29

Physiology of the Mammalian Circadian System

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ABSTRACT

Our understanding of the physiology of the mammalian circadian system has increased enormously in just the past few years. Although it has been known for many years that the circadian clock has a period of approximately 24 hours (hence the name *circadian*, "about a day"), and that the light–dark cycle synchronizes ("entrains") the clock to the solar day through a special tract of nerves to the anterior hypothalamus, called the *retinohypothalamic tract*, only in the past few years have the functional retinal photoreceptors been identified as a unique subset of melanopsin-containing ganglion cells. Embedded in the hypothalamus are the bilaterally paired suprachiasmatic nuclei (SCN), first identified in the early 1970s as critical for the normal expression of circadian rhythms, and long considered to be the master circadian pacemaker in mammals. Only in the past few years, however, has the SCN given up the secret of how it generates circadian rhythmicity, a process that is now known to be intrinsic to individual SCN neurons and to involve a transcriptional–translational feedback loops (s) comprising a number of "clock genes" and their protein products. In addition to photic signals from the retina, the SCN also receives inputs from a number of other sources conveying functional information about various aspects of the internal and external environments that are integrated to regulate the overall temporal organization of the animal. Just as the mystery of the molecular timing mechanism is being unraveled, new findings have revealed that the circadian timing system at the organismal level is much more complex and interesting than previously thought. The circadian system is now seen as hierarchically organized, such that the master clock in the SCN synchronizes multiple circadian oscillators distributed throughout the brain and body. Indeed, most, if not all, tissues and organs of the body appear to contain the core molecular circadian clock machinery, such that the molecular clock in the SCN entrains similar molecular clocks throughout the body to ensure overall internal temporal organization. Thus, two revolutionary developments in the circadian clock field—(1) the elucidation of the core molecular clock machinery, and (2) the demonstration that most, if not all, tissues can themselves express the 24-hour molecular clock—have opened up an entirely new area of biomedical research on the importance of internal timing for health and disease. With hundreds and even thousands of genes oscillating in any given organ system, under the control of both central (i.e., SCN) and local (i.e., within the tissues) self-sustained circadian oscillations, the circadian clock system clearly plays a central role in regulating cellular function in many ways. Indeed, the balance between health and disease may be highly dependent on the proper synchrony within and between oscillating systems.

A great deal of new information has been obtained in just the past few years about the mammalian circadian timing system at the molecular, cellular, neural systems, and behavioral levels. These advances have led to improved understanding of the neuroanatomy, neurochemistry, and molecular neurobiology of the circadian pacemaker, how this pacemaker is synchronized (entrained) to the external environment, and how it regulates a variety of peripheral oscillating systems. This chapter reviews recent findings on the structure and function of the mammalian circadian timing system, with an emphasis on the newly emerging multioscillatory nature of this system, how the master circadian clock in the hypothalamus receives inputs from the internal and external environment, and how the clock regulates the wide diversity of biochemical, molecular, cellular, physiologic, and behavioral rhythms.

THE SUPRACHIASMATIC NUCLEUS, "STILL" THE MASTER CIRCADIAN PACEMAKER IN MAMMALS

As described later in this chapter, exciting new results demonstrating that the molecular circadian clock exists in many tissues and organs are revolutionizing our understanding of how the circadian clock system is organized in mammals. Nevertheless, there is still substantial evidence from a variety of different experiments that the hypothalamic suprachiasmatic nucleus (SCN) is the site of a "master" circadian clock in mammals that is responsible for regulating, directly or indirectly, most, if not all, circadian rhythms in mammals.[1,2] Over the years, a variety of studies involving SCN lesions, recordings of SCN neural activity in vivo and in vitro, functional metabolic mapping, fetal tissue transplantation, and molecular rhythm analysis, have revealed that the SCN not only is capable of sustained rhythmic activity, but is responsible for maintaining circadian rhythmicity in central and peripheral tissues.[3-5]

Although early mathematical models raised the possibility that the circadian pacemaker could be constructed from an ensemble of coupled, high-frequency (i.e., noncircadian) oscillatory units, it is now clear that the generation of circadian signals by the SCN is fundamentally a cellular process.

Studies using a variety of in vitro models, including long-term SCN cell culture,[6] simultaneous recording of multiple single units using multielectrode plates,[7-10] and optical monitoring of calcium flux[11] or gene expression[12] in individual SCN neurons, have now provided compelling evidence that circadian oscillation is indeed a cell-autonomous process, expressed in many, and possibly all, individual SCN neurons. Nevertheless, this multitude of cellular circadian oscillators normally interacts to produce coherent circadian patterns of behavior.[13] Although the mechanisms underlying intra-SCN oscillator coupling have not been identified completely, early studies using tetrodotoxin revealed that individual neuronal oscillators in the SCN apparently remain synchronized even in the absence of sodium-dependent action potentials, both in vivo and in vitro.[14,15] Thus, it has been suggested that gap junctions, glial coupling, calcium-dependent action potentials, or local diffusible signals may be responsible for maintaining interoscillator synchrony (for reviews, see Shirakawa et al.[16] and Miche and Colwell[17]).

