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INTERACTIONS BETWEEN PHOTIC AND NONPHOTIC STIMULI TO SYNCHRONIZE THE MASTER CIRCADIAN CLOCK IN MAMMALS

Etienne Challet and Paul Pévet

Laboratory of Neurobiology of Rhythms, Centre National de la Recherche Scientifique UMR7518, University Louis Pasteur, Strasbourg, France

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1. ABSTRACT

The master circadian clock is located in the suprachiasmatic nuclei (SCN) in mammals. The most powerful synchronizer of the SCN clock is the daily variation in light intensity. Several other nonphotic cues are well known to be able to shift or synchronize the circadian clock in the absence of photic cues. Some results obtained at systems, cellular and molecular levels provide evidence in contrast to the view that nonphotic signals reset the SCN clock independently of the mechanisms of photic synchronization. Rather, the SCN appear to integrate a wide range of information from the environment to fine-tune photic synchronization. The neuronal mechanisms underlying this integration are far from being understood. Nevertheless, in real-life situations, multiple interactions between photic and nonphotic cues could be of importance for the daily phase adjustment of the circadian clock and its control of the 24-h temporal organization of the whole organism.

2. INTRODUCTION

Circadian (i.e., near 24 h) rhythmicity is a common feature in living organisms, from cyanobacteria to humans. This well-conserved function is considered to be critical because it generates an internal temporal organization of physiology and behavior, and allows the organisms to be in phase with daily and seasonal changes in their physical environment. In mammals, the suprachiasmatic nuclei (SCN) of the hypothalamus are the site of the master circadian clock (1-3). The major environmental synchronizer (zeitgeber) of the SCN clock is the daily light-dark cycle. A variety of other nonphotic cues are also capable of phase shifting and/or modulating photic synchronization. These nonphotic factors include, for example, behavioral arousal which induces both hyperactivity and sleep deprivation, food shortage, and social interactions such as mating-oriented behaviors (2-4). In the natural environment, therefore, daily resetting of the circadian clock results from the integration of several environmental and homeostatic cues. The following is a review of the evidence for interactions between photic and nonphotic stimuli to achieve the daily synchronization of the circadian timing system in mammals.

3. BEHAVIORAL STUDIES

3.1. Photic synchronization

"Photic" and "photic-like" factors are defined here as cues that share similar phase shifting properties, as described later. Photic synchronization occurs by means of

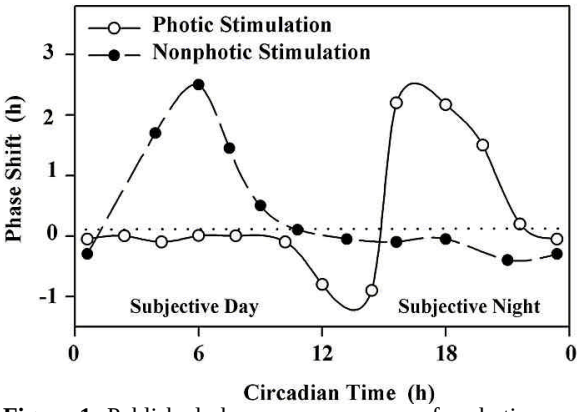

Figure 1. Published phase response curves for photic cues (i.e., 1 h light pulses; adapted from Ref. 6) and nonphotic (i.e., 3 h pulses of novely-induced running; adapted from Ref. 4) cues. The data were obtained in Syrian hamsters housed in constant darkness.

resetting of the phase and period of the SCN clock on a daily basis. The phase shifting effects of light, associated with transient changes in the endogenous period, depend on the time of the circadian cycle when light is applied (5, 6; see Figure 1). Phase response curves to discrete light pulses in constant darkness are characterized by phase delays during the early subjective night (i.e., the active and resting period in nocturnal and diurnal animals, respectively) and phase advances during the late subjective night. In contrast, light exposure has no phase resetting effect during most of the subjective day, defining a "dead zone" in the phase response curve to light in rodents. Light-induced phase shifts also depend on the properties of the light pulse (i.e., duration and irradiance). Compared to "image forming" visual networks, the circadian timing system is more sensitive to photic stimuli of longer duration (7). Moreover, the threshold for circadian responses to light is relatively high. These properties are of adaptive significance to optimize photic synchronization of circadian oscillations (7).

3.2. Nonphotic synchronization

Phase resetting cues as different as behavioral arousal, food shortage or treatment with chronobiotic drugs have been defined as "nonphotic", which only means that they differ in nature from light stimulation. From a physiological point of view, most of them share the property of inducing phase advances when applied during the subjective day (Figure 1). In view of the variety of so-called nonphotic factors, further knowledge of the physiological and neurochemical mechanisms underlying their effects on the SCN circadian clock will be helpful to classify them more rigorously. The phase shifting/synchronizing properties of the nonphotic factors mentioned in the following section have been studied in free-running animals maintained in constant darkness.

