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METABOLISM AND THE CONTROL OF CIRCADIAN RHYTHMS

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■ **Abstract** The core apparatus that regulates circadian rhythm has been extensively studied over the past five years. A looming question remains, however, regarding how this apparatus is adjusted to maintain coordination between physiology and the changing environment. The diversity of stimuli and input pathways that gain access to the circadian clock are summarized. Cellular metabolic states could serve to link physiologic perception of the environment to the circadian oscillatory apparatus. A simple model, integrating biochemical, cellular, and physiologic data, is presented to account for the connection of cellular metabolism and circadian rhythm.

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HYPOTHESIS

Billions of years ago, primordial life was subjected to rhythmic metabolic fluctuation—heating up with solar energy in the day and cooling down at night. An early paradigm of differential gene expression endowed these ancestral

microorganisms a means of activating the right genes during day or night to adapt metabolic flux to the rhythmic fluctuation in ambient temperature. This primordial regulatory system employed a transcription factor capable of sensing a key chemical metabolite whose abundance reflected overt metabolic rate. So regulated, this transcription factor ensured that the proper gene products were produced at the proper time—day or night—to facilitate adaptation to the cyclic change of sun rays. Might this same paradigm of metabolic sensing persist today in humans, lying at the heart of the regulatory apparatus responsible for the control of circadian rhythm?

INTRODUCTION

Evolution has given mammals a robust and effective system for perceiving changes in light conditions. Mammals also maintain a less obvious system for anticipating regular changes in light and other extrinsic stimuli that fluctuate as a function of the earth's rotation around its axis (1). This latter system is called circadian rhythm, so named because it creates a period of "about one day." When advantageous to the organism, it is used to synchronize the timing of organismal processes with cyclic changes in the external environment. This system imposes a daily oscillation on most physiologic functions, including circulating hormones (2, 3), cardiac and circulatory function (4, 5), and core body temperature (6). The importance of this anticipatory system linking physiology with the day/night cycle is underscored by its presence in species from every branch of the phylogenetic tree (7).

The study of circadian rhythm in many diverse organisms has led to the elucidation of a common mechanistic strategy for the generation and organization of daily rhythms. In all cases, the core oscillatory mechanism is composed of a negative transcriptional feedback loop wherein positive transcription factors activate the expression of their own inhibitors. Whereas the molecules differ from one organism to the next, they execute the same feedback cycle from cyanobacteria to humans. This review focuses on circadian rhythms in mammalian organisms and how such rhythms are maintained in synchrony with the environment. In particular, we focus on the connection between the circadian regulatory apparatus and intracellular metabolism. For a thorough analysis of circadian rhythms in nonmammalian species, we refer the reader to the many outstanding reviews on the subject (7–10).

Core Circadian Oscillator

The central mechanism responsible for the regulation of circadian rhythms is a transcriptional negative feedback cycle. In mammals, the positive acting transcription factor is a heterodimer composed of two bHLH-PAS transcription factors, CLOCK and BMAL1 (11–13). Takahashi and colleagues cloned the

mouse *Clock* gene as the culmination of a heroic forward genetic study to identify mutations altering circadian locomotor activity (14, 15). The discovery of *Clock*, the first mammalian circadian rhythm gene to be identified, initiated studies of the molecular basis of mammalian circadian rhythm. Paralogs of CLOCK and BMAL1 have been discovered that are likely to perform similar functions. The NPAS2 protein is very similar in sequence to CLOCK (16) and has been shown to behave similarly in biochemical and cell-based experiments (17–20). Likewise, the MOP9 protein is very similar in sequence to BMAL1 and performs similarly in transcriptional activation assays (21). Throughout this review arguments made and interpretations offered for the CLOCK:BMAL1 heterodimer are likely to apply to heterodimers in which NPAS2 replaces CLOCK or MOP9 replaces BMAL1.

The primary negative components of the mammalian transcriptional feedback loop are the products of the *Cry* genes, which are specifically activated by the CLOCK:BMAL1 heterodimer (17, 22). The CRY proteins, in turn, potently repress the activity of the CLOCK:BMAL1 heterodimer (23, 24). The mechanism of CRY-mediated inhibition is unclear, further mystified by the changing function of CRY over evolutionary time. The bacterial homolog of CRY, DNA photolyase, is a DNA repair enzyme composed of intrinsic FAD and methenyltetrahydrofolate (MTHF) prosthetic groups (25). Using these chromophores, photolyase captures a photon and uses its energy to repair thymine dimers in DNA. The CRY protein of *Drosophila melanogaster* retains the photosensing function of bacterial photolyase but has acquired a new output mechanism. Instead of repairing DNA, dipteran CRY serves as a circadian photoreceptor, involved in resetting the molecular cycle to correspond with the external light/dark cycle (26–28). Recent studies have provided evidence that mammalian CRY has assumed yet a different function, serving as the key negative element in the transcriptional feedback loop (23, 29, 30). We hereafter refer to the oscillatory system composed of CLOCK:BMAL1 and CRY as the core circadian oscillator (CCO).

A functional, albeit unstable, temporal rhythm might be generated simply by the CCO negative feedback loop. Nature, however, provides other tools to stabilize the CCO, causing it to oscillate with the proper period and conferring susceptibility to adjustment when necessary. In mammals, the products of three period genes (*Per1, Per2, Per3)*, whose expression is also induced by the CLOCK:BMAL1 heterodimer (31), specify part of this second tier of functionality. In flies, *Per* is equally important to *Cry*—forming half of the negative component of the transcriptional feedback loop [(32); reviewed in (33)]. Mutations in the single *Per* gene of fruit flies can either entirely abolish circadian rhythm or lengthen or shorten its period (34, 35). Mammalian *Per* is also necessary for proper circadian rhythm. Mice homozygous for mutations in both *Per1* and *Per2* exhibit completely arrhythmic locomotor activity (36). Unlike the situation in flies, however, the mammalian PER proteins may not be directly involved in CLOCK:BMAL1 repression (37). Although the mammalian PER

proteins clearly play an important role in maintaining or organizing circadian rhythm, the mechanistic nature of PER action remains to be elucidated.

A second mechanism contributing to the robustness of the mammalian CCO is afforded by the circadian regulation of *Bmal1* gene expression. This phenomenon is probably due to repression of *Bmal1* gene expression by the CLOCK:BMAL1 heterodimer (17) and the potentially related effect of PER2-mediated activation of *Bmal1* gene expression (37, 39). Cycling BMAL1 protein levels naturally reinforce the CLOCK:BMAL1 activity cycle conferred by the rhythmicity of CRY-mediated inhibition. Casein kinase Iϵ (CKIϵ) is also an important component of the circadian rhythm system in flies, rodents, and humans (40–42). Another elegant and heroic effort by Takahashi and colleagues (41) identified CKIϵ as the product of the gene affected in the hamster *tau* mutant, which exhibits an anomalously short circadian period (43). CKIϵ is a serine/threonine kinase that phosphorylates the PER proteins, thereby posttranslationally regulating their stability and activity (44). It is likely that more clock-stabilizing components and phenomena will be uncovered as the molecular and biochemical functions of the various clock genes and gene products are further defined.