Although early studies demonstrated that protein synthesis was involved in the generation of circadian signals,[18,19] analysis of the fundamental circadian oscillatory mechanism has more recently been extended to the molecular genetic level (see Chapter 30). The discovery of the first mammalian circadian clock gene, *Clock*,[20,21] was followed quickly by the identification of a number of other "core" clock genes, many of which showed clear homology to previously or later discovered circadian clock genes in the fruit fly.[22] At present, putative mammalian clock genes include three *Per* genes (*Per1*, *Per2*, *Per3*), *Tim*, *Clock*, *Bmal*, *CK1e*, and two plant cryptochrome gene homologs (*Cry1* and *Cry2*), all of which are expressed in SCN neurons.[23-25] These genes and their protein products interact to form interlocking autoregulatory transcription–translation feedback loops that define the molecular core of the circadian oscillator[26-28] (Fig. 29–1). These basic molecular feedback loops are modulated by posttranslational biochemical processes, leading eventually to the display of remarkably precise 24-hour rhythms in metabolic, physiologic, and behavioral processes.[24,25]

CLOCK and BMAL form protein heterodimers that exert positive drive on transcription of the *Per* and *Cry* genes, whereas the PER and CRY proteins form both homodimers and heterodimers that negatively regulate *Clock* and *Bmal* activity. This feedback loop results in rhythmic transcription of specific clock genes in the in vivo[29-31] and in vitro SCN.[32,33] In addition, molecular outputs from the core oscillator result in rhythmic expression of various clock-controlled genes (i.e., genes that are controlled by, but not part of, the core circadian oscillator loop), which in turn serve as the basis for rhythmic outputs to myriad other cellular processes.[28] Ultimately, these molecular processes are reflected in circadian behavioral rhythmicity, as amply documented by analysis of altered circadian pacemaker function in mice carrying a mutation of the *Clock* gene[20,34] or null or loss-of-function mutations of *Per* and *Cry* genes,[35-37] as well as in *tau*-mutant hamsters carrying a mutation of the *CK1e* gene.[38,39] Interestingly, mutation or deletion of circadian clock genes has also been shown recently to affect sleep–wake homeostasis, suggesting a possible molecular link between the circadian and sleep-regulatory systems.[40,41]

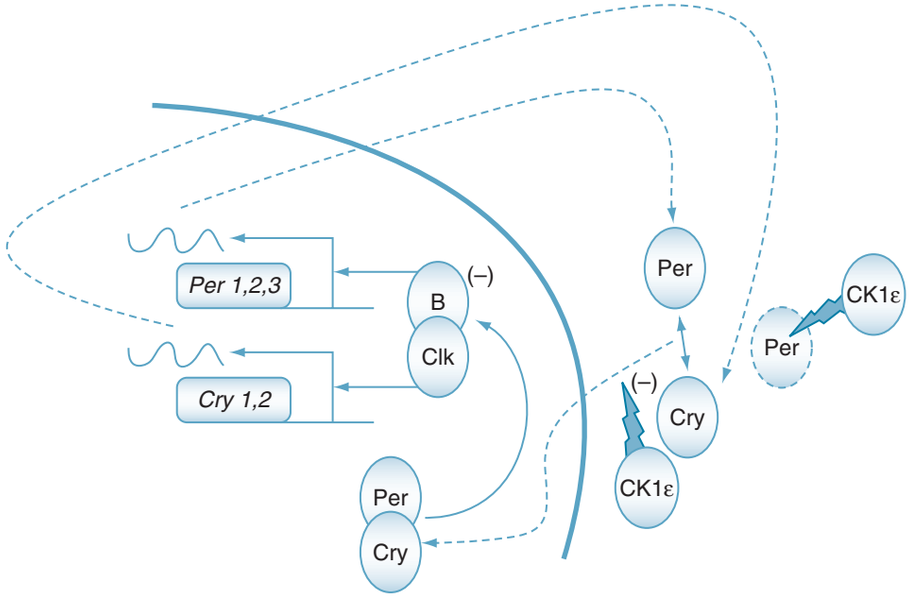

Figure 29–1. Essential elements of the core molecular loop underlying circadian timing at the cellular level in mammals. The transcription factors CLOCK (Clk) and BMAL1 (B) form protein heterodimers exerting positive drive on the transcription of several clock genes, including the *Per* ("period") genes *Per1*, *Per2*, and *Per3*, and the *Cry* (cryptochrome) genes *Cry1* and *Cry2*. The protein products of these genes dimerize in several different combinations, including PER-CRY (as shown here) and PER-PER pairings. After nuclear translocation, PER and CRY inhibit the transcriptional effects of CLOCK-BMAL1 through direct protein–protein interaction, and thus exert negative autoregulation of their own transcription. This negative feedback results in circadian expression of *Per* and *Cry* transcripts. CK1ε acts posttranslationally to degrade PER and inhibit its nuclear translocation, thus regulating the period of the rhythm. At the behavioral level, this model predicts (1) the shortening of free-running period seen in *tau*-mutant hamsters carrying a mutation of the *CK1ε* gene, (2) the lengthening of free-running period seen in *Clock*-mutant mice, and (3) the loss of coherent free-running rhythms seen in *Clock* mice and in *Per*- and *Cry*-knockout mice.

FUNCTIONAL NEUROANATOMY AND NEUROCHEMISTRY OF THE SUPRACHIASMATIC NUCLEUS

Traditionally, the SCN has been characterized as comprising distinct ventrolateral and dorsomedial subdivisions. Recently, however, this scheme has been reconceptualized to include SCN "core" and "shell" subnuclei, a concept that may better accommodate species differences in the anatomic distribution of neuropeptides and afferent terminal fields in the SCN[42,43] (Fig. 29–2). Although SCN neurons express a large number of neuropeptides, core and shell subnuclei have been most commonly identified by the concentration of arginine vasopressin–positive neurons in the SCN shell, and by vasoactive intestinal peptide– and gastrin-releasing peptide–positive neurons in the SCN core.[42,44] Beyond this basic organization, however, clear species differences have been noted, even among nocturnal rodents. For example, the hamster SCN core contains a very distinct and compact cluster of photoresponsive calbindin-positive cells, which is absent in the rat.[43]

Although the specific functions of these chemically defined SCN cell populations are not fully known, a reasonable heuristic is that the SCN core serves to collect and collate pacemaker inputs, whereas the shell is primarily responsible for generation of the circadian timing signal. These suggestions are consistent with findings that (1) major SCN efferent systems converge in the core subnucleus[42,44]; (2) spontaneous circadian rhythmicity in neuronal activity, neuropeptide release, and *cFos* and *Per* gene expression is seen more reliably in the SCN shell than in the core[45-49]; and (3) administration of SCN core peptides such as vasoactive intestinal peptide and gastrin-releasing peptide can mimic both light-induced phase shifting and *Per* gene expression in the SCN, in vivo and in vitro.[50-53] On the other hand, the view that SCN core and shell functions reflect circadian entrainment and circadian pacemaking functions, respectively, is probably too simplistic because (1) light-evoked responses are seen in SCN shell as well as in core neurons; (2) certain nonretinal SCN afferent systems converge within the SCN shell; and (3) in vitro studies have revealed independent circadian rhythmicity in secretion of both SCN core and SCN shell peptides, which may free-run with different periods in the same tissue, implicating separate core and shell oscillators.[49,54]

