February 10, 2012

BIOLOGICAL TIMEKEEPING

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This work was supported by the following past and present grants from the National Institutes of Health: HL67007, HL086870, HL092571Z ARRA, NS22155, and NS35859 (MUG), F30 NS047802 and GM07143 (SMA), and GM007283 (JMA).

Keywords: biological rhythms, sleep, circadian, suprachiasmatic nucleus

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The daily transitions between light and darkness have significantly shaped the evolution of most living species, from unicellular organisms to mammals. Superimposed upon the daily light-dark cycle is a seasonal influence that changes the relative durations of day and night over the course of a year. Be they day-active or night-active, all organisms organize their behaviors in the 24-hour world, adapting to the availability of food and changing temperature, rearing their young, and avoiding predators. To optimize survival, they must be able to anticipate environmental transitions and to adjust to changes in night-length or transition times that may occur.

Adaptation to these needs occurred through the emergence of a circadian system capable of aligning behavioral, physiological, and metabolic processes with this light-dark cycle. The circadian system organizes body systems so that they occur in 24-hour rhythms. Rather than simply reflecting the external day-night cycle, these rhythms in behaviors persist in the absence of exogenous timing cues, such as light, food availability, or social cues. Every organism expresses an endogenous rhythm that varies slightly from 24 h, making it *circadian*, or 'about a day.' Uninterrupted, this circadian rhythm persists.

These circadian rhythms can be observed in outputs, such as the patterning of the sleep-wake cycle. In humans, core body temperature is often used as a marker of circadian phase. In addition, numerous endogenous hormones oscillate with a predictable phase relationship to day and night (reviewed by Van Cauter[1]). Hormonal rhythms exhibit complex waveforms due to combined effects of the circadian pacemaker, organismic state, such as activity level, sleep and feeding, and the pulsatile nature of secretion. Nevertheless, clear diurnal patterns of secretion have been reported[2]. Plasma melatonin[3,4], growth hormone[5], prolactin[6], thyrotropin-releasing hormone[7], luteinizing hormone[8] and leptin[9-11] are all elevated during the night, in antiphase to adrenocorticotropic hormone and cortisol[12,13]. These oscillations in hormone secretion continue in a constant environment, and, therefore, are clock-regulated. Circadian rhythmicity appears to be present at virtually every level of function studied. In fact, maintenance of a constant *milieu interior* may be a consequence of a balance among rhythmic, mutually opposed control mechanisms[2].

This review will explain the neurobiology of circadian timekeeping, describing what is known about the master pacemaker for circadian rhythmicity, how various biological systems can provide input to the endogenous biological timing, and how the pacemaker can, in turn, influence the physiology and behavior of the individual. We will discuss how the circadian system can adapt to a changing environment by resetting the circadian clock in the face of a variety of inputs, including changes in light, activity and the sleep-wake cycle. We will then discuss the genetics of circadian time-keeping, highlighting what is currently known about heritable disorders in circadian timing and how circadian genetics have been utilized to study timekeeping. Finally, we will discuss the clock's role in peripheral tissues.

I. THE CIRCADIAN CLOCK

In mammals, circadian rhythms are regulated by a paired set of nuclei located at the base of the hypothalamus, directly above the optic chiasm, hence their name – the suprachiasmatic nuclei (SCN) (Fig 1). Multiple experiments have demonstrated the role of the SCN as a central pacemaker for circadian rhythms. Lesioning studies found that damage to the SCN disrupts rhythmicity in corticosterone levels, drinking, and wheel

running behavior[14,15]. This provided the initial evidence that the central pacemaker for the mammalian clock lay within the SCN.

In later work, it was found that transplanting fetal SCN tissue into the third ventricle of animals in which the SCN had been lesioned could restore rhythmicity[16]. Furthermore, if fetal SCN tissue from a wild-type hamster was implanted into the third ventricle of a hamster with a genetic alteration that shortened free-running period, the new free-running period resembled that of the SCN donor rather than the host animal. This evidence suggested that not only was the SCN necessary for generating rhythms, but also the period of this rhythmicity was an intrinsic property of the SCN cells—and the presence of SCN was sufficient to drive the rhythms for the entire animal[17].

In the mouse, each SCN measures approximately 300 μm medial to lateral, 350 μm dorsal to ventral, and spans approximately 600 μm from rostral to caudal end. One SCN contains a total of approximately 10,500 cells[18]. The rodent SCN has several peptidergic subregions (Fig. 1). The central region of the SCN contains small neurons that show positive staining for gastrin-releasing peptide (GRP) colocalized with γ-amino butyric acid (GABA), and the newly discovered peptide, little SAAS[18-21]. The ventrolateral region of the SCN contains neurons that stain predominately for vasoactive intestinal peptide (VIP), but a population of calretinin (CALR) cells is also seen here. The dorsomedial region of the SCN contains larger neurons that contain arginine vasopressin (AVP), met-enkephalin (mENK), and angiotensin II (AII)[18,20,21]. There are topographic connections between all regions of the nucleus, as well as communication between the two nuclei of the animal[22].