Although a rough outline of how a molecular cycle is created and maintained has begun to emerge, we are largely ignorant as to how this CCO translates into the physiologic changes necessary for cells or organs to execute the right biochemistry at the right time of day. In addition to activating production of the CRY and PER proteins, CLOCK:BMAL1 also activates the expression of other genes, including DBP (45, 46), TEF (45), and Rev-Erbα (47). The products of these "output" genes represent effectors of the CCO, and their cycling activities create, directly or indirectly, the daily oscillations observed in the myriad of physiologic parameters that fluctuate as a function of the day/night cycle. To avoid confusion, one must recognize an important variable in regard to organismal and physiological oscillations. It is unclear how much of this oscillation is controlled directly by the CCO and how much is a secondary effect of other clock-controlled behaviors such as sleeping, feeding, or locomotor activity. A confounding problem in the field of circadian biology is the difficulty of uncoupling the various circadian behaviors and influences. For example, it is impossible to isolate the direct effects of light on the circadian clock in rodents because light potently inhibits locomotor activity and feeding (48, 49), which themselves have strong influence on the clock. Although complex, we show that this interplay has also led to progress in understanding how the clock is coordinated throughout the animal.

As is discussed in more detail later in this review, it has long been appreciated that the suprachiasmatic nucleus (SCN) of the hypothalamus contains the central circadian pacemaker. The SCN was interpreted to serve as a master regulator that directly controls the physiological oscillation observed throughout the body. Surprisingly, we have learned over the past five years that various tissues and cell types employ independently oscillating cellular clocks. Cells from most tissues examined to date maintain a functional CCO that directs circadian expression of

the *Per* and *Cry* genes as well as genes involved in tissue-specific circadian physiology. The cycling of these "peripheral" oscillators in vivo has been extended to in vitro assays wherein cycling tissue explants are now used to study the behavior of clocks in isolation (50, 51). The autonomous cycling of tissues has recently been refined to the single cell level. Individual cells from many tissue sources maintain a functional and reasonably stable cycle in culture independent of native cell contacts (52, 53). Indeed, synchronized rhythms can be generated in cultured fibroblasts by serum shock (47), glucocorticoid administration (54), or pharmacological inducers of a wide variety of intracellular signaling pathways (55). This resetting of rhythms in culture is reminiscent of a fundamental property of the clock in vivo. As with cells in culture, the in vivo clock adapts its cycle to coincide with environmental cues, a process termed entrainment. Whereas a detailed understanding of the component parts of the CCO has begun to emerge, clock entrainment remains obscure. This review focuses on recent work that reveals clues as to how the CCO might, in response to appropriate stimuli, be modified to remain in synchrony with environmental cues.

ENTRAINMENT OF CIRCADIAN RHYTHMS

What Is Entrainment?

Entrainment is the process by which extrinsic stimuli change or reset the phase of a circadian clock. Confusion regarding entrainment resulted from the presence of multiple independent oscillators throughout the body that are controlled by different inputs and are responsible for the control of different outputs. Some clocks, for example, respond to light, while others are completely unaffected by light. Some clocks respond to alterations in feeding schedule, while others are unaffected by food. Likewise, the clock that directly controls body temperature rhythm is different from the clock that controls the cyclic levels of circulating melatonin. Often a stimulus or treatment is said to entrain the circadian clock or CCO if it creates an alteration in the phase of circadian locomotor activity. Certainly a phase change in the cycle of a complex circadian output, such as locomotion, must represent the consequence of phase changes in multiple cellular oscillators. For the purpose of this review, however, we define entrainment in a more restricted manner—specifically as a phase change in the rhythmic expression of circadian genes (i.e., genes that are direct transcriptional targets of the CCO itself).

A defining feature of entrainment is that the circadian phase change must outlast the entraining stimulus. Therefore, entrainment involves permanently changing the intrinsic phase of the clock. More specifically, entrainment as defined herein must alter the activity cycle of the CLOCK:BMAL1 transcription factor. Such an effect might be achieved by any of a number of different mechanisms, but they all must coalesce at this transcription factor. A corollary of

this argument is that any stimulus or treatment that affects the periodicity of activity of the CLOCK:BMAL1 heterodimer will execute entrainment.

Entrainment of Multiple Independent Oscillators

The realization that clocks are ticking all over the body and that they respond variably to different environmental or physiological cues raises three fundamental questions. Exactly what constitutes entrainment at a cellular level? Are the mechanisms of entrainment different for different cell types? Finally, is there a unifying theme for entrainment, or has nature independently evolved a wide variety of mechanisms for the entrainment of the CCO in different tissues and cell types? Some insight regarding these questions is gained by examining available data regarding entrainment in a few of the best-characterized sites of circadian gene expression.

SUPRACHIASMATIC NUCLEUS The suprachiasmatic nucleus (SCN) is composed of a pair of bilaterally symmetric structures located in the anterior hypothalamus, immediately dorsal to the optic chiasm (56, 57). This structure exhibits a very robust circadian rhythm and plays a critical role in the maintenance of peripheral oscillators and the execution of rhythmic behavior (58–60). Neural connections from the retina to the SCN, known as the retino-hypothalamic tract, mediate a close coupling between the SCN and the light/dark cycle as perceived by photopigments in the eye (61). Through this close functional connection with the retina, SCN gene expression responds rapidly to light, entraining to a shifted light/dark schedule within one day (51). This fast molecular response to light in the SCN is thought to underlie the fairly rapid (two – five days) adaptation of behavioral rhythms to a shift in the light/dark schedule. Interestingly, while various nonphotic stimuli, including a forced feeding schedule alteration or forced wheel running, can dominate light in entraining behavioral and peripheral rhythms, the SCN is hard-wired to the light/dark rhythm. Indeed, the rhythm of gene expression in the SCN is not affected by restricted feeding (RF) even when the light/dark cycle is removed as a zeitgeber (zeitgeber = "time keeper" or entraining stimulus) (50, 51, 62).

The mechanism of photic entrainment in the SCN is poorly understood. The products of the *Per1* and *Per2* genes were inferred to play a direct role in the SCN clock due to the observation that mice harboring null alleles of these genes display abnormal circadian rhythm (36). Indeed, *Per1*/*Per2* double mutant mice display no overt circadian rhythm at the molecular or behavioral level (36). A clue to the function of the *Per* genes, and potentially to the mechanism of SCN entrainment, came from experiments designed to analyze the response of the *Per* genes in the SCN to light. Correlating with their necessity for response to light (63, 64), the *Per1* and *Per2* genes are induced transcriptionally in the SCN by light perceived by the retina (65, 66). Importantly, the effect of light on *Per1* expression in the SCN correlates well with its effect on behavioral rhythms (67).