LIGHT INPUT TO THE PACEMAKER: THE RETINOHYPOTHALAMIC TRACT

Numerous studies have established that a specialized retinal projection system, referred to as the *retinohypothalamic tract*

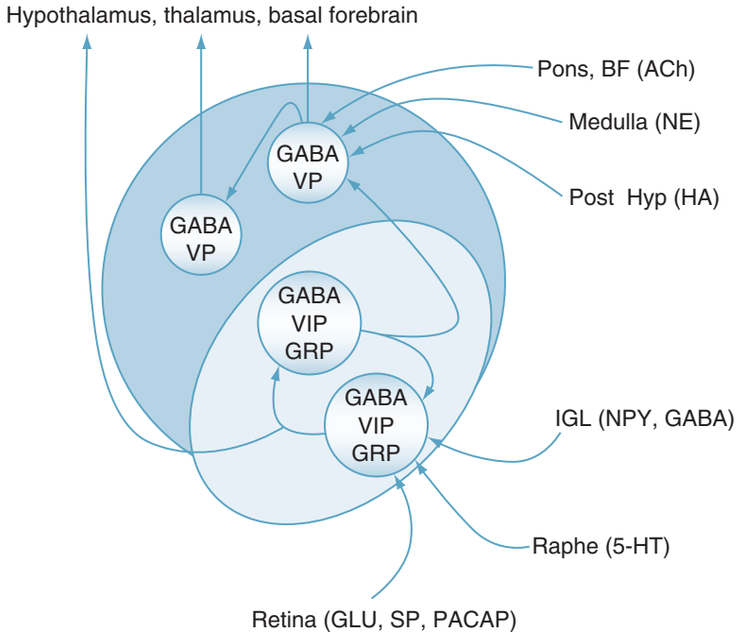

Figure 29–2. Core and shell organization of the suprachiasmatic nucleus (SCN). The vast majority of SCN neurons release the inhibitory amino acid transmitter gamma-aminobutyric acid (GABA). In the SCN core (*light blue*), GABA is commonly colocalized with one or more neuropeptides, including vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP), whereas neurons of the SCN shell (*darker blue*) frequently contain GABA colocalized with arginine vasopressin (VP). SCN core neurons project to other core neurons, to SCN shell neurons, and to extra-SCN targets, most prominently in the diencephalon and basal forebrain; SCN shell neurons project to other shell neurons, and to extra-SCN targets, but not to SCN core neurons. This anatomic organization implies that the flow of information within the SCN is generally from core to shell. Consistent with this suggestion, the three best-characterized SCN afferent systems, originating in the retina, the intergeniculate leaflet of the thalamus (IGL), and the mesencephalic raphe nuclei, converge in the SCN core. Retinal afferents contain the excitatory amino acid transmitter glutamate (GLU) as well as the neuropeptides substance P (SP) and pituitary adenyl cyclase–activating peptide (PACAP); raphe afferents contain 5-hydroxytryptamine (5-HT; serotonin); and IGL afferents contain neuropeptide Y (NPY) and GABA. Beyond these core afferents, several less well-characterized afferent systems converge in the SCN shell, including acetylcholine (ACh)-containing projections from the basal forebrain (BF) and pons, medullary norepinephrine (NE)-containing projections, and histamine (HA)-containing projections from the posterior hypothalamus (Post Hyp). A number of other anatomically identified but functionally uncharacterized SCN afferent systems have been omitted from this figure, and are not discussed in the chapter.

(RHT), is both necessary and sufficient for photic entrainment of the circadian pacemaker.[55] The RHT originates from a distinct subset of retinal ganglion cells separate from those giving rise to the primary visual pathways,[56] and terminates mainly in the SCN, as well as more sparsely in the anterolateral hypothalamus, subparaventricular zone, and supraoptic region.[57,58] In addition, RHT axon collaterals also project to the thalamic intergeniculate leaflet (IGL; Fig. 29–3), which, as discussed later, is itself an important component of the circadian system.

Remarkably, retinally degenerate strains of mice, in which nearly all classic photoreceptors (i.e., rods and cones) are lost by early adulthood, exhibit normal circadian responses to light.[59] More recently, similar findings have been reported in genetically engineered mice with a total developmental absence of both rods and cones, demonstrating conclusively that circadian light entrainment can be mediated by a novel, nonrod, noncone photoreceptor system.[60] Recent studies indicate that the protein melanopsin, found specifically within the small subset of retinal ganglion cells giving rise to the RHT, serves as a circadian photoreceptor molecule in a novel population of photosensitive RHT retinal ganglion cells.[61-63] Thus, light entrainment of the circadian pacemaker is mediated by a dedicated system of photoreceptors, retinal neurons, and central pathways, entirely distinct from those mediating visual perception: a startling finding still not appreciated by most of the biomedical community outside of the fields of sleep and circadian rhythmicity.