The human SCN is not as compact as the rodent, but has a similar peptidergic organization. The dorsal and medial regions contain neurophysin/vasopressin neurons. The central region contains calbindin, synaptophysin, and VIP neurons, while the ventral and rostral regions contains synaptophysin, calbindin, and substance P[23].

Inputs

In conjunction with its ability to regulate circadian timing, the SCN is also positioned to receive information about environmental and behavioral states of the animal in order to ensure proper alignment of the circadian clock. This information is conveyed to the SCN by projections from a variety of different brain regions.

One of the most extensively studied inputs to the SCN comes from a subpopulation of retinal ganglion cells whose central projections form the retinohypothalamic tract (RHT). Lesions of the SCN disrupt the development of these neurons[24], and disruption of the RHT results in an inability to respond to resetting light signals[25,26]. The class of retinal ganglion cells that comprise the RHT contain a blue-light photopigment, melanopsin[27]. These melanopsin-containing cells are photosensitive at the same wavelengths that are most effective for circadian resetting[28]. Additionally, the terminals of the melanopsin-positive retinal ganglion cells collocalize glutamate (GLU) and pituitary adenylate cyclase-activating polypeptide (PACAP)[29], the neurotransmitters of the RHT[30,31].

The RHT also sends projections to the thalamic intergeniculate leaflet (IGL), which, in turn, sends projections back to the SCN through the geniculohypothalamic tract (GHT). The GHT contains neuropeptide Y (NPY) and GABA. NPY is believed to be involved in activity-induced phase shifts during the daytime in nocturnal animals, but also appears to be able to modulate light-induced phase shifts[32-34]. However, while the GHT pathway can transmit photic signals, disruption of this pathway does not prevent entrainment[35].

The SCN also receives serotonergic input, primarily from the median raphe, that is primarily involved in activity-induced phase shifts during the daytime. Activation of the median raphe results in an increase in serotonin (5-HT) release at the SCN[36-38]. 5-HT release also shows a strong circadian release pattern in the SCN, with 5-HT release peaking at CT 14, and 5-hydroxyindole acetic acid (5-HIAA), the major metabolite of 5-HT, peaking at CT 16[39].

Cholinergic projections to the SCN originate both in the brainstem and basal forebrain in brain nuclei with identified roles in sleep and arousal[40], and were recently demonstrated to also be present in diurnal animals[41]. Within the brainstem, these cholinergic projections arise from three nuclei. The parabigeminal nucleus (PBg) is considered a satellite region of the superior colliculus, which appears to play a role in generating target-location information as part of saccadic eye-movements[42]. The laterodorsal tegmental (LDTg) and pedunculopontine tegmental (PPTg) nuclei both are important for regulating the sleep-wake cycle[43]. In the basal forebrain, the substantia innominata (SI) within the nucleus basalis magnocellularis (NBM) in the basal forebrain contributes to arousal and focused attention[44]. The LDTg, PPTg, and NBM are interconnected, and all play roles in regulating the sleep and arousal states of the animal. This would suggest that the cholinergic input to the SCN is providing a signal regarding the sleep and arousal states of the animal, and may provide a link between the sleep-wake cycle and circadian rhythms.

Additional sleep-wake input to the SCN may come from the tuberomammillary nucleus (TMN). Studies have shown histaminergic input to the SCN from the TMN[45]. Histamine is a regulator of the sleep-wake cycle, primarily providing a signal of wakefulness.

Outputs

The SCN exerts its influence on the body primarily at the level of the hypothalamus. Neurons from the ventral regions of the SCN project to the lateral region of the hypothalamic subparaventricular zone (sPVHz), the peri-suprachiasmatic area (PSCN), and the ventral tuberal area (VTU). The dorsal region of the SCN projects to medial preoptic area (MPOA), medial sPVHz, dorsal parvocellular paraventricular nucleus (dPVN), and the dorsal medial hypothalamus (DMH), also all within the hypothalamus[46]. The targets of efferents to the dPVN consist of endocrine neurons, autonomic neurons, or intermediate neurons that potentially serve to integrate a number of hypothalamic signals[47].