As mentioned previously, the transcription factor responsible for the circadian pattern of *Per1* and *Per2* gene expression is the CLOCK:BMAL1 heterodimer. The identity of the transcription factors that mediate photic induction of *Per1* and *Per2* in the SCN is not completely clear, although some evidence suggests that the cAMP response element binding protein (CREB) transcription factor may fulfill this role (68, 69).

The issue of nonphotic entrainment in the SCN has not been satisfactorily addressed. As mentioned previously, administration of a restricted feeding schedule changes behavioral and peripheral oscillators but does not affect the CCO rhythm within neurons of the SCN (50, 51, 62). One informative study by Menaker and colleagues (70) demonstrated a clear inverse correlation between multi-unit neural activity (MUA) in the SCN and locomotor activity. Nightly wheel running causes a marked increase in neural activity in the majority of the brain but causes a clear decrease in MUA activity in the SCN. Transcriptional activity of the CCO in the SCN correlates with neural activity as forced wheel running causes an SCN-specific decrease in *Per* gene expression (71). The alteration of circadian rhythms elicited by forced wheel running might be caused by neural activity changes in the brain oscillator controlling locomotor activity. We return later to the potential relationship between neural activity and entrainment.

LIVER Unlike the SCN, the liver CCO does not rapidly entrain to changes in the light/dark schedule. Even after 16 days of an altered light/dark regimen, the liver does not completely adapt to the new schedule (51). Light, therefore, exerts only a weak entraining influence on the liver, which is probably indirect via changes in activity or feeding schedule. Food, on the other hand, is a very potent zeitgeber for the liver circadian cycle. One paradigm to examine the effect of food on circadian rhythm is a restricted feeding (RF) protocol wherein mice are allowed food for a limited period during the light phase of a light/dark cycle. Being nocturnal, mice prefer to eat during the dark phase and will do so almost exclusively even if food is provided ad libitum. Two recent studies (50, 62) have shown that RF almost immediately resets the phase of circadian gene expression in the liver. Of the six tissues examined in these studies, the liver CCO is the fastest to entrain to an RF schedule, suggesting that it is more directly coupled to food intake than other peripheral or central oscillators. Importantly, the phase change induced by RF in the liver constitutes bona fide entrainment, as it is maintained after removal of the stimulus by fasting (50).

OTHER PERIPHERAL ORGANS These same two studies (50, 62) also examined the effect of RF on various other peripheral organs, including kidney, heart, pancreas, skeletal muscle, and lung. Each of these tissues maintains a functional CCO that drives the rhythmic expression of circadian genes, such as Per and Cry, and each oscillatory rhythm is phase shifted in response to the imposed RF regimen. Unlike with liver, however, the phase resetting of these other tissues is

not immediate but instead occurs over a 5–10 day period. A shift in the light/dark cycle also causes phase shifting in peripheral oscillators with a delay similar to RF (51). Interestingly, the kinetics of the molecular response of these peripheral tissues to RF or light shift correlates precisely with the kinetics of locomotor activity shifting induced by either treatment (50, 51).

A related observation is that the *Per* expression rhythm in the lung is phase shifted in an RF regimen in which the food is available for 4 h each day but not in an 8-h RF regimen. This differential CCO response mimics the locomotor activity response to the two RF regimens; 4-h RF causes a phase shift in locomotor activity whereas 8-h RF does not. The authors suggest that the direct zeitgeber in these tissues is neither light nor food, but instead the alteration in locomotor activity (50). In other words, an organ such as the lung changes its CCO to match the time of its optimal activity—the time of maximal physical exertion. This synchrony of CCO and organ activity emerges as a simple and defining paradigm that may be instructive to the core underpinnings of entrainment. It appears, more specifically, that the apex of CCO activity always matches the apex of organ activity.

EXTRA-SCN REGIONS OF THE BRAIN The SCN exhibits a high-amplitude circadian oscillation in neural activity with a peak during the light phase. In rodents, the majority of other brain areas also exhibit a circadian fluctuation in MUA, but peak activity occurs during the dark phase, corresponding to the peak of locomotor activity (72). Indeed, behaviors such as wheel running depress SCN activity while increasing neural activity in other brain areas (70). The phase of MUA rhythms correlates with the phase of CCO-dependent gene expression in both the SCN and other brain areas. *Per1* and *Per2* gene expression is elevated in the SCN during daylight. By contrast, *Per* gene expression peaks nocturnally in the cerebral cortex, hippocampus, and other brain areas (73). Moreover, RF shifts the rhythm of *Per* gene expression in these extra-SCN brain nuclei by roughly 12 h. As with peripheral tissues, the kinetics of CCO entrainment in the cortex and hippocampus strongly correlate with the kinetics of alteration in wheel running rhythms.

In most tissues, the positive component of the CCO feedback loop consists of the CLOCK:BMAL1 heterodimer. In large areas of the forebrain, including the somatosensory cortex, the secondary motor cortex, the entorhinal cortex, parts of the hippocampus, caudate putamen, and accumbens nucleus, however, the CLOCK paralog NPAS2 seems to predominate (74). Temporal analysis of mouse forebrain mRNA indicates that *Cry* and *Per* gene expression exhibit robust circadian oscillations in the forebrain. Relative to wild-type mice, homozygous *NPAS2* mutant mice show a markedly dampened oscillation of *Per2* expression in brain areas where *NPAS2* would normally be expressed (17). Because conditional expression of NPAS2 and BMAL1 can stimulate *Per* and *Cry* gene expression in tissue culture models, these data suggest that NPAS2 serves as a critical CCO component in the forebrain.

As in the case of cells kept in culture (55), many treatments other than food and light cause a phase shift in locomotor activity rhythms. These include restricted exercise (75, 76), social interaction (77), and exposure to certain odors [(77); reviewed in (78)]. Perhaps the most fundamental observation, however, is that sleep deprivation can rapidly reset behavioral rhythms (79). The variety of stimuli that shift behavioral rhythms, including simple arousal, suggests that merely being awake and processing sensory information is sufficient to impinge upon the oscillator controlling rhythmic behavior. This clock must be composed of some constellation of neuronal oscillators whose cellular rhythms are entrained by exposure to sensory stimuli. As with photic entrainment in the SCN, it is possible that neuronal activity itself may lie at the heart of nonphotic entrainment of extra-SCN oscillators (see below).

Coupling of Independent Oscillators

A variety of observations identified the SCN as playing a special role in the organization of circadian locomotor activity and led to its designation as the master pacemaker (56, 57). Perhaps the most informative experiment to this end was conducted by Menaker and colleagues utilizing the *tau* mutant hamster. The *tau* hamster exhibits a short period of 20 h as assessed by locomotor rhythms. Using *tau* and wild-type hamsters, SCN tissue of one genotype was transplanted into hamsters of the other genotype whose SCN had been ablated (59). Regardless of the direction of the transplant, the phenotype of the donor SCN dictated the locomotor activity cycle of the recipient animal. It was later demonstrated that this effect on the recipient hamster is attributable to a diffusible signal and independent of direct cell-cell contact (80). As an aside, SCN explanted cells in culture are also able to entrain otherwise noncycling cells via a diffusible signal (81).