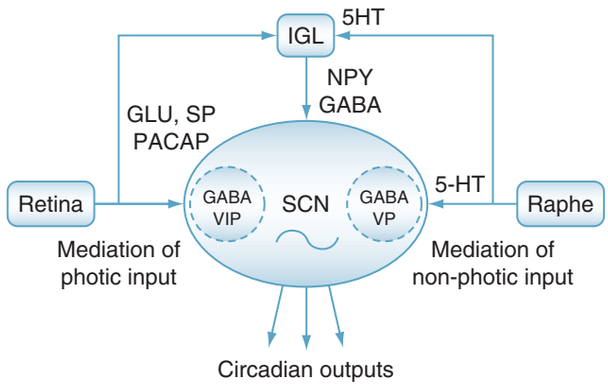

Figure 29–3. Overview of functional neuroanatomic pathways in the mammalian circadian system. Major suprachiasmatic nucleus (SCN) afferent systems originating in the retina and raphe nuclei also target the intergeniculate leaflet of the thalamus (IGL), which in turn projects to the SCN. Retinal projections to the SCN and IGL mediate photic input to the circadian system, raphe projections to the SCN and IGL mediate the effects of certain nonphotic, behavioral state–related signals, and IGL-SCN projections are involved in mediation of both photic and nonphotic signaling to the SCN pacemaker. As described in the text, photic and nonphotic pathways generally interact to produce mutually antagonistic effects on the circadian pacemaker. Thus, photic signals evoke circadian phase shifting during subjective night and antagonize nonphotic phase shifting during subjective day, whereas signals related to arousal and wakefulness evoke phase shifting during subjective day and antagonize photic phase shifting during subjective night. These antagonistic interactions are mediated in part at the level of the SCN, but the scheme presented here suggests that the IGL is also a probable locus for interaction between photic and nonphotic signals—a hypothesis that is largely unexplored. GABA, gamma-aminobutyric acid; GLU, glutamate; 5-HT, 5-hydroxytryptamine (serotonin); NPY, neuropeptide Y; PACAP, pituitary adenyl cyclase–activating peptide; SP, substance P; VIP, vasoactive intestinal polypeptide.

Although these surprising findings represent the first evidence for non–rod/cone-mediated photic input to the mammalian nervous system, it has been known for many years that the circadian clock of *non*-mammalian vertebrates could be entrained even in the absence of the eyes, and that such extraretinal entrainment is mediated by both pineal and "deep-brain" (encephalic) photoreceptors.[64] Even though non–rod/cone-mediated entrainment in the mammalian system is nevertheless based on a retinal photoreceptor, the recent findings on the nature of these photoreceptors do highlight the evolutionary continuity between nonmammalian and mammalian vertebrates, and reveal that circadian entrainment depends on "nonvisual" photoreceptive mechanisms in all vertebrates.

RHT terminals release the excitatory amino acid neurotransmitter, glutamate, in response to photic stimulation. Extensive evidence from in vivo and in vitro studies indicates that glutamate acts through both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors and a variety of intracellular signaling molecules (e.g., Ca^{2+}, nitric oxide, calmodulin/calmodulin kinase, protein kinase C, protein kinase G, cyclic adenosine monophosphate–responsive element-binding protein [CREB], and others)[65] and immediate early-response genes including *c-fos*,[66] leading to increased expression of *Per1* and *Per2*, and possibly other core clock genes.[67,68] The protein products of these genes represent state variables of the molecular oscillator, such that alterations in their transcription levels, when superimposed on the ongoing circadian transcription cycle, correspond functionally to phase shifts of the oscillator[69] (Fig. 29–4).

In addition to glutamate, RHT terminals also release two identified peptide cotransmitters, substance P (SP) and pituitary adenyl cyclase–activating peptide (PACAP). SP appears to play an important role in RHT transmission because selective SP antagonists block light-induced phase shifting and early-response gene expression in vivo,[70-72] as well as glutamate receptor–mediated phase shifting in vitro.[73] By itself, SP can mimic at least one component of the photic phase–response curve (phase delays during early subjective night) both in vivo and in vitro.[74,75] At least in vitro, the phase-shifting effects of SP appear to depend on SP-evoked glutamate release, and can be blocked by the NMDA antagonist, MK-801.[74] In contrast, PACAP administration has been reported either to antagonize or mimic the effects of glutamate on circadian phase shifting and *Per* gene expression in vitro, depending on dose and on circadian phase.[76-80] Specifically, when administered at relatively high doses, PACAP blocks the effects of glutamate during subjective night and evokes phase advances during subjective day, but when administered at much lower doses, PACAP actually mimics or potentiates the effects of glutamate on the SCN pacemaker.

OTHER FUNCTIONAL INPUTS TO THE CIRCADIAN CLOCK

An additional major SCN afferent system arises from the IGL, a distinct retinorecipient region of the lateral geniculate complex, intercalated between the dorsal and ventral lateral geniculate nucleus.[81-83] The projection from the IGL to the SCN is referred to as the *geniculohypothalamic tract* (GHT),