Many SCN projection sites are regulators of sleep and arousal. The DMH projections are especially interesting, as many of these neurons appear to project to neurons containing hypocretin/orexin, a peptide well known for its role in arousal[48,49]. In addition, evidence exists for a multi-synaptic pathway between the SCN and locus coeruleus (LC), an important arousal center in the brain, mediated by orexin[50], with the DMH as a relay[51]. A minor set of SCN efferents project to the ventrolateral preoptic nucleus (VLPO), a region which, if lesioned, produces prolonged reduction in sleep duration and amplitude[52]. The SCN projects to the paraventricular nucleus (PVT) and intergeniculate leaflet (IGL) of the thalamus. Both nuclei project back to the SCN. The PVT loop is proposed to provide assessment of sleep/arousal states and SCN modulation, whereas the IGL loop is thought to provide the SCN with information from higher, integratative visual centers[53-55]. The PVN appears to act as a relay between the SCN and the amygdala, which may provide a link between the circadian system and affective

disorders[56]. Overall, the SCN appears to be uniquely situated within a network that allows it to interact closely with the regions controlling sleep and arousal states.

II. CIRCADIAN RESETTING

Despite the circuit-based organization of neural function, there is a consensus that timekeeping is a cellular process[57]. Indeed, the expression of independently-phased circadian firing rhythms from individual neurons dissociated from neonatal rat SCN cultured on an electrode array provides compelling evidence for the cellular nature of this clock[58]. It follows that gating of sensitivity to resetting stimuli and phase resetting must be cellular properties. Moreover, the clock must be able to restrict the range of responses in the cellular repertoire so that activation of select signaling pathways can occur only at the appropriate time in the circadian cycle[59]. We have endeavored to determine how the clock temporally regulates the responsiveness of specific signaling pathways.

In an attempt to define and understand the underlying control mechanisms subserving clock-gated windows of sensitivity, SCN-bearing brain slices are exposed *in vitro* to treatments that activate elements of specific signaling pathways. Treatments are administered at various discrete points in the circadian cycle, and effects on the time-of-peak in the spontaneous rhythm of neuronal activity assessed over the next one or two circadian cycles *in vitro*. If the time-of-peak appears earlier during cycle(s) after treatment compared to controls, the phase of the rhythm is advanced. If the time-of-peak appears later than in controls, then the phase is delayed by the treatment. By assessing the changing relationship between the circadian time of treatment and its effect on phase, a phase-response curve (PRC) can be generated. This relationship graphically presents the temporal pattern of SCN sensitivity to activation of specific signaling pathways and, in fact, defines the window of sensitivity to phase resetting via this pathway. The permanence of the phase shift is examined by evaluating the time of the peak in neuronal activity over one or two days after a treatment. Timing of the peak after experimental reagents are administered at the maximal point of sensitivity is compared with the time of the peak in media-treated controls.

Temporal spheres identified as sensitive to phase resetting via specific first and second messenger pathways coincide with discrete portions of the circadian cycle. In terms of these temporal restrictions, the circadian cycle can be divided into several discrete temporal states, or domains, of the clock: day, night, dusk and dawn[59,60]. These studies not only contribute to defining the properties of the clock's temporal domains, they emphasize the complexity of control that the clock exerts over signal integration and phase resetting within the SCN. These properties have been incorporated into putative clock-gated regulatory pathways. Each will be discussed in the context of the clock domain that is regulated.

Subjective day and night are distinct with respect to their sensitivities and response characteristics. Furthermore, each correlates with discrete periods of sensitivity to specific neurotransmitter systems that are demonstrated to impinge upon this hypothalamic site as evidenced by a large body of neuroanatomical studies[61]. This permits speculation regarding the nature of pathways that gain access to and regulate the biological clock at different points in the circadian cycle. We will now consider, in turn, the major identified domains of clock sensitivity.

Circadian Clock Regulators

Daytime

A number of signaling molecules appear to be important in resetting circadian rhythms during the daytime, including 5-HT, PACAP, NPY and GABA (Fig. 2). The majority of these experiments have been performed in nocturnal rodents, so daytime is defined as the time in which the lights are on, and/or the animal is inactive. As a result, the functional context of this regulation seems to be tied to arousal-induced resetting, often referred to as non-photic resetting[62,63]. Non-photic signals cover a wide variety of phenomenon, including sleep deprivation, activity associated with exposure to a novel wheel, or even cage changes. The unifying factor in non-photic signals is that they involve arousal during a time when the animal would normally be inactive.

5-HT is believed to play a role in non-photic, activity-induced phase shifts during the day. Increasing 5-HT in the SCN during subjective day induces an advance in peak electrical firing rate *in vitro* or onset of wheel-running *in vivo*[36,64]. *In vivo*, 5-HT levels in the SCN are increased by electrical stimulation of the dorsal or median raphe[36,65]. Forced wheel-running or sleep deprivation during the day also increases 5-HT in the SCN[66,67], which suggests a role for 5-HT in non-photic phase-shifting. However, depleting 5-HT from raphe projections does not prevent this non-photic daytime shift[68], and serotonergic antagonists are not able to attenuate this phase shift[69], providing mixed evidence for the role of 5-HT. This suggests modulation by additional messengers, possibly neuropeptides.