A molecule that might meet the criteria for a periphery-entraining signal is retinoic acid. Recent work has identified the retinoic acid receptors, RXR and RAR, as NPAS2 and CLOCK interacting proteins (20). Importantly, this interaction is potentiated up to 15-fold by retinoic acid and is dependent on the region of the receptor, the AF2 domain, that is required for other ligand-dependent interactions (82). Retinoid receptors also affect NPAS2 and CLOCK functionally. They inhibit DNA binding in cell-free assays and inhibit NPAS2- and CLOCK-mediated transactivation in cell-transfection assays. Finally, timed injection of mice with RAR ligand (all-*trans* retinoic acid) causes subtle phase shifts in the heart and aorta (20). The RXR/RAR -CLOCK/BMAL1 interaction brings to mind an underappreciated relationship between these disparate classes of transcription factors. Many of the nuclear hormone receptor coactivators are members of the bHLH-PAS family of transcription factors, including SRC1 and TIF2. Indeed, the interaction between RXR and NPAS2 is dependent on a LXXLL sequence in the C-terminal half of NPAS2. The same LXXLL motif is found in nuclear

hormone receptor coactivators and is responsible for mediating their interaction with nuclear receptors, typically facilitating transcriptional activation (83).

Adrenocorticotropic hormone (ACTH), corticotropin-releasing hormone (CRH), and glucocorticoid output, although not necessarily regulated by diffusible factors from the SCN, exhibit a strong circadian pattern. Furthermore, the synthetic glucocorticoid, dexamethasone, can induce circadian gene expression in cultured fibroblasts. Dexamethasone also produces a phase shift response in peripheral tissues, including liver, kidney, and heart, in intact animals (54). This phase shift of peripheral CCOs by synthetic glucocorticoids depends on a functional glucocorticoid receptor, which is not present in the SCN. Thus, glucocorticoids represent a second signal whose ultimate production may be under the control of the SCN and which serves as a means of synchronizing the CCOs of peripheral tissues (54).

In addition to being sufficient for generation and control of circadian locomotor activity, the SCN is also necessary. Ablation of the SCN causes a complete loss of behavioral rhythms under dark/dark conditions [see (80)] as well as disorganization of all peripheral oscillator rhythms (58). A third observation implicating the supremacy of the SCN is that rhythms of SCN-derived tissue are much more stable in culture than those from other sources. Whereas peripheral tissues lose all detectable CCO-regulated rhythmicity within 2 to 7 days following explantation, the SCN can maintain a robust rhythm for as long as 32 days in culture (51).

Despite the critical role of the SCN, the simple model of a hierarchical organization wherein the SCN is the master pacemaker enforcing a cycle upon slave oscillators may be inaccurate. The challenge to this appealing and parsimonious model derives from experiments showing that the RF phase shifts both locomotor activity rhythms and the CCO rhythm of peripheral tissues without affecting the CCO of the SCN (50, 62). Moreover, SCN-ablated animals can still be entrained by RF (73) or by activity-inducing treatments such as amphetamine injection (84, 85). Circadian rhythms of peripheral gene expression and behavior can, therefore, be completely uncoupled from the SCN. How does one rationalize the apparent dominance of the SCN in certain paradigms and the irrelevance of the SCN in others? Invoking an intermediary between the SCN and peripheral and behavioral rhythms might answer the question. Perhaps the primary function of the SCN is not to directly coordinate rhythms throughout the body but to control the cycle of feeding behavior. Rhythmic food intake and the resultant impact on locomotor activity could then exert an entraining influence more broadly. As described earlier, existing evidence suggests that oscillators outside the SCN are maintained in phase with the activity and food-intake cycle of the animal. If the SCN rhythm and feeding rhythm are forced out of synchrony, the SCN influence is superseded to allow the animal to avoid starvation.

METABOLISM AND ENTRAINMENT

Ample experimental evidence has long favored a link between metabolism and circadian rhythms. It is well documented that the circadian clock controls the levels of many cellular and circulating metabolites and fuels. Substantial data indicate, however, that the coupling is more complex than the rhythm simply controlling metabolism. Numerous studies have pointed to a cyclic relationship wherein the rhythm impacts metabolic activity and metabolism feeds back to impinge upon the rhythm (86). The acceptance of a causal effect of metabolism on circadian rhythms could unveil a unification of a disparate literature of data regarding entrainment. The validity of the hypothesis that metabolism directly affects the CCO is best evaluated by considering its ramifications in the context of what is known about the entrainment of various oscillators.

Metabolism and Entrainment in the Periphery

The recent identification of a tissue culture model capable of recapitulating circadian rhythmicity (47) has allowed the study of various treatments in a uniform and tractable experimental system. Surprisingly, the CCO rhythm of these cells is altered by treatment with a diverse array of pharmacologic agents, including phorbol-12-myristate-13-acetate (PMA), forskolin, and calcimycin. These drugs cause, respectively, PKC activation, adenylate cyclase activation, and calcium release. In spite of the different pathways activated, these treatments all induce expression of the *Per* and *Cry* genes and synchronize a reasonably stable circadian oscillation (55). Could it be that a unifying component of all these diverse stimulatory effects is the activation of cellular metabolism? If cellular metabolism is able to directly impinge upon the CCO, this could account for the ability of diverse stimuli to entrain circadian rhythms via a common mechanism.

Alternative sites of entrainment in which to evaluate the hypothesis are peripheral oscillators operating in heart, lung, muscle, and other organs. As described above, each of these tissues exhibits a circadian pattern of gene expression controlled by the CCO, and the phase of these rhythms is shifted by treatments that alter locomotor activity rhythms (50, 62). These tissues are integral to the energy and force production that enable locomotor activity, and ample evidence has demonstrated a tight coupling between locomotor activity and the CCO. Cellular metabolism in these tissues must be impacted by vigorous locomotor activity such as wheel running and could underlie the coordination between activity and CCO rhythms in these tissues.

Perhaps the best test case to evaluate the link between metabolism and circadian rhythms is the liver. A large number of genes in the liver are under control of the CCO, including the genes encoding many metabolic enzymes. These include cytochrome P450s (87–89) as well as enzymes involved in heme biosynthesis and mitochondrial function (36). To complete the cycle of influence, there is also a clear effect of RF on circadian phase in the liver (50, 62).

Obviously, the liver is critically involved in the primary food response. Cellular metabolism in the liver is markedly affected by changes in feeding status and therefore fluctuates as a function of the day/night cycle in rodents (90, 91). We speculate that the connection between metabolism and circadian rhythms in the liver is not merely a coincidence but instead is indicative of a mechanistic link between the two.