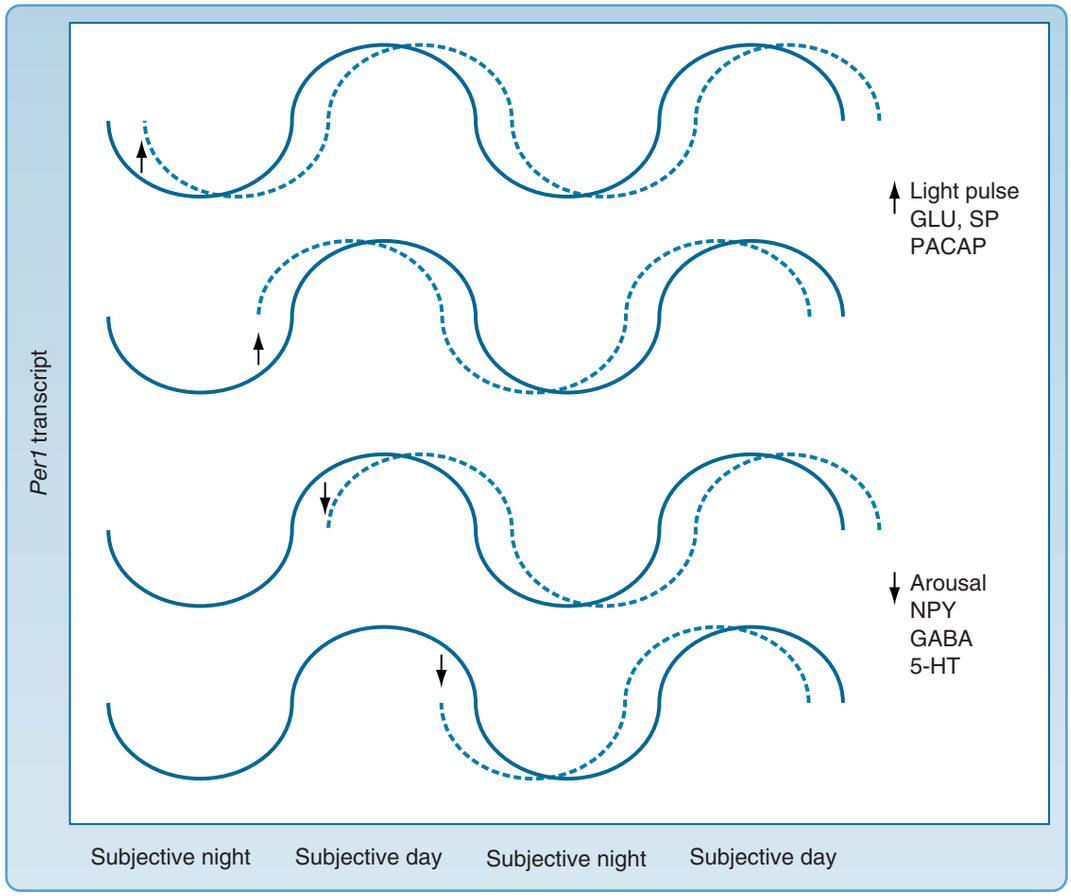

Figure 29–4. A simple qualitative-molecular model for circadian phase shifting by photic and nonphotic signals, and for their mutually antagonistic interaction. In this "phase-only" model, amplitude is fixed and the underlying state variable (here, *Per1* transcript level) can oscillate only within predetermined upper and lower bounds, such that the *Per1* level represents the phase of the molecular oscillator. During the subjective night, *Per1* levels are relatively low (*solid line*), and light pulses (or corresponding neurotransmitters or intracellular messengers) induce an abrupt increase in transcript level (*arrow*). Early in the night, when *Per1* levels are normally decreasing, this increase in transcription essentially forces the oscillator to repeat part of its normal trajectory, and is thus equivalent to resetting the oscillator to an earlier phase, resulting in a permanent phase delay (*dashed line*). In contrast, late in the night, *Per1* levels are normally increasing, such that a light-induced increase in transcription forces the oscillator to omit part of its normal trajectory, equivalent to resetting the oscillator to a later phase, and resulting in a permanent phase advance. Opposite to light pulses, arousal-related signals (or corresponding neurotransmitters or intracellular messengers) induce abrupt *decreases* in *Per1* transcription, resulting in phase delays during early subjective day and phase advances during late subjective day. Thus, the model predicts that photic and nonphotic phase–response curves (PRCs) should have essentially identical shapes, but should be phase-displaced by 180 degrees (12 circadian hours) along the horizontal axis; these predictions are at least roughly consistent with experimental observations.[69] Further, this model accounts for the general insensitivity of the circadian pacemaker to photic phase shifting during mid-subjective day and to nonphotic phase shifting during mid-subjective night: Because the underlying state variable can vary only within a predetermined range, stimuli that increase *Per1* transcription are ineffective when transcript levels are already maximal, and stimuli that decrease *Per1* transcription are ineffective when transcript levels are already minimal. Nevertheless, despite these periods of insensitivity, nonphotic signals would remain capable of counteracting light-evoked increases in transcription, and photic signals would remain capable of counteracting arousal-evoked decreases in transcription. Finally, the exact waveform and phasing of the photic and nonphotic PRCs would obviously depend on the exact waveform and phasing of the underlying spontaneous transcription cycle, here presented arbitrarily as two interlocking circular arcs centered over mid-subjective day and mid-subjective night. GABA, gamma-aminobutyric acid; GLU, glutamate; 5-HT, 5-hydroxytryptamine (serotonin); NPY, neuropeptide Y; PACAP, pituitary adenyl cyclase–activating peptide; SP, substance P.

and GHT neurons release both neuropeptide Y and gamma-aminobutyric acid (see Fig. 29–3). Retinal signals are conveyed to the IGL in part by axon collaterals of RHT neurons,[84] and GHT and RHT terminal fields are largely coextensive within the SCN core.[42,44] It is thus not surprising that early functional studies emphasized the possible role of the IGL/GHT system in providing a secondary, indirect pathway for photic entrainment of the circadian pacemaker.[81] Although the IGL is clearly not necessary for photic entrainment, lesions of the IGL/GHT do produce subtle modifications in the ability of light signals to effect phase and period control of the circadian clock.[81]

In addition to its role as a secondary source of photic signaling to the circadian clock, the IGL also plays a preeminent role in the nonphotic regulation of the circadian system. Thus, IGL lesions abolish the phase-shifting effects of novelty-induced wheel running[85,86] and benzodiazepine administration in hamsters,[87-91] as well as the period-shortening effect of running-wheel access in rats[92] and the entrainment effect of scheduled daily treadmill activity in mice.[93] Further studies

on the role of the RHT and IGL in mediating photic and nonphotic inputs to the SCN are reviewed in Rosenwasser.[5]

Another major SCN afferent system converging on the SCN core originates from the serotonergic midbrain raphe, especially the median raphe nucleus.[94,95] In addition, ascending serotonergic projections originating in the dorsal raphe nucleus innervate the IGL, providing a second potential route for serotonergic regulation of the SCN circadian pacemaker (see Fig. 29–3). Extensive evidence has implicated serotonergic projections to the SCN (and IGL) in two distinct functions: (1) modulation of photic effects on the circadian pacemaker during the subjective night,[94-96] and (2) mediation of nonphotic, behavioral state–related effects on the pacemaker during subjective day.[97-100] In addition, whereas light during the middle of the subjective day is normally thought to have no effects on the circadian clock, light at this time can block the phase-shifting effects of a 5-hydroxytryptamine (5-HT; serotonin) agonist.[101] These latter results indicate that in addition to 5-HT inputs having a modulatory effect on light input to the SCN, the reverse is also true.