Behavioral activation of Syrian hamsters during the subjective day (i.e., during their resting period) is one of the best studied nonphotic time cues. A common procedure is to place a hamster in a novel clean wheel. This usually makes the animal run (i.e., so-called novelty-induced activity) and the SCN clock is phase shifted. When the animal did not run enough or not at all, the subsequent phase shift was most likely of small amplitude, if not undetectable (8,9). In contrast to the phase shifting effects of light, wheel running hyperactivity in hamsters induced large phase advances only when it occurred during the subjective day, while small phase delays were obtained after behavioral activation in late subjective night (8-10; see Figure 1). Forced treadmill running and confinement to running wheel during the resting period also produced phase advances in mice, but of lower amplitude than those observed in Syrian hamsters (11, 12).

Besides novelty-induced wheel running, other stimuli that induce transient hyperactivity and/or arousal during the usual resting period can also produce significant phase advances of the SCN clock. These factors include injections of triazolam, a benzodiazepine, in Syrian hamsters (e.g., 13,14), morphine treatment in mice (15). Saline injections in hamsters can induce phase advances (16,17) in a smaller window of sensitivity (i.e., in late subjective day only) than for other stimuli associated with marked behavioral activation.

A recent study has challenged the nature of nonphotic phase shifting cues involving high intensity wheel running activity. In Syrian hamsters, a 3 h period of sleep deprivation without major exercise, as induced by gentle handling during the middle of the resting period, led to reliable phase advances of the locomotor activity rhythm as large as those produced by novelty-induced wheel running (18). This effect seems to be due to sleep loss rather than nonspecific arousal, given that a 3 h period of restraint stress (i.e., a stressful, arousing situation) started during the mid-subjective day had no phase shifting effect in the same species (19). It remains unclear, however, why a 24 h period of sleep deprivation using a rotating treadmill produced no phase shift (20). Mistlberger and colleagues have raised the possibility that intense locomotor activity by itself is not necessary for behavioral nonphotic phase shifting. These authors suggest that hamsters prevented from running and those that do not run, do not shift because they do not stay awake during the experimental paradigm. This novel hypothesis could be further tested by EEG recording during nonphotic manipulations.

Melatonin, a hormone produced during the night by the pineal gland, plays a critical role in seasonal timing. When injected in the late subjective day in nocturnal (21, 22) and diurnal rodents (23), daily administration of exogenous melatonin, a pharmacological nonphotic manipulation that does not induce transient hyperactivity, is capable of synchronizing the circadian clock.

Periodic feeding without undernutrition affects the circadian timing system differently to other nonphotic stimuli. In animals maintained under constant lighting conditions, schedules of restricted feeding (i.e., supplying food for a limited daily period) usually only synchronize a bout of locomotor activity anticipating the time of feeding, called food-anticipatory activity, while the other circadian

components of locomotor activity continue to free-run (24,25). The food-anticipatory activity is considered to be driven by a food-entrainable clock outside the SCN. Detailed reviews on the food-entrainable system and its coupling with the light-entrainable SCN can be found elsewhere (24,25). In contrast to schedules of restricted feeding that enables the animals (rats, in particular) to eat normal amounts of food, periodic feeding coupled with calorie restriction (i.e., when a hypocaloric diet is given and the animals loose weight) is a more potent zeitgeber. In this paradigm, a timed hypocaloric feeding can synchronize circadian rhythms in rats kept in constant darkness (26). Furthermore, prolonged fasting in free-running rats induced phase shifts of circadian rhythms, indicating that metabolic cues can, directly or indirectly, impact on SCN function (27).

Stimulation of two pathways within the circadian timing system, namely serotonergic and NPYergic inputs to the SCN, evokes nonphotic-like responses. These effects will be considered later in section 4.2.

3.3. Interactions between synchronizing cues

The daily variation in light intensity is the most powerful synchronizer of the circadian timing system. Moreover, light exposure during the day can modify the processing of nonphotic signals by the SCN clock. Conversely, several nonphotic factors have been found to modulate, to various degrees, circadian responses to light pulses during the night and/or the phase angle of synchronization to the light-dark cycle.