A second daytime modulator of the SCN clock is the peptide, PACAP. PACAP is not intrinsic to the SCN, but instead is released from the RHT, where it colocalizes with GLU[70]. Levels of PACAP have been found to oscillate throughout the day in SCN samples, which include synaptic terminals of the RHT, but not in other brain regions[71]. If PACAP is applied to the SCN brain slice in micromolar quantities, it elicits an advance in peak neuronal firing during the day, but has little effect during night[29]. *In vivo* findings, however, conflict with this, as the long-term effect of PACAP injection into the SCN seems to be a delay in onset of wheel-running[72]. This conflicting data suggests that further study of PACAP's effects on the clock during the day are warranted.

A third daytime regulator of the clock, NPY, also appears to play a dual role in the SCN, resetting the circadian clock both during the daytime and at night. NPY is released from the GHT, the projection from the IGL to the SCN. When NPY was applied during the daytime either to an SCN brain slice *in vitro*[32] or directly to the SCN *in vivo*[73,74], it induced a phase advance. Additional *in vivo* studies stimulated the IGL, presumably inducing the release of NPY at the SCN. These stimulations also produced advances in wheel-running behavior during the daytime[75]. Interestingly, it has been found that exposing an animal to light[76] or applying GLU to the brain slice[77] were both capable of blocking the response to daytime application of NPY. The addition of the $GABA_A$ antagonist, bicuculline, is also capable of inhibiting the effects of NPY[78], suggesting that the effects of NPY are linked to GABAergic signaling.

One factor that daytime signaling pathways have in common is that they all may be mediated by cyclic adenosine monophosphate (cAMP). In the hypothalamic brain slice, cAMP or cAMP analogs applied during the daytime induce phase advances in the circadian clock, while at night they have little effect[79,80]. In addition, endogenous cAMP is high during late day and late night[81], suggesting a role for cAMP in the transition periods

between day and night. It can be hypothesized that by increasing cAMP, these daytime resetting signals are moving the animal to a state that resembles late day, thus resetting the clock.

Dawn and Dusk

The primary resetting signal associated with dawn and dusk is melatonin (Fig. 2). This "hormone of darkness" is produced at night in the absence of light, providing a means by which the animal can measure night-length. Photoperiod is an important measure for animals, such as hamster and sheep, that are seasonally reproductive. Melatonin is produced by the pineal gland, and in lower vertebrates, such as fish, lizards, and some birds, the pineal is actually the primary regulator of circadian rhythms, rather than the SCN. However, in mammals this timekeeping mechanism has moved to the SCN, as demonstrated by the fact that removal of the pineal does not significantly disrupt circadian rhythms of rats[82].

While the pineal is not necessary for maintenance of mammalian circadian rhythms, it is possible to entrain free-running rats with daily injections of melatonin. Entrainment appears to work best if the melatonin injections are timed to occur shortly before the onset of the animal's active period. This entrainment appears to be working through the SCN, as lesioning the SCN, but not the pineal, abolishes the ability of a rat to entrain to melatonin injections[83].

Evidence that melatonin can entrain circadian rhythms led to a number of studies looking at the direct effect of melatonin on the SCN. Melatonin application immediately before dusk in rat or hamster tissue *in vitro* decreases SCN activity, measured by 2-deoxy-[1-^{14}C]glucose (2-DG) uptake or neuronal firing rate[84-86]. Additionally, melatonin applied to SCN brain slices at either dawn or dusk advances the peak in neuronal firing. Melatonin is ineffective when applied at other times of day[87,88]. This resetting pattern is reproduced by direct activation of protein kinase C (PKC), and can be blocked by inhibitors of PKC, suggesting that PKC is a downstream component of this resetting pathway[88]. In addition, melatonergic resetting is inhibited with antagonists specific for the MT-2 type melatonin receptor[89]. In humans, circadian sensitivity to melatonin also occurs at dawn and dusk, but the effect is to advance the circadian system at dusk and to delay it at dawn, opposite to the effects of light at night.

Nighttime

In the nighttime domain there are two known key players, GLU and acetylcholine (ACh), as well as a number of modulatory substances associated with these signals (Fig. 2). As was discussed previously, considerable evidence supports GLU as the neurochemical signal transmitting photic stimuli from the retina to the SCN, but the functional context of the cholinergic resetting signal is still unknown.

The GLU signaling pathway is similar to many of the pathways that already have been discussed in that it resets the circadian clock at a discrete time of day and in a specific direction. The GLU signaling pathway can either advance or delay the clock, depending on what time of day the signal is presented[30,90]. The GLU resetting pathway has been demonstrated both *in vitro* and *in vivo* to be mediated through an N-methyl-D-aspartate (NMDA) receptor-mediated rise in intracellular calcium, followed by nitric oxide synthase (NOS) induction and resultant production of nitric oxide (NO)[30,91-94]. Beyond this point, the early and late night pathways diverge. During the early night GLU induces delays in the circadian clock through ryanodine receptor (RyR)-mediated calcium release[95]. GLU exposure

during the late night, however, advances the circadian clock through a cyclic guanosine monophosphate/protein kinase G (cGMP/PKG) signaling cascade followed by cAMP response element-binding protein (CREB)-activated transcription[95-97].

