrane potential, calcium signaling, or vesicular release in astroglia dramatically reduces the proportion of circadian flies and, interestingly, circadian rhythms in neuropeptide signaling (Ng et al. 2011).

In mammals, there is indirect evidence that glia contribute to circadian behaviors. GFAP knockout mice show longer periods of activity and more arrhythmicity in constant light conditions compared to wild type (Moriya et al. 2000). Manipulations of gliotransmission have been implicated in the regulation of sleep homeostasis, but not yet in circadian biology (Halassa and Haydon 2010). Here, we review the evidence for neural and glial control of glial circadian cycling in mammals.

2.1 Neuron-to-Glia Signaling

There is strong evidence for neuronal coordination of glial circadian rhythms in mammals. Glia cultured from mouse motor cortex with a knock-in bioluminescent reporter of Period2 expression show circadian rhythms that damp out over a week (Prolo et al. 2005). When cocultured with an SCN explant, glia express sustained circadian rhythms, suggesting that SCN neurons can coordinate glial rhythms through a diffusible signal (Prolo et al. 2005). In vivo, circadian rhythms in ATP release appear to derive primarily from astrocytes within the SCN (Womac et al. 2009). Interestingly, astrocytes in the SCN respond to photic stimulation with an

increase in cFOS expression (Bennett and Schwartz 1994), suggesting they may participate in the response to light and, perhaps, entrainment. Future work will likely focus on the role of glia in different aspects of circadian behavior and whether glia in different brain areas have different circadian functions.

2.1.1 VIP/VPAC2R

In addition to the well-established role of VIP in communication between circadian neurons (Vosko et al. 2007), VIP has been implicated in neuron-to-glia daily signaling. Cortical astrocytes respond to agonists for VPAC2R, but not VPAC1R (Zusev and Gozes 2004). Cultured astrocytes respond to nanomolar concentrations of VIP with clock gene induction, ATP release, and shifts in their circadian rhythms (Marpegan et al. 2009, 2011). Daily administration of VIP to cultured astrocytes sustains and entrains rhythmic circadian expression of Period2 (Marpegan et al. 2009). Gerhold and Wise have provided in vivo evidence for VIP-mediated circadian rhythms in glia. By suppressing expression of VIP in the SCN, they disrupted the diurnal rhythms in surface area observed in astrocytes that ensheathe gonadotropin-releasing hormone (GnRH) neurons (Gerhold and Wise 2006; Gerhold et al. 2005). They also showed mRNA expression of VPAC2 receptors in these astrocytes, indicating a direct interaction between VIP and glia (Gerhold and Wise 2006). This fluctuation in astrocyte surface area is thought to modulate stimulatory, neural inputs to GnRH neurons, thus modulating GnRH synthesis and release (Cashion et al. 2003). Further in vivo studies will be required to elucidate other potential roles of VIP in regulating astrocyte function in the SCN.

2.1.2 ATP/Purinergic Receptors

In addition to providing energy to cells, ATP acts as a transmitter to send signals to neighboring glial and neuronal cells in the nervous system (Haydon 2001; Suadicani et al. 2006). Extracellular accumulation of ATP in the SCN fluctuates in a circadian fashion, peaking in the middle of the night, or subjective night, in rats (Womac et al. 2009; Yamazaki et al. 1994). Cultured cortical astrocytes also display circadian rhythms in extracellular ATP accumulation (Womac et al. 2009; Burkeen et al. 2011; Marpegan et al. 2011). A circadian role for this extracellular ATP has not been identified.

ATP can act directly on purinergic receptors or be degraded into biologically active ADP or adenosine. The nucleoside adenosine has a predominantly inhibitory effect on neuronal activity in the CNS (Dunwiddie and Masino 2001). Adenosine has been implicated in the regulation of sleep (Chikahisa and Séi 2011). Adenosine receptor antagonists (i.e., caffeine) disrupt inhibitory effects caused by extracellular adenosine, leading indirectly to a stimulatory effect (Fredholm et al. 1999). In the retina, circadian changes in extracellular adenosine levels appear to arise from non-neuronal sources to regulate rod-cone coupling (Ribelayga et al. 2008). Thus,

ATP and other nucleosides can carry time-of-day information to regulate sensory processing.

2.2 *Glia-to-Neuron Signaling*

There is indirect evidence for astrocytes communicating circadian timing information to other cells. For example, because circadian changes in SCN ATP likely derive primarily from astrocytes, there is potential for glia to regulate SCN activity. Additionally, rhythms in excitatory amino acids (EAAs) including glutamate in the SCN may be due to astrocyte release (Shinohara et al. 2000). Evidence includes the few, if any, glutamatergic neurons in the isolated SCN, the calcium independence of EAA rhythms, and the persistent EAA rhythms in the presence of L-trans-pyrrolidine-2,4-dicarboxylic acid, a glutamate/aspartate uptake inhibitor (Shinohara et al. 2000). Because astrocytes can act as a source of calcium-independent neurotransmitter release (Malarkey and Parpura 2008), glia may regulate extracellular glutamate levels in the SCN. A recent study found that the circadian clock in cortical glia, however, does not regulate their glutamate reuptake on a daily basis (Beaulé et al. 2009). Future studies will likely focus on whether glia regulate EAA levels through circadian release.

3 Common Drugs and Their Effects on Circadian Signaling

This review has identified several signaling pathways involved in generation and regulation of daily rhythms in behavior and physiology. For example, neuropeptides within the SCN are involved in synchronizing circadian cells to each other, amplifying their daily cycling, and adjusting their rhythms to local time. Thus, drugs that impinge on neuropeptidergic receptors or signaling pathways can potently shape daily schedules. We must consider the likely effects of many drugs on the circadian timing system (see also Musiek and FitzGerald 2013; Antoch and Kondratov 2013; Ortiz-Tudela et al. 2013). One striking example is the drug most commonly taken by humans—caffeine. Caffeine adjusts circadian timing of electrical activity in the isolated SCN and of clock gene expression in cultured mammalian cells and modestly lengthens the circadian period of locomotor activity in mice (Oike et al. 2011; Wyatt et al. 2004; Ding et al. 1998). Despite an extensive literature on caffeine's effects on sleep and vigilance (Wright et al. 1997; Fredholm et al. 1999; Landolt et al. 1995), there have been no studies of the effects of caffeine on human circadian biology. Thus, the circadian effects of common pharmaceuticals are understudied and now can be easily assayed in vivo and in vitro.

We have stressed that some ligands have different effects at different times of day, for example, phase shifting the clock during the night, but not during the day.

This can arise from circadian changes in the abundance or activity of receptors or downstream, intracellular second messengers. In pharmacology, the consequences of this must be emphasized (see Musiek and FitzGerald 2013; Ortiz-Tudela et al. 2013). Time-dependent administration may determine the efficacy of a drug based on known circadian rhythms. For example, angiotensin II receptor antagonists, used in the treatment of hypertension, may be more effective at times when their target pathway is available for modulation (Portaluppi et al. 2012). Thus, chronopharmacology is the strategic use of drugs to affect the circadian clock or clock-regulated pathways.

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