Double-pulse experiments have been conducted in constant darkness by firstly applying a nonphotic stimulus, either confinement to a novel wheel or triazolam injection, during the mid-subjective day followed by a second, photic stimulus (28,29). Both studies revealed that the synchronizing effects of nonphotic factors could be altered by subsequent exposure to light. The combination of nonphotic and photic phase shifts, however, are complex, being partially but not fully additive. Conversely, several studies have shown a modifying effect on light-induced phase shifts by exposure to nonphotic cues during or before nocturnal exposure to light. For example, nocturnal wheel running activity in hamsters or nighttime injection of morphine in mice decreased the amplitude of light-induced phase advances (30-32), but both treatments did not significantly modify light-induced phase delays (31, 32). Moreover, daytime sleep deprivation has been shown to attenuate light-induced phase delays in both hamsters (20) and mice (33). In addition, a decrease in glucose availability can reduce photic phase resetting in mice (34). Although not all of the combinations have been tested in detail (e.g., exposure to light before nonphotic cues during the subjective day), the various results suggest that the relationship between photic and nonphotic synchronizing cues is probably not linear (35).

Other studies have strengthened the potential importance of nonphotic effects in influencing the steady-state phase of photic synchronization, as well as the rate of re-synchronization to a new light-dark cycle. In hamsters synchronized to a light-dark cycle (with light levels of 10 lux during the light phase), a single 3 h confinement to a new wheel during the mid-day was able to phase advance transiently the phase angle of photic synchronization by 30 min (36). This effect, however, has not been clearly demonstrated in hamsters housed under a light-dark cycle (with 30 lux intensity during the light phase) and exposed to 3 h access to a wheel daily at mid-day for 2 weeks (37). Using the same paradigm followed by a transfer of the animals into constant darkness on the last day of nonphotic behavioral stimulation, however, Mrosvosky and Janik (38) observed a split of the locomotor activity rhythm in hamsters that ran a lot during scheduled daytime activity, while another study noticed large phase delays and longer free-running periods in good “runners” (35).

In rodents, another nonphotic procedure, namely a schedule of restricted feeding, has been shown to slightly affect the phase angle of photic synchronization estimated by the onset of wheel running activity (37, 39, 40). The phase of the SCN as assessed by the acrophase of neuropeptide release (e.g., the daily rhythm of vasopressin release from the SCN) was not phase shifted by restricted feeding schedules in rats kept under a light-dark cycle (41). A more marked alteration in the phase angle of photic entrainment was observed when rodents including rats, mice and Siberian hamsters, but not Syrian hamsters, were subjected to a daytime restricted feeding coupled with calorie restriction (42-44).

Repeated daily injections of triazolam to Syrian hamsters exposed to a short photoperiod induced clear changes in the phase angle of photic synchronization (45). Moreover, several nonphotic treatments, like novelty-induced running in hamsters (46), triazolam administration to hamsters (47) or melatonin injections to rats (21) accelerated re-entrainment to a new light-dark cycle. In contrast, limited access to food during the daytime has been shown to slow photic re-synchronization in rats (48). In addition to synchronizing cues, the actual phase of the daily rhythms expressed under a light-dark cycle can also be modulated by the direct masking effects of light on behavior. For example, exposure of nocturnal rodents to bright light has an inhibitory effect on the expression of locomotor activity (see Ref. 49 for review).

4. NEUROCHEMICAL PATHWAYS

4.1. Pathways conveying photic cues

4.1.1. Retinohypothalamic glutamatergic pathway

The retinal ganglion cells innervating the SCN have been shown to be photosensitive and express melanopsin (50). From the retinal photoreceptors, light signals reach the SCN clock via at least two neuronal pathways (Figure 2), the direct retinohypothalamic tract and the geniculohypothalamic tract via the intergeniculate leaflet (IGL). Excitatory amino acids released from the retinohypothalamic terminals are the most likely transmitters of photic signals to the SCN (review in Ref. 51,52). *In vivo* injections of NMDA in the SCN region mimicked the phase shifting effects of light (53). Similar effects have been observed in isolated SCN slices *in vitro*