While GLU alone is capable of resetting circadian rhythms, there are many substances that modulate this resetting. These can be divided into two categories: those that decrease the amplitude of the phase-resetting effect of GLU during both the early and late night, which include NPY and GABA[33,64], and those that have differing effects on GLU-induced phase shifts, depending on what time of night they are applied.

This second category of time-dependent modulators include 5-HT and PACAP. If animals are depleted of 5-HT, they show increased phase delays in response to light[98,99]. Co-application of a PACAP antagonist, however, either *in vitro* or *in vivo*, decreases the phase delay seen with application in early night, and when applied during late night, increases the amplitude of the phase advance in both rat and hamster[100,101]. When PACAP is administered in conjunction with GLU in early night, it increases the delays, but in late night it decreases phase advances. This is similar to the effects seen following application of cAMP analogs to the hypothalamic brain slice, suggesting that the effects of PACAP may be mediated via a cAMP pathway[102].

The role of ACh in resetting circadian rhythms has been unclear, with much of the confusion arising from the fact that its effects vary depending on the site of application. The first evidence that ACh might play a role in resetting the circadian clock came in 1979, when Zatz and Brownstein examined whether pharmacological manipulation of the SCN could affect circadian rhythms. It was found that injections of the ACh agonist carbachol into the lateral ventricle of Sprague-Dawley rats at CT 15 caused phase delays that were similar to, but not as large as, the phase delays produced by light[103]. Carbachol injections into the lateral ventricle were also later repeated in mice[104] and hamsters[105], where it was found that administration of carbachol during early night caused phase delays, while late night administration caused phase advances.

This pattern of sensitivity and response is similar to that previously demonstrated in response to light or GLU. Support for the involvement of ACh in the light response came from studies looking at ACh levels in the rat SCN using a radioimmunoassay (RIA)[106]. Using this technique, no significant oscillation in ACh levels was found under constant conditions, but light pulses administered at CT 14 were found to increase ACh levels in the SCN. However, only one time-point was examined, so it is not known whether this increase was simply a response to exposure to light or if there was actually a circadian pattern to the light-stimulated release. The implication of these studies, however, is that ACh might be the primary neurotransmitter providing the signal of light to the clock.

However, significant evidence began to emerge indicating that ACh was not likely to be the primary signal of light. First of all, whereas it had previously been determined that the RHT transmitted the signal of light from the eye to the SCN, it was found that choline acetyltransferase (ChAT) was not present in this projection[107], making it anatomically unlikely that ACh was the primary neurotransmitter involved in this signal. This evidence might warrant reconsideration, however, as recent studies have found an alternative splice variant of ChAT present in ganglion cells that was not picked up using previous antibodies[108].

Additional evidence against ACh being the signal of light came from experiments that found intracerebroventricular (*icv)* injections of hemicholinium, which significantly depletes ACh stores in the brain, did

not block the ability of the animal to phase shift in response to light[109]. There was also evidence that injecting NMDA receptor antagonists could block carbachol induced phase shifts, suggesting that although ACh may play a role in the light response, it must be upstream of a glutamatergic signal[110]. Finally, Liu and Gillette[111], using extracellular recording *in vitro*, found that microdrop applications of carbachol directly to the SCN caused only phase advances, regardless of whether the carbachol was applied early or late in the night.

In an attempt to explain these contradicting data, it was hypothesized by our lab that the dual response pattern of the SCN to cholinergic stimulation was a result of the location of application. Note that in the initial *in vivo* studies, carbachol was injected into the lateral or third ventricle, where the drug could have a diffuse effect, while in the *in vitro* studies carbachol was applied in microdrops directly to the SCN. As was predicted, if the *in vivo* experiments were performed by injecting carbachol directly into the SCN rather than into the ventricle, a phase response pattern similar to that observed in the *in vitro* experiments using microdrop applications resulted[112]. This evidence suggests that ACh has at least two different effects on the circadian clock, depending upon the site of application. There is an indirect response, working through ventricular pathways, that is likely upstream of a glutamatergic signal, and a direct response that is mediated by the M_1AChR[113]. Based on the anatomical studies looking at cholinergic projections to the SCN that originate in the LDTg and PPTg, as well as the (NBM), the current hypothesis is that this cholinergic signal may be involved in linking the sleep-wake and circadian cycles together.