Metabolism and Entrainment in the Brain

Any potential connection between metabolism and the brain CCO is less obvious than the connection of metabolism with the liver CCO. Global blood glucose supply to the brain is homeostatic and does not fluctuate substantially as a function of feeding state. There are, however, a number of lines of evidence that point to a functional link between metabolism and circadian rhythms in the brain. First, the expression of certain genes encoding metabolic enzymes and transport systems for energy metabolites is under circadian control. These include glycogen phosphorylase (92, 93), cytochrome oxidase (94), lactate dehydrogenase (19), and the monocarboxylate transporter MCT2 (P. J. Magistretti, personal communication). Second, glucose uptake in the SCN and other brain areas fluctuates as a function of the light/dark cycle (95–97). Finally, the concentration of the primary cellular metabolic currency, ATP, exhibits marked fluctuation as a function of the light/dark cycle in the SCN and in numerous other brain nuclei [(98); see also (99)]. As with neural activity, the rhythmic fluctuation in intracellular ATP concentration is shifted roughly twelve hours in the SCN relative to the other brain areas assayed.

The correlation of metabolic activity and circadian rhythm in the brain raises the possibility of a functional link between the two. The remaining question is how an entraining stimulus perceived elsewhere is translated into altered metabolic activity in neurons of the brain. Clues pertinent to this question have arisen from analysis of neural activity as a function of the light/dark cycle (70). As mentioned earlier, the SCN of rodents exhibits high-amplitude neural activity oscillations with a peak during the light phase. Most other brain areas also exhibit cyclic patterns of activity yet peak during the dark phase. Yamazaki and colleagues further reported a differential effect of wheel running on neural activity in separate brain regions. Locomotor activity stimulates neural activity in the majority of the brain while depressing electrical activity in the SCN (70). In summary, the retina-responsive SCN has higher neural activity during the light phase, whereas the remainder of the brain, which responds to other stimuli including smell, touch, sound, and emotion, is electrically active during the active locomotion phase of animal behavior. A relationship between neural activity rhythms and metabolic activity rhythms might provide a link between stimuli such as light, food, running, or other sensory stimuli and entrainment of circadian oscillators in the brain.

Physiologically, the relationship between metabolic activity and neural activity has been well characterized by various methods of functional brain imaging.

Correlations between neural activity and changes in cerebral glucose utilization were first shown by autoradiographic detection of 2-deoxy-D-[^{14}C]glucose (2-DG) uptake (100). Following the discovery that the SCN exhibits strong circadian variation in 2-DG uptake, autoradiographic measurement of 2-DG uptake was used as a marker of rhythmic circadian neuronal and metabolic activity (95). For higher temporal resolution, positron emission tomography (PET) has been used to detect changes in blood flow, oxygen consumption, and glucose uptake in response to neuronal activity (101, 102), and changes in intermediate metabolites such as lactate or glucose have been measured by magnetic resonance spectroscopy [(103); reviewed in (104)].

Whereas coupling of neural activity with metabolic activity does not require changes in cerebral blood flow (CBF) (105, 106), they usually go hand in hand with changes in neural activity and glucose utilization. Of the many signals that have been implicated in the control of CBF, at least three vasoactive agents, arginine vasopressin, vasoactive intestinal peptide (VIP), and nitric oxide (NO), exhibit circadian variation in or around the SCN (107). It is notable that VIP can directly affect the phase and the period length of circadian oscillations within the SCN by signaling through the VIP receptor VPAC2 (108, 109). NO may prove to be of particular interest for several reasons. First, NO is thought to account for most of the circadian variation and regulation observed in CBF (110). Second, NO is a vasodilator that induces increased flow in capillary beds (110). Third, both NO and nitric oxide synthase (NOS) activity exhibit robust circadian fluctuations around in the SCN, the pineal gland, as well as in cortical areas of the brain (111–113). Finally, NO production depends on the availability of reduced NAD cofactors, which in turn depends on the availability of glucose and oxygen (114).

As discussed below, the ratio of reduced to oxidized NAD cofactors may play a central role in the regulation of the CCO. Therefore, output of vasoactive substances (circadian, or in response to neural activity) that cause changes in blood flow, as well as concomitant changes in glucose and oxygen availability, may feed back onto the CCO by affecting intracellular redox state.

At the cellular level, coupling between neuronal activity and energy substrate uptake and utilization is regulated by astrocytes. Astrocytes are ideally suited for this function because astrocytic endfeet cover the endothelial bed, whereas other astrocytic processes are located in close proximity to the synaptic cleft (115). It has been demonstrated that the duration of glutamate action on postsynaptic neurons is limited by active removal of glutamate by specific transporters present on astrocytic processes (116). Based on this observation, as well as their findings that glutamate stimulates glycolysis in glial cells in a Na^+-dependent manner, a model developed by Magistretti & Pellerin (104) delineates how neural activity induces glucose uptake and glycolysis in astrocytes (Figure 1). According to this model, neural activity and concomitant synaptic transmission involve the release of excitatory neurotransmitters, such as glutamate. Glutamate is then actively absorbed by astrocytes via specific glutamate transporters by cotransport with

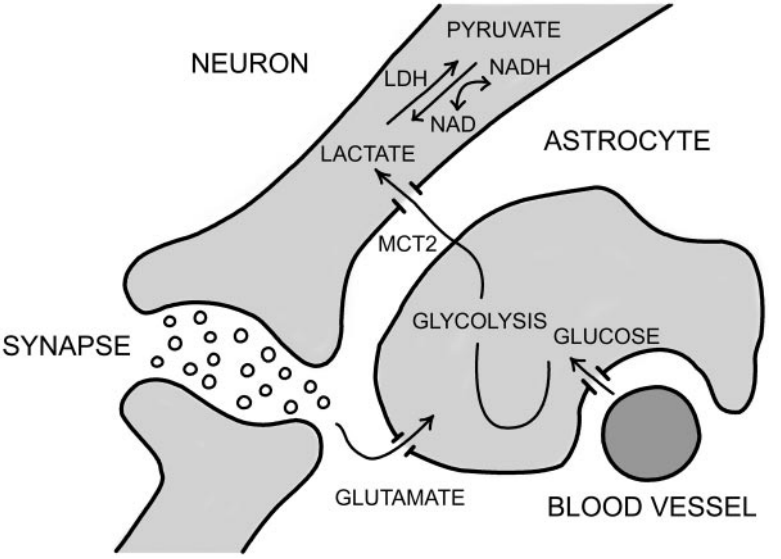

Figure 1 Model for electrical activity-induced change in neuronal redox status. An increase in synaptic glutamate stimulates glycolytic flux and lactate export in astrocytes. The monocarboxylate transporter, MCT2, facilitates neuronal uptake of lactate, which increases the NADH:NAD ratio via the lactate dehydrogenase (LDH) reaction. Adapted from (104).