During the subjective night, photic effects on the circadian pacemaker are attenuated by electrical stimulation of the raphe nuclei or by systemic or intra-SCN administration of serotonergic agonists active at the 5-HT_{1A}, 5-HT_7, or 5-HT_{1B} receptors, whereas conversely, photic signaling in the SCN is potentiated by neurotoxic lesions of serotonin projections or by targeted serotonin antagonists.[94,95] In addition, photic entrainment is inhibited in the presence of high levels of arousal or locomotor activity, apparently through arousal-related release of endogenous serotonin.[97] During the subjective day, the circadian pacemaker can be phase shifted by electrical stimulation of the midbrain raphe nuclei[102,103] as well as by in vivo[104,105] or in vitro[106] administration of 5-HT_{1A}/5-HT_7 receptor agonists. The ability of direct 5-HT application to the in vitro SCN to evoke circadian phase shifts indicates that stimulation of intra-SCN 5-HT receptors is sufficient to phase shift the pacemaker. Nevertheless, in vivo experiments using direct intracerebral 8-OH-DPAT [8-hydroxy-2-(di-*N*-propylamino)tetralin] administration have identified several potential loci in the circadian system for serotonergic phase shifting, including the SCN, the IGL, and the median and dorsal raphe nuclei.[71,104,107]

The phase-shifting effects evoked by serotonergic stimulation closely resemble those seen with other nonphotic phase-shifting stimuli, including novelty-induced activity, sleep deprivation, and benzodiazepine and neuropeptide Y administration.[5,108] Thus, several studies have directly examined the potential role of serotonergic afferents to the SCN and IGL in mediating the effects of behavioral state on the circadian pacemaker. Arousal, wakefulness, and motor activity are all associated with increased forebrain serotonin release,[109-111] and serotonin content in the rat SCN is correlated positively with spontaneous activity level and negatively with free-running period.[112] Indeed, state-dependent variations in serotonin release appear to mediate (1) the effects of activity level on free-running period,[111] (2) phase-shifting by activity or sleep deprivation,[110] (3) entrainment by restricted daily running wheel access[113] or scheduled daily treadmill activity,[93] and (4) activity-dependent inhibition of photic phase shifting in hamsters.[114]

Several other chemically identified pathways provide afferent input to the circadian system, including noradrenergic projections from the locus coeruleus, cholinergic projections from the basal forebrain and pontine tegmentum, and histaminergic projections from the posterior hypothalamus.[95,115,116] The cholinergic inputs, and their unknown function, are particularly intriguing because it was studies with the cholinergic agonist, carbachol, that were among the first to use a pharmacologic approach to study the neurochemistry of the circadian clock.[117] However, the significance of the cholinergic system in the circadian organization is still not understood. In addition, noradrenergic and cholinergic projections both innervate the IGL, providing an alternate pathway by which these transmitter systems could alter SCN circadian pacemaker function. Unlike the retinal, geniculate, and raphe projections described previously, which form generally overlapping terminal fields in the SCN core, these afferents target preferentially the SCN shell[42,44] (see Fig. 29–2). Although less studied than the SCN core afferents, sufficient data exist to suggest that these SCN shell afferents also contribute to circadian pacemaker regulation.

MULTIPLE OSCILLATOR NATURE OF CIRCADIAN SYSTEM

To this point, the review of current circadian neurobiology presented in this chapter has treated the SCN as the locus of *the* circadian pacemaker, but in fact, the circadian system comprises a multiplicity of circadian oscillators and pacemakers. As reviewed earlier,[118,119] circadian systems may exhibit complex dissociations among multiple rhythmic subcomponents. For example, two or more discrete daily activity epochs may emerge from the single normally consolidated activity period, a phenomenon known as *splitting*.[120,121] Such phenomena at the behavioral and endocrine level strongly imply the existence of an underlying multioscillatory neurobiologic circadian system. Interest in these complex phenomena appears to have been deprioritized for several years, coincident with the ongoing maturation of molecular approaches to the core pacemaker mechanism. However, in the last few years, these same molecular approaches, and especially the finding that mammalian clock genes are expressed not only in the SCN but in other brain regions[122] and in many peripheral tissues as well,[123,124] have spurred renewed interest in the identification and functions of multiple circadian oscillators in the circadian timing system.

At the level of the SCN itself, the observation that individual SCN neurons express the molecular mechanisms responsible for generating a circadian time signal demonstrates that the SCN pacemaker is *itself* composed of numerous, potentially autonomous but normally coupled, cellular circadian oscillators. Further, the clock genes *Per1*, *Per2*, and *Per3* exhibit a significant degree of functional specialization in the SCN,[37,125-127] and according to one hypothesis, *Per1* and *Per2* may represent state variables of Pittendigh's "morning" and "evening" oscillators, respectively.[128,129] Ultimately, it may be possible to integrate this molecular model with other recent findings suggesting that morning and evening oscillators may be represented by different subpopulations of SCN neurons.[49,130] In addition, it is not known if hypothesized intra-SCN morning and evening oscillators are related to the separate intra-SCN oscillators capable of driving independent secretion of core and shell peptide rhythms.[54] An important challenge for circadian biologists over the next few years will be to integrate

modern insights into the molecular nature of the circadian clock with the earlier and equally important era of the field when many of the formal properties and basic principles underlying circadian organization were initially defined.[131,132]

Outside the SCN, recent evidence suggests that several non-SCN neural and neuroendocrine tissues are capable of expressing autonomous (although generally damped) circadian oscillations. Thus, cultured mammalian retinae display persisting circadian rhythmicity in melatonin secretion,[133] whereas more recent studies have demonstrated self-sustaining oscillations of *Per* gene expression in cultured endocrine tissues (pineal, pituitary), diencephalic nuclei (e.g., hypothalamic arcuate and paraventricular nuclei, thalamic paraventricular nucleus), and the olfactory bulbs[122] as well. Although the relationships between these extra-SCN neural oscillators and the SCN pacemaker have not been fully elucidated, it appears that at least certain types of behavioral rhythm splitting may involve dissociations between intra-SCN and extra-SCN central clocks.[134,135]