III. GENETICS OF CIRCADIAN RHYTHMS

Much research effort has focused on determining how a biological system keeps 24-hour time. With the discovery that single, dispersed cells can exhibit circadian rhythms[114], the focus turned towards understanding cellular processes that generate a near 24-hour timebase. A molecular clockwork generates a ~24-hour rhythm through a feedback cycle involving a set of core clock genes, their mRNAs, and proteins[115,116]. This cycle consists of a set of interconnected positive and negative feedback loops, and their regulatory elements. Positive elements, which include *Clock* and *Bmal1,* are transcribed into mRNA, which is then translated into proteins that heterodimerize and are translocated into the nucleus. In the nucleus, they activate continued transcription of their own genes, as well as activating transcription of negative elements. The negative elements, which include *Period*, *Cryptochrome* and *Rev-erbα,* are then transcribed and translated. Proteins of the negative elements also associate in complexes and are translocated to the nucleus, where they feed back to inhibit transcription of the positive elements[115,116]. Additional genes that have been proposed to be involved in the circadian clock include *Rorα*[117], *Timeless (Tim)*[118], *Dec1* and *Dec2*[119], and more recently *SIRT1*[37,120,121]. These feedback loops are further affected by regulatory enzymes, including casein kinase 1 epsilon (CKIε) and glycogen synthase kinase (GSK)[122-124], and small intracellular regulatory molecules, such as calcium and cAMP with established roles in signal transduction[37,125]. The cycle of these feedback loops takes approximately 24 hours to complete, providing a means by which cells can maintain a circadian rhythm.

Core clock elements have been found to play a critical role in human sleep disorders. For example, inherited forms of advanced sleep phase syndrome (ASPS) have been associated with a mutation in the *Per2* gene that interferes with a normal phosphorylation site of CKIδ/ε[126] or with a mutation in CKIδ[127]. Delayed sleep phase

syndrome (DSPS), on the other hand, has been found in some cases to be associated with a specific polymorphism of hPER3[37,128,129]. PER3 expression patterns in human leukocytes correlate with sleep-wake timing, particularly in those individuals with a preference for morningness[130]. Finally, morningness or eveningness preferences have been associated with polymorphisms of the human *Clock* gene[37,131,132].

The clock genes have proven useful for studying rhythms as well. Several of these genes, including *Bmal1* and two *Per* genes, have been fused to reporter molecules, such as green fluorescent protein (GFP) and firefly luciferase, which enables study of the reporter as a marker of the transcription or translation of the gene. Both by transfecting cell cultures with a construct containing one of these fusion genes[133-136] and by creating transgenic rodents that express these fusion products[137-140], new insights in clock dynamics have emerged. Among the most surprising is that all cells express them in a circadian pattern, even in dispersed cell culture. This established that circadian clocks are components of nearly all cells.

IV. MOLECULAR CLOCKS IN DIVERSE MAMMALIAN CELLS

Although the SCN is necessary as the central circadian pacemaker, the discovery of autonomous clocks driven by oscillations in clock genes focused attention on extra-SCN clocks. Some non-SCN tissue, such as the mammalian pineal gland[141] and retina[142], express circadian oscillations in metabolites or melatonin when cultured independently. The first oscillations of clock genes outside of the SCN were found using a Rat-1 fibroblast cell line. These immortalized cells express clock gene mRNAs, such as *rev-erbα*, *per1,* and *per2*, which oscillate in cell culture with a period near 24 hours[143]. When primary mouse embryonic fibroblasts also showed clock gene oscillation, the possibility was raised that individual cells throughout the body might express the molecular components of a clock[135,144-147].

With the advent of clock gene reporter systems, studies emerged supporting the evidence that peripheral, non-SCN tissues in the body contain functional clocks. The olfactory bulb oscillates in a transgenic rat that contains the promoter sequence for *mPer1* linked to luciferase[139]. The olfactory bulb maintains rhythmic luciferase expression when the SCN is surgically ablated[148], the rat is made arrhythmic by placing it in constant light[149], or when the olfactory bulb is isolated in tissue culture[148,150]. Many brain regions[150,151] and peripheral tissues, including skeletal muscle, liver, and lung, also exhibit oscillations in clock genes, which remain rhythmic in culture for up to a week[139,151,152].

Further technical advancements lead to the creation of a mouse containing a reporter of the PER2 protein fused to luciferase (PER2::LUC). This knock-in approach, which enabled direct assessment of PER2 protein expression, is more physiological than transcriptional reporters and allows for study of peripheral tissues in culture for much longer periods[140]. Many tissues from this mouse, including cornea, kidney, liver, lung, pituitary, and tail, show clear, robust oscillations in PER2::LUC for up to 1 week *in vitro*, although SCN, liver, and lung tissues continue to show circadian rhythms for up to 3 weeks. Unexpectedly, tissue taken from SCN-lesioned mice 3-5 weeks following surgery still demonstrated robust near-24-hour rhythms in cellular luciferase luminescence, though the phasing of the rhythms were widely dispersed amongst the various tissues[140]. Individual fibroblasts from these PER2::LUC mice also maintain an oscillation in culture[135]. Experiments carried out with these reporter animals,

along with those carried out using the transcriptional reporters, demonstrate that nearly all peripheral cells in the body and some in the brain contain the molecular machinery of a functional circadian clock.