Na^+. The resulting intracellular increase in Na^+ concentrations activates the astrocytic Na^+,K^+ -ATPase, which in turn activates glucose uptake and glycolysis (117, 118). Lactate, the product of glycolysis, is not metabolized by astrocytes but is shuttled to neurons through two monocarboxylate transporters, MCT1 and MCT2 (119). Both the cell-type specific distribution of LDH-isoforms (120) and the specific localization of the lactate transporters MCT1 and MCT2 (119) on astrocytes and neurons, respectively, support this model. Interestingly, the mRNA transcript of a fly MCT-like gene is rhythmically expressed in the head, which could be indicative of a more intimate connection between rhythms and lactate transport (120a). In the neuron, lactate then serves not only to meet the metabolic requirements resulting from excitatory activity but also to elevate the ratios of reduced to oxidized forms of NAD cofactors.

Thus, mechanisms exist by which signals originating from active neurons induce increased blood flow, increased glucose uptake by astrocytes, and ultimately increased availability of energy metabolites in the neuron itself. Many aspects of the metabolic coupling between neuronal activity and energy substrate availability exhibit circadian variation. The question then arises whether changes in energy availability and utilization induced by neuronal activity feed back on to the CCO directly or whether they merely represent a homeostatic mechanism

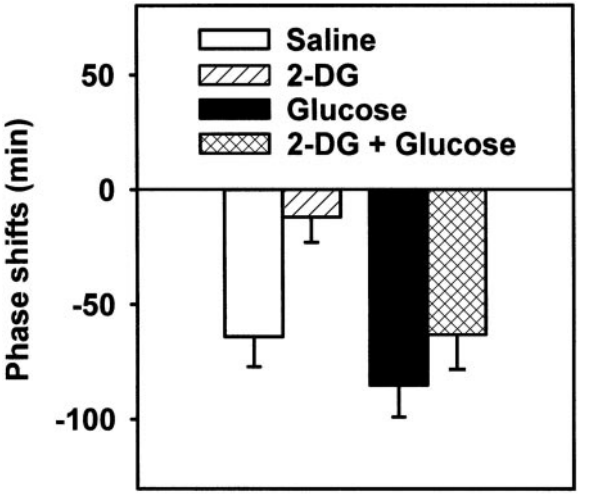

Figure 2 Phase shifts in circadian activity rhythms of mice housed in constant darkness after intraperitoneal injections of saline, 500mg/kg 2-DG, 2g/kg D-glucose, or a mixture of 500mg/kg 2-DG and 550mg/kg D-glucose at CT17.5 followed by a light pulse 30 min later. Negative values are delays. Values are means ± SE; $n = 6$ mice per group. Reprinted with permission from (121). Copyright 1999 American Physiological Society.

that happens to exhibit some circadian component. Two observations suggest that availability of energy metabolites may indeed play a significant role in the regulation of circadian rhythms. First, light-induced phase shifts can be blocked in rats by injection of 2-DG, which inhibits glucose utilization (121, 122) (Figure 2). Second, calorie-restricted, periodic feeding can entrain the rhythm of peripheral oscillators and modify the phase angle of photic entrainment (50, 62, 122).

Mechanisms of Activity-Induced Entrainment

Given correlative evidence that cellular metabolism might impinge upon the CCO, it is useful to consider what types of metabolic parameters could be read by the clock (86). Essentially any parameter that reliably changes as a function of metabolic activity could potentially qualify as a candidate for the direct entrainment of the CCO. One candidate, cellular redox status, has recently been proposed (19). In vitro experiments have shown that the reduced nicotinamide adenine dinucleotide cofactors, NADH and NADPH, stimulate the DNA binding activity of both CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers. By contrast, the oxidized forms of both cofactors, NAD+ and NADP+, were observed to inhibit the activating transcription factor of the CCO. The result is a dimeric transcription factor with a redox-response curve bearing a Hill coefficient of 15,

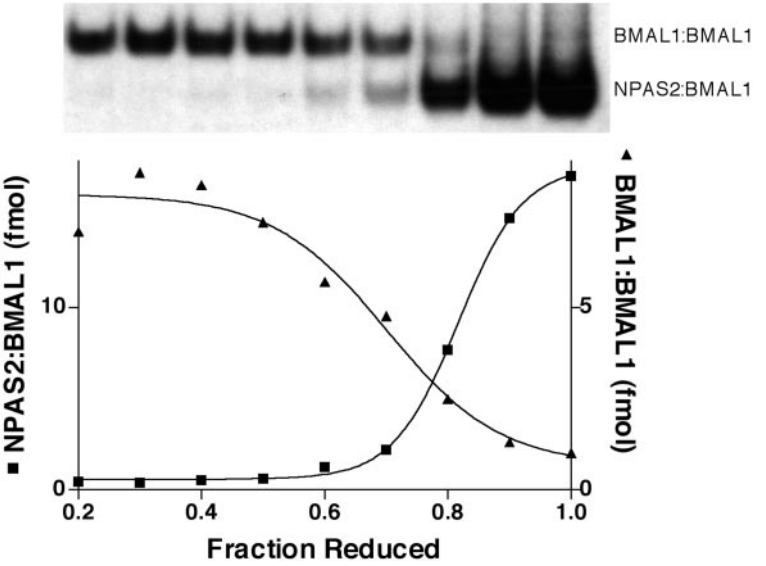

Figure 3 The NPAS2:BMAL1 heterodimer is exquisitely responsive to NADP(H) redox state. NPAS2 and BMAL1 proteins were subjected to electrophoretic mobility shift assays in the presence of varied ratios of NADP and NADPH. The total NADP(H) concentration was held constant at 5mM with the mixture composed of a 20% NADPH:80% NADP molar ratio on the far left to 100% NADPH on the far right and varying in 10% increments for intervening data points as indicated on the *x* axis. Reprinted with permission from (19). Copyright 2001 American Association for the Advancement of Science.

indicative of near switch-like behavior. Specifically, a small change in the ratio of reduced to oxidized cofactor (from 60:40 to 80:20) causes a transition from almost undetectable to near maximal DNA binding activity (Figure 3). Subtle changes in cellular metabolic state could thereby substantially alter the activity of CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers, which represent the key transcriptional activators of the CCO. As articulated earlier in the review, if entrainment can be simply defined as a stable change in the activity cycle of CLOCK:BMAL1 or NPAS2:BMAL1, then alterations in cellular redox state could qualify as a bona fide entraining stimulus (Figure 4).