Similar techniques have also been used to reveal rhythmic *Per* expression in liver, lung, kidney, and other peripheral tissues.[136-138] Initial studies found these peripheral rhythms to be highly damped, and dependent on periodic input from the SCN for their continuous expression.[136,139] However, a more recent and highly elegant series of studies using mice with a reporter gene that could measure real-time circadian dynamics[124] revealed that circadian oscillations could persist for many days in peripheral tissues in vivo (Fig. 29–5). In addition, tissues from different SCN-lesioned animals were found to be rhythmic in vitro, although no longer in phase with one another as they are when taken from SCN-intact animals entrained to a light–dark cycle, indicating that the SCN synchronizes rather than drives these peripheral rhythms (see Fig. 29–5). Peripheral oscillators may also dissociate from the SCN pacemaker under certain conditions, such as after light–dark cycle phase shifts (i.e., simulated jet lag)[123] or during restricted feeding schedules, which entrain peripheral but not SCN *Per* gene oscillations.[4] These observations indicate that the SCN pacemaker normally serves to entrain both central and peripheral secondary oscillations generated by a broadly distributed population of autonomous cellular oscillators, but that under certain conditions, these downstream

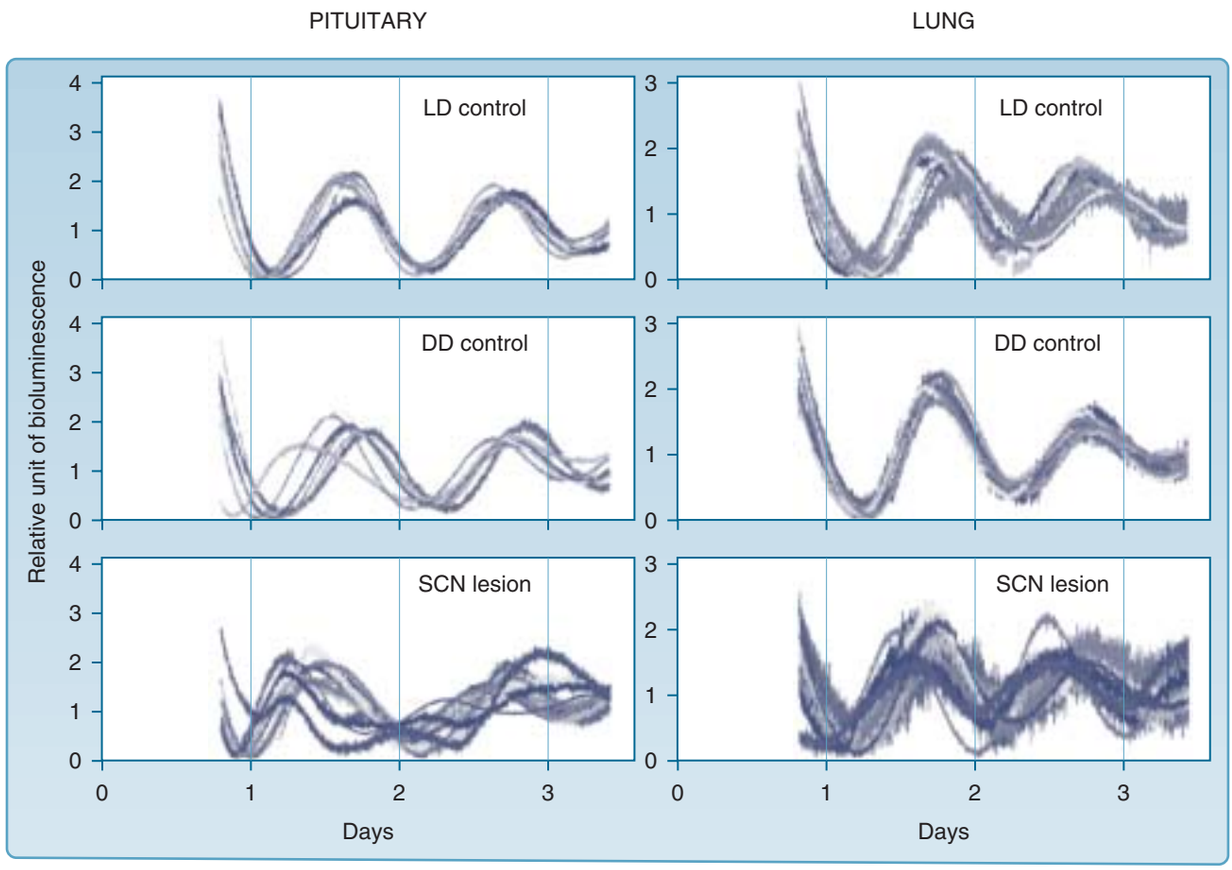

Figure 29–5. Superimposed plots of bioluminescent data from pituitary and lung tissues from individual animals that were intact and maintained on a light–dark cycle (LD controls) or in constant darkness (DD controls), as well as from suprachiasmatic nucleus (SCN)-lesioned animals. The tissue was maintained in vitro and made use of a Period2:Luciferase fusion protein as a real-time reporter of circadian dynamics. The first three cycles in culture are represented; each animal's record is a different shade. Although tissues collected from individual animals on an LD cycle, and relative to activity onset in DD control animals, were in phase with one another, phase desynchronization is evident in individual records of the SCN-lesioned animals for both tissues. (From Yoo SH, Yamazaki S, Lowrey PL, et al: PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A 2004;101:5339-5346.)

oscillators are capable of adaptive (as well as maladaptive?) disengagement from SCN control.