V. COUPLING OF CENTRAL AND PERIPHERAL CLOCKS

The above discussion emphasizes the myriad individual oscillating clocks in the body. In animals with a functional SCN, these clocks are aligned so that each individual tissue maintains a stable phase relationship to the SCN so that clock genes are expressed at the same time each day. When SCN rhythmicity is removed or the phase is shifted, the various tissues maintain their individual circadian rhythms, but they quickly fall out of phase with each other[139,140,153]. This indicates a hierarchical relationship in which the SCN is the master regulator that synchronizes and aligns the rest of the body's clocks. Much study of the coupling of extra-SCN clocks to the central pacemaker has been undertaken, and several examples will be highlighted in the following discussion.

The earliest SCN isolation studies established the SCN's role as the master clock, but these studies also hint at the various means by which this clock exerts control over peripheral structures. When the SCN is surgically isolated from the rest of the hypothalamus in rats, serum corticosterone oscillations continues, while locomotor rhythms are lost[154]. Additionally, surgical cuts to rodent brains between the SCN and PVN abolish reproductive rhythms in hamsters, but rhythmic locomotor activity is maintained in hamsters[155,156] and rats[157]. These findings provide early evidence for both synaptic coupling of SCN to output tissues, as well as the possibility that humoral signals entrain peripheral tissues. This idea was furthered by transplant studies in which an encapsulated fetal SCN is transplanted into an animal with an SCN lesion. Fenestrations in the encapsulating polymer were too small to permit neurite passage, and, indeed, no neural connectivity to the recipient brain could be found. The transplant restores locomotor, feeding, drinking, body temperature, and sleep/wake, but not endocrine, rhythms to the lesioned animal[158]. Clearly, some non-SCN rhythms require physical connections and some do not.

Many rhythm-generating tissues are coupled to the SCN by synaptic connections. Anatomical studies have shown SCN projections that extend to several hypothalamic nuclei, including the organum vasculosum of lamina terminalis (OVLT), medial preoptic area, and PVN, seemingly forming direct synapses with gonadotropin-releasing hormone (GnRH) and corticotropin-releasing hormone (CRH) neurons in these regions[159-161]. Additionally, the neuronal networks connecting the SCN to the IGL and PVT of the thalamus provide the SCN and these sleep/arousal modulatory regions with bi-directional communication[53-55].

The SCN is one of many regulators of opposing sympathetic and parasympathetic autonomic signals to peripheral organs. Anatomical studies using retrograde tracers injected into peripheral organs, such as liver, adrenal gland, pancreas, and adipose tissue, demonstrate a multi-synaptic pathway connecting these tissues to autonomic centers in the spinal cord, brain stem, PVN and DMH, and finally, to the SCN and other hypothalamic regions[162-165]. Discriminative tracing of either sympathetic or parasympathetic tracts identify SCN neurons in overlapping areas of the nucleus, but these neurons seem to be involved in signaling to one or the other of these pathways[163,164]. Light from the external environment can affect these two pathways through SCN-mediated control. For example, exposure of rats to light at night results in increased sympathetic activity, but suppression of parasympathetic activity. When the SCN is abolished, this effect is lost[166]. Also, heart rate decreases after light exposure at night in a nocturnal rodent, whereas SCN-lesioned animals do not exhibit this response[167]. Clearly, the SCN plays a role in modulating

autonomic signals to the periphery, but it works in concert with many other regions of the brain, including those regulating body temperature, metabolism, reproductive state, and other physiologic functions.

A growing body of evidence supports a role for humoral signaling in the coupling of rhythms between the SCN and other regions. In brain-slice cultures containing PVN tissue, an electrical rhythm emerges in the PVN only after co-culture with an SCN brain slice. The lack of neuronal connections between the two slices *in vitro* strongly supports a diffusible factor as cause of the electrical oscillation of the PVN[168]. Additionally, parabiosis connecting the circulatory system of an intact mouse to that of a SCN-lesioned mouse indicates that diffusible signals from the intact animal can entrain peripheral tissue in the lesioned recipient. Peripheral rhythms of clock gene expression are restored after parabiosis in kidney and liver[169]. Co-culturing functional SCN tissue with peripheral cells or tissue induces rhythms in these cells that follow the SCN under culture conditions that prevent synaptic connections[146,170,171].

These studies indicate that diffusible signals can modulate rhythms between tissues. Neuropeptides are abundant in the SCN, and are good candidates for humoral signals. As described previously, major neuropeptides found in the SCN include VIP, GRP, little SAAS, and AVP, among others. These peptides are released from the SCN in a circadian fashion[20,172,173], and each has been implicated in a physiological role in some aspect of circadian biology[20,172,174-180]. Identification of the diffusible signals that couple other tissues to the SCN is the current subject of intense study, with high therapeutic potential.