It is important to note that among the many metabolic substrates that exhibit circadian oscillation in the liver, the NADP+:NADPH ratio exhibits one of the highest amplitudes of fluctuation (91). It is likely that similar oscillations occur in the brain given that *Ldh* expression, glucose uptake, and ATP content all fluctuate as a function of circadian rhythm. The causal link between in vivo metabolic and redox state and circadian rhythm, however, remains to be established. Preliminary evidence suggests that the CCO in tissue culture systems can

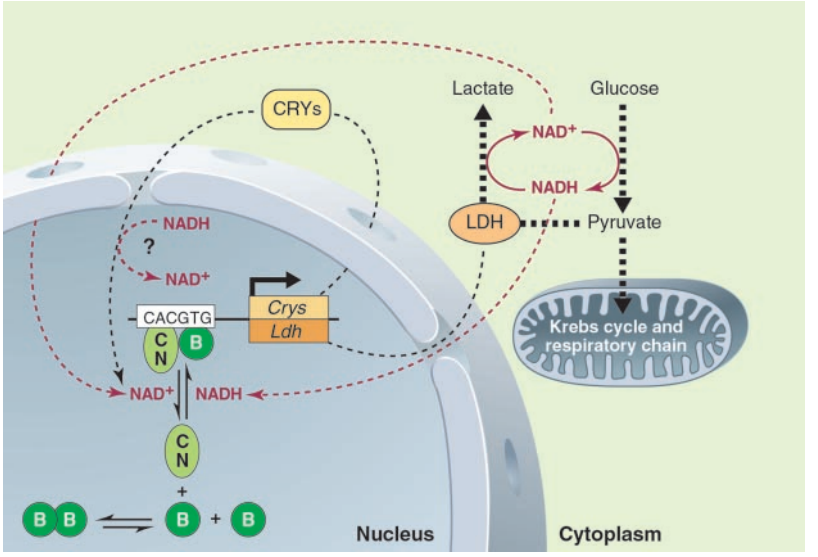

Figure 4 Metabolic states and circadian oscillators. The mammalian circadian feedback loop in gene expression is established by the BMAL1 (B) and CLOCK (C) transcriptional activator proteins and by the CRY transcriptional repressor proteins. In some brain regions, NPAS2 (N) substitutes for CLOCK. The formation of the CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers and their binding to DNA is stimulated by reduced NADH and inhibited by oxidized NAD+. These heterodimers enhance the expression of the clock genes *Cry* and *Per* (not shown) and the clock output gene *Ldh*. CRY proteins repress CLOCK/NPAS2-mediated gene activation, possibly by oxidizing the NAD+ cofactors associated with these proteins. Conceivably, the negative action of CRY proteins on CLOCK/NPAS2 could be reinforced by lactate dehydrogenase (LDH), which may increase the cellular concentration of NAD+. Reproduced with permission from (124). Copyright 2001 American Association for the Advancement of Science

respond to metabolic manipulation, possibly via changes in cellular redox. Direct application of lactate to the medium of cultured neuroblastoma cells was shown to activate NPAS2:BMAL1-dependent gene expression (19). A primary effect of lactate, via lactate dehydrogenase, should be to decrease the ratio of NAD+:NADH, which is coupled to NADP+:NADPH ratios through nicotinamide nucleotide transhydrogenases (123).

Genome-wide expression analysis in flies has yielded another potential link between circadian rhythm and nicotinamide nucleotides. Young and colleagues have identified the *Zw* gene as one of many that exhibit circadian variation in transcript levels in fly heads (120a). *Zw* encodes glucose-6-phosphate 1-dehydrogenase, which catalyzes the rate-limiting step in the pentose-phosphate pathway. This pathway is the major producer of reduced nicotinamide nucleo-

tides, potential positive regulators of CLOCK:BMAL1. Rhythmic *Zw* expression should lead to rhythmic levels of reduced nicotinamide nucleotides, with peak *Zw* activity being coincident with the peak of reduced nucleotide. The cycle of *Zw* expression, exhibiting a peak at ZT11, slightly precedes the cycle of expression of bona fide CLOCK:BMAL1 target genes such as *per* and *tim* (peak at ZT14-ZT16). This temporal relationship is perfectly organized to suggest a role for *Zw* in activating the expression of *per* and *tim* through an indirect positive effect on CLOCK:BMAL1. It is also notable that the CG10611 gene encoding an enzyme with the opposite metabolic effect, fructose bisphosphatase, is also rhythmically expressed, but with an opposing phase (peak at ZT 0) (120a).

The identification of the redox cofactors, NAD(H) and NADP(H), as potential regulators of the CCO offers a speculative model for the mechanism of CRY-mediated inhibition of the CLOCK:BMAL1 and NPAS2:BMAL1 heterodimers. FAD is critical to the electronics of DNA repair by DNA photolyase and is likely to be involved in the photosensing function of the CRY protein encoded by *Drosophila melanogaster*. A potential mechanism for CRY function in mammals could involve CRY-bound FAD oxidizing CLOCK/NPAS2-bound NADPH, thus replacing the activating effect of the reduced nicotinamide cofactor with the inhibiting function of the oxidized cofactor (124). The discovery that NPAS2 (and presumably CLOCK) binds heme via its PAS domains adds another potential layer to this model (J. Rutter, S. L. McKnight, unpublished observations). This observation potentially embellishes the hypothesis that the biochemistry of CLOCK:BMAL1 and NPAS2:BMAL1 regulation involves the regulated movement of electrons. Notably, the Nernst potentials of the involved cofactors are properly aligned for a NAD(P)H $\rightarrow$ FAD $\rightarrow$ heme electron transport chain (Figure 5). This redox chain could provide a mechanism for CRY to effect reasonably stable inhibition of the key activating transcription factors of the CCO. The functional relevance of this hypothetical electron transport chain remains to be established. An alternative function for PAS domain-associated heme might be as a sensory regulator of NPAS2/CLOCK activity. PAS-bound heme could interact with a gas, resulting in either a positive or negative effect on NPAS2/CLOCK activity. The relevant gas could, for example, catalyze exchange between an intramolecular PAS-PAS interaction and an intermolecular PAS-PAS interaction, thereby altering the levels of the NPAS2:BMAL1 or CLOCK:BMAL1 heterodimer (Figure 5).

The ability of many seemingly unconnected stimuli to entrain circadian rhythm was addressed previously. It is either the case that many disparate pathways have evolved independent access to the CCO or that a common mechanism links this penetrance. We offer the speculative model wherein intracellular redox state serves as the common mediator. In this model, redox acts to directly entrain the CCO by regulating the DNA binding activity of the CLOCK:BMAL1 and NPAS:BMAL1 heterodimers.