With the thought-provoking title "Circadian lessons from peripheral clocks: Is the time of the mammalian pacemaker up?," Brandstaetter suggested that perhaps "... the hypothalamic SCN of a rodent has to resign from its major function."[140] To paraphrase from a "live speech" of the American humorist, Mark Twain, "The recent announcement of my death is premature." We maintain that the death of the SCN is premature. For years the clock community has referred to the SCN as a "master pacemaker," and many speculated that such a pacemaker may drive circadian rhythms or regulate "slave oscillators."[131,141] Although it is now clear that many (most, all?) tissues and organs can produce circadian rhythms (using the same or similar molecular clock machinery as the SCN cells) in the absence of the SCN (e.g., in vitro), that does not mean these slave oscillators are independent of the SCN. Indeed, what is emerging in the field is the hypothesis that the SCN is still (in 2004) the "master oscillator" regulating all circadian rhythms either directly or indirectly. Rhythms are regulated directly when circadian information from the SCN directly controls a particular rhythm (presumably, by imposing circadian timing on the neural and physiologic systems regulating that function). However, "indirect" control is also important, as, for example, when the SCN's direct control of behavioral rhythms (e.g., feeding, sleep–wake cycle) sets in motion various metabolic, endocrine, and physiologic processes, which in turn control (entrain?) downstream rhythms. Indeed, many circadian rhythms are controlled by the behavioral states of sleep and wake in that the circadian expression of many rhythms depends on whether the animal is awake or asleep.[141] In animals, direct and indirect control of circadian rhythms by the SCN are normally in synchrony with each other. After all, it is only humans (and perhaps their live-in domestic pets) that routinely override their biologic clock with respect to the control of behavioral rhythms such as the sleep–wake cycle or the feeding cycle. Only humans sleep (or eat) at inappropriate times with respect to the adaptively appropriate environmental time. Whether direct or indirect, the SCN in mammals still appears to control the expression of all circadian rhythms, and in 2004 it is not time (yet) for the SCN pacemaker to give up its preeminent role in the circadian timing system.

This enriched appreciation of the multioscillatory nature of the mammalian circadian system has opened up new approaches for understanding the temporal organization of mammalian physiology and behavior, and raises a number of questions about the adverse effects associated with lack of normal synchronization between central and peripheral oscillations. Although such circadian dysregulation rarely occurs in nature, it certainly occurs quite often in humans, who can override their circadian clock and exert substantial volitional control over their sleep–wake cycles. Under such circumstances, abnormal phase relationships are expressed between sleep–wake behaviors (and other rhythmic processes tightly linked to sleep or wake states) and the circadian clock (and rhythmic processes tightly linked to it). Although the internal desynchronies that occur with jet lag and shift work may be the most dramatic, they are not the only examples of such dyschrony. Regardless of work or travel schedules, humans in our modern, around-the-clock society are becoming increasingly nocturnal despite millions of years of evolutionary pressure to be diurnal.

SUMMARY AND CONCLUSIONS

The primary pacemaker for the mammalian circadian system is contained in the SCN, and the mechanisms underlying the pacemaker function of this structure are rapidly being elucidated at the molecular, cellular, and neuroanatomic levels. The SCN contains a large number of normally coupled but potentially autonomous cellular oscillators that generate a circadian time base through the expression of a complex molecular feedback loop. The activity of the core molecular loop results in the circadian expression of a large number of clock-controlled genes, which in turn regulate coordinated circadian rhythms in the metabolism, electrical activity, and neurotransmitter and neuropeptide release of SCN neurons. These processes result in the transmission of circadian timing signals to both passive targets and inherently rhythmic secondary oscillators throughout the brain and periphery.

The core molecular loop is entrained by a number of convergent SCN afferent pathways. Photic signals are transmitted from a specialized set of photoreceptive retinal ganglion cells by a dedicated neural pathway (the RHT) to the SCN, and activity in this pathway results in the release of glutamate as well as multiple peptidergic cotransmitters. Other major SCN afferents arise from the IGL and raphe nuclei, which form terminal fields that largely overlap the RHT terminal field in the SCN core. These afferents serve to regulate photic signaling in the SCN during subjective night and to mediate the phase-shifting effects of nonphotic stimuli, including behavioral activity and arousal, during subjective day.

Two revolutionary developments in the circadian clock field—(1) the elucidation of the core molecular clock machinery, and (2) the demonstration that most, if not all, tissues/organs can themselves generate the 24-hour molecular clock—when coupled with recent studies indicating that 5% to 10% of all genes being expressed in any particular tissue/organ are under circadian regulation,[123,142,143] have opened up an entirely new area of biomedical research on the importance of internal timing for health and disease. Indeed, it can be argued that we are at the beginning of a new era in understanding human health and disease. With hundreds and even thousands of genes oscillating in most, if not all, tissues and organs that are under the control of central (i.e., SCN) and local (i.e., within the tissues) self-sustained circadian oscillations, normal health and well-being undoubtedly depend on

Clinical Pearl

Most, if not all, organs/tissues contain the core molecular circadian clock machinery, and can produce circadian rhythms in vitro. This greatly heightens the theoretical importance of internal synchronization for normal physiologic function. Abnormal circadian timing between and within tissue/organ systems could be just as important as the overproduction or underproduction of key cellular processes to overall health. Internal temporal dysfunction may thus be at the root of many physical and mental diseases. Although jet lag and shift work have been the primary ways in which circadian dyschrony were thought to occur (see Section 8, this volume), it is probable that dyschrony may turn out to play an important role in many human pathologic processes as well.

internal synchronization. Further, this internal synchronization can occur on two levels, between separate oscillating systems and within each self-sustained system, and the balance between health and disease is likely to be highly dependent on proper synchrony at both levels.

Recent findings that there are different alleles of circadian clock genes that can influence the timing of human sleep and wake[144,145] surely represent the early understanding of human variability in the molecular clock machinery. The importance of this variability at the level of the organism, as well as at the tissue/organ level, and the implications of this variability for different human populations and for understanding disease states are not known, but surely will be the subject of extensive future research.

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