Another role for diffusible factors from the SCN appears to be to provide a signal inhibitory to activity. Two candidate factors for communicating such signals include transforming growth factor-α (TGF-α) and prokineticin 2 (PK2). Under normal conditions, TGF-α peptide is expressed rhythmically in the SCN with a peak during the animal's inactive period, and a trough during the active period. When infused continuously into the ventricles, TGF-α fully inhibits locomotor activity. Conversely, mice lacking the cognate receptor, epidermal growth factor (EGF) receptor, are unable to respond to TGF-α and show an excessive amount of daytime activity[181]. PK2 also is expressed rhythmically in the SCN, again showing peak expression during the animal's inactive period, and can inhibit locomotor activity when infused continuously[182]. This suggests a role for output signals from the SCN in promoting an inactive state that would be permissive for sleep.

Some tissues appear to require both synaptic and humoral signals to synchronize to the SCN. When autonomic nerve connections to the liver are severed, plasma insulin and corticosterone levels remain rhythmic, but plasma glucose levels do not[183]. However, liver tissue from an SCN-lesioned mouse with surgical parabiosis to an intact animal recovers and continues to maintain rhythmicity from that point onward[169]. This suggests that control of liver timing requires both neuronal and diffusible signals that coordinate separate physiological functions. Dissecting the intricacies of circadian regulation among peripheral tissues will require careful study.

Coupling of the SCN to peripheral targets, regardless of the manner of this connection, has important implications for health. This interaction allows for synchronization of internal systems to environmental light signals, both on a day-by-day basis and to adjust the animal to seasonal changes. Modern human activities, such as shift work and transcontinental flight, result in significant desynchronization of the central internal clock and various body tissues. This circadian disarray can have dire consequences for human health, including increased risks of

various cancers, reproductive health, stroke, metabolic syndrome, cardiovascular disease[184-186], and overall mortality in aged individuals[187].

IV. CONCLUSION

Circadian rhythms, the near 24-hour oscillations in brain and body functions, such as core body temperature, hormone release, and the sleep-wake cycle, are embedded in the physiology of cells and tissues. The master pacemaker regulating these rhythms, the suprachiasmatic nucleus (SCN) in the hypothalamus, is optimally situated to receive input about environmental light, sleep-wake state and activity status. It can be reset in response to changes in environmental conditions and internal state. These stimuli, in turn, provide output signals to regulate the timing of rest/activity and behavioral cycles. The core mechanisms providing this timekeeping property are provided through transcription/translation feedback loops, consisting of both positive and negative elements, coupled with other intracellular elements associated with signaling events. Clock gene proteins are now being utilized as molecular tools to further study clocks in all tissues, and how the SCN synchronizes and aligns these various body clocks to environmental cycles and imposed work schedules. Circadian rhythm sleep disorders as well as sleep phenotypes are correlated with abnormalities in the genes regulating circadian rhythms. Internal desynchrony of peripheral tissues and the SCN can have negative consequences for human health and longevity. Research to date has revealed surprising complexity in the ordering of body functions. Much remains to be discovered regarding the roles of the SCN and peripheral clocks in coordinating the brain and body in health and disease.

ACKNOWLEDGEMENTS: The authors gratefully recognize present and past support from the National Institutes of Health: HL67007, HL086870, HL092571Z ARRA, NS22155, and NS35859 (MUG), F30 NS047802 and GM07143 (SMA), and GM007283 (JMA).

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Figure Legends

Figure 1. Anatomy of the mammalian suprachiasmatic nucleus (SCN). This medial, transverse section of the rat anterior hypothalamus shows the bilateral SCN stained darkly with an antibody to an endogenous peptide. The paired SCN are at the base of the brain, flanking the third ventricle (3V) and positioned directly above the optic chaism (OC). The major subdivisions of the SCN are delineated. The dorsomedial SCN (DM) is marked by neurons expressing arginine vasopressin (AVP), whereas neurons of the ventrolateral SCN (VL) express vasoactive intestinal peptide (VIP). A central region contains neurons that express gastrin-releasing peptide (GRP) and little SAAS.

Figure 2. Circadian organization of temporal windows of SCN sensitivity to phase-resetting signals transmitted from various brain sites. Time-of-day specific signals are presented together with the major sources of SCN innervation by projections bearing these neurotransmitters and neuropeptides. Daytime is marked by sensitivity to serotonin (5-HT), pituitary adenylate cyclase-activating peptide (PACAP), neuropeptide Y (NYP) and GABA. During dusk and dawn, the pineal hormone melatonin can stimulate resetting of the

SCN clock. At night, the SCN is sensitive to phase adjustment by glutamate and PACAP from the eye, as well as by cholinergic inputs from brain regions that regulate sleep and wakefulness.