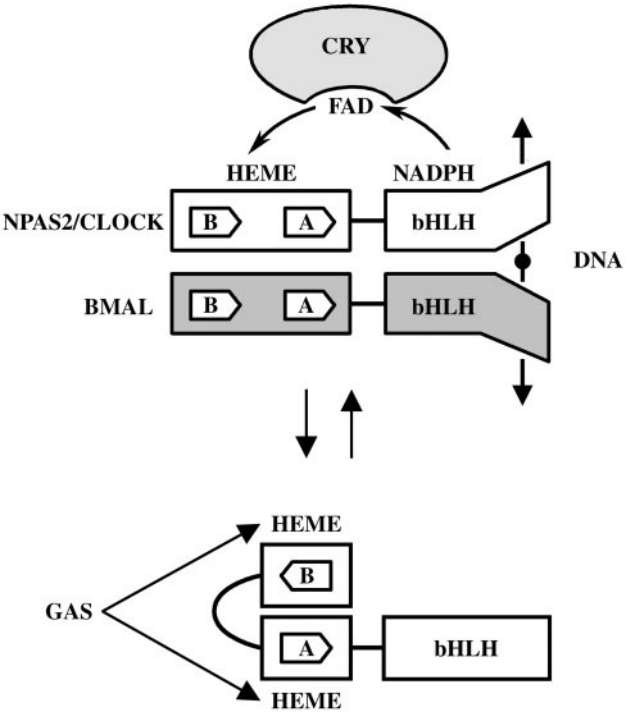

Figure 5 Two potential roles for heme in regulating the activity of NPAS2/CLOCK. (*Top*) Model for the inhibitory effect of CRY on CLOCK:BMAL1 or NPAS2:BMAL1 activity. An electron is transferred from bHLH-bound NADPH to PAS domain-bound heme. CRY, by virtue of its FAD cofactor, mediates this pair of redox reactions and thereby inhibits CLOCK:BMAL1 or NPAS2:BMAL1 DNA-binding activity. (*Bottom*) The PAS domains of NPAS2 or CLOCK bind gas via an associated heme and undergo a conformational change. This conformational change causes an alteration in the activity of NPAS2/CLOCK, potentially by changing PAS domain dimerization state.

THE DEAD ZONE AND PHASE RESPONSE CURVES If redox flux is indeed capable of direct biochemical regulation of the CCO, what rules and properties of the phenomenon of entrainment must it obey? It is well established that light-induced shifts in the phase angle of rhythmic locomotor activity are not uniform throughout the circadian cycle. In rats, light pulses have almost no effect early in their subjective day, between circadian time (CT) 4 and CT8. This dead zone is followed by a 12-h period during which light pulses cause ever-increasing phase delays, up to a maximum delay (2+ h) at CT14 (125–127). Subsequently, light-induced phase delays decrease until the cross-over point at CT20. Between CT20 and CT4, light pulses cause phase advances with maximal effects around CT22-CT24. A similar phase response curve (PRC) has been observed for mice,

albeit shifted by about 2–4 h (128). These light-induced changes in the phase angle of rhythmic locomotor activity correlate relatively well with the response curves of the photic induction of *Per* gene expression (127). As such the rules that govern the phenomenon of entrainment would appear also to govern regulation of the CCO itself.

What do we know about the molecular and biochemical status of the CCO during the dead zone (CT4-CT8)? In situ hybridization experiments carried out on rodent brain sections indicate that this period of time corresponds to the forward edge of the apex of *Per* and *Cry* gene expression in the SCN (127, 129). It is therefore reasonable to assume that this 4-h interval also corresponds to the point of maximal activity for the CLOCK:BMAL1 component of the CCO. Conversely, this interval should represent a nadir for the presence of negatively acting components of the CCO (functional CRY and PER proteins). Finally, we speculate that intracellular redox balance should be tipped in favor of the reduced state of NAD cofactors during this interval. We offer the latter interpretation based upon studies of NADP(H) redox flux in the rodent liver (90, 91). Such studies have clearly shown that the NADP:NADPH ratio is lowest (most reduced) concomitant with maximal activity of the CCO, and highest (most oxidized) when *Per* and *Cry* gene expression levels are at their lowest (50, 62, 91). If these assumptions are tenable, the CLOCK:BMAL1 activity component of the CCO should be fully de-repressed during the CT4-CT8 dead zone, both by virtue of the absence of negative regulators and by the presence of a favorable redox state. Under such conditions we propose that the CCO would be immune to redox-mediated stimulation (entrainment). Although neuronal activity via the retino:SCN pathway is expected to enhance metabolism as depicted in Figure 1, any enrichment of the reduced state of NAD cofactors would take place well to the right side of the CLOCK:BMAL1 induction switch (Figure 3). Given the steepness of this induction curve, we argue that any further enrichment of reduced NAD cofactors would have no effect upon a fully induced system, thus giving rise to a dead zone during which the system should not respond to entraining stimuli.

If correct, a redox-based interpretation of entrainment would also predict that at other times, before or after this dead zone, ratios of oxidized to reduced NAD cofactors should be higher and closer to the crossover point between the induction and inhibition curves governing CLOCK:BMAL1 and NPAS2: BMAL1 DNA binding activity. During such times, when the proportion of reduced NAD cofactors is lower and DNA binding activity diminished, changes in redox ratios resulting from photic stimuli and concomitant neuronal activity might be expected to shift the cellular redox balance toward the reduced state (by means of coupling of neuronal activity and metabolism as discussed previously). As a result, CLOCK:BMAL1 DNA-binding activity and transactivation should be enhanced, resulting in a phase shift.

This simplistic model further predicts that inhibitors of glucose uptake and utilization should interfere with light-induced phase changes of circadian rhythms.

Experimental evidence supportive of this interpretation was recently published. Mice injected with 2-DG, a nonmetabolizable glucose analog, exhibit a significantly reduced phase response to photic stimuli in comparison to mice injected only with saline. Simultaneous injection of glucose and 2-DG reverses the inhibition by 2-DG (121, 122) (Figure 2). Conversely, conditions that increase the proportion of reduced NAD cofactors, at times when cellular redox is tilted toward the oxidized state, should enhance CLOCK:BMAL1 DNA binding activity and induce the transcription of genes regulated by the CCO. Experiments using lactate to increase the proportion of reduced NAD cofactor support this hypothesis. When neuroblastoma cells stably transfected with a luciferase reporter driven by three optimal NPAS2:BMAL1 binding sites are infected with adenovirus constructs encoding NPAS2 and BMAL1, luciferase activity is increased less then twofold. However, when this cell line is simultaneously treated with lactate, luciferase activity is elevated sixfold (19). Lactate-induced increases of luciferase expression in this experimental paradigm depend on the expression of NPAS2 and BMAL1 and may therefore reflect an increase of NPAS2:BMAL1 DNA binding activity resulting from higher proportions of reduced NAD cofactor.

CONCLUSION

Forward genetic studies of model organisms have provided a compelling description of the regulatory apparatus controlling circadian rhythms. Now that we understand the molecular components of the central circadian oscillator (CCO), the challenge arises as to how it integrates at biochemical and physiological levels in complex animals. We have emphasized the correlation between the activity state of the CCO and cellular metabolic activity. Transcriptional output of cellular oscillators throughout the body seems to move in concert with cellular energy flux. As we hypothesized at the beginning of the review, the coupling of metabolic state and differential gene expression might be an ancient paradigm that underlies the daily physiological oscillation we observe today. If redox state represents a bona fide input regulator of the CCO, it is almost certainly the case that output genes controlled by the CCO will likewise regulate redox balance as well as the myriad of pathways that adapt cells, organs, and animals to the diurnal cycle. Might it be that when our brain or body tires at daily intervals, this state can be traced all the way back to a shortage of electrons on NAD cofactors, and when alert—a matched abundance?

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