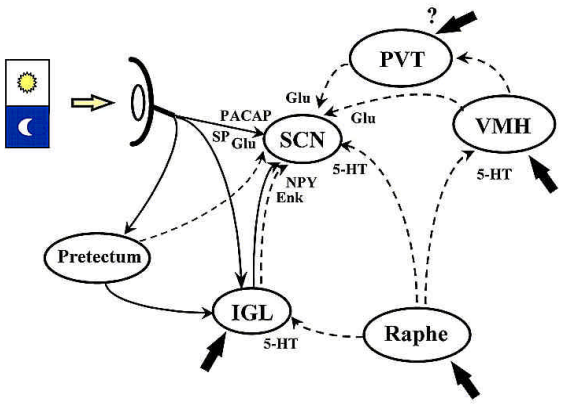

Figure 2. Proposed neuronal pathways within the mammalian circadian system. For clarity, a few connections and neurotransmitters/neuropetides have been omitted. Solid and dotted lines represent pathways conveying photic and nonphotic cues, respectively. The thick black arrows indicate target structures receiving nonphotic signals. IGL: intergeniculate leaflets of the thalamus, PVT: paraventricular thalamic nuclei, SCN: suprachiasmatic nuclei, VMH, ventromedial hypothalamic nuclei, 5-HT: serotonin, Enk: enkephalin, Glu: glutamate, PACAP: pituitary adenylate cyclase-activating peptide, NPY: neuropeptide Y, SP: substance P.

after optic nerve stimulation and following injections of NMDA and non-NMDA agonists and antagonists (51,52). In addition to excitatory amino acids, there is some indication that pituitary adenylate cyclase-activating peptide (PACAP; 54, 55, 56) released from retinal fibers participates in mediating photic input to the circadian clock. Moreover, substance P within the SCN or in afferent fibers may modulate light-induced phase resetting (56, 57).

Within the SCN, the retinohypothalamic terminals project to the ventrolateral region of the nucleus. This region contains numerous neurons synthesizing vasoactive intestinal (VIP) and/or gastrin releasing peptide (GRP). Under a light-dark cycle, the levels of both peptides displayed daily variations in the SCN, with a peak of VIP and GRP observed during the nighttime and at the light-dark transition, respectively (58). These variations disappeared in the SCN of animals transferred to constant darkness, demonstrating that both VIP and GRP rhythms are not endogenously regulated, but result from direct, albeit distinct, responses to ambient light (58). These and other observations point to a differential involvement of VIP and GRP in the photic synchronization of the SCN (58,59,60).

4.1.2. Geniculohypothalamic NPYergic pathway

The IGL receive retinal inputs from the ganglion cells, some of them projecting to both the SCN and the IGL (61). The IGL relay photic information to the SCN through the geniculohypothalamic tract (Figure 2). These fibers release neuropeptide Y (NPY), gamma-aminobutyric acid (GABA), and enkephalin into the ventrolateral region of the SCN (review in Ref. 62). The IGL is thought to influence photic synchronization of the SCN clock. Firstly, cells in the hamster IGL displayed changes in the firing rate depending upon light intensity (63). Secondly, electrolytic lesions of the lateral geniculate thalamic/IGL region reduced light-induced phase shifts in hamsters (64,65), but not in mice (66). Moreover, damage to the lateral geniculate thalamus has been shown to slow re-synchronization to a new light-dark cycle (62). These lesions, however, did not modify the phase angle of photic synchronization in hamsters (65), mice (67) and rats when fed ad libitum (68).

Nocturnal rodents can be synchronized to 1 h light pulses given at dawn and dusk, a laboratory lighting schedule considered to mimic light exposure they receive in the field. Under this so-called skeleton photoperiod, rats with IGL lesions were unable to be synchronized and exhibited “drifting” rhythms (69; in this case, the rhythms were not strictly speaking “free-running” because a photic zeitgeber was present and the animals were not exposed to constant conditions).

Reciprocal connections between the IGL and the deep superior colliculus, that also receives direct retinal fibers, could participate in modulating photic phase shifting. In support of this hypothesis, electrical stimulation of the deep superior colliculus has been shown to attenuate light-induced phase shifts (70).

IGL activation is thought to induce NPY release from the geniculohypothalamic terminals into the ventrolateral SCN. In rats exposed to a light-dark cycle, the level of NPY in the SCN showed daily bimodal variations with peaks at both the light-dark and dark-light transitions (58). Once rats were transferred into constant darkness, only the NPY peak around circadian time 12 (i.e., close to the onset of locomotor activity) persisted, demonstrating its circadian control. These data have led to the hypothesis that NPY released from the geniculohypothalamic terminals conveys photic information during twilight times (Review in Ref. 58). The lack of this NPY input in IGL-lesioned rats may explain why their circadian timing system cannot be synchronized to a skeleton photoperiod (69; see above).

4.2. Pathways conveying nonphotic cues

4.2.1. Raphe-SCN serotonergic pathway

A major afferent pathway that conveys nonphotic inputs to the SCN is the serotonergic innervation from the midbrain raphe nuclei (Figure 2). In Syrian hamsters, serotonergic modulation comes from a direct projection to the SCN originating in the median raphe nucleus and an indirect projection originating in the dorsal raphe nucleus to the IGL (71). Serotonin (5-hydroxy-tryptamine; 5-HT) release in the SCN shows circadian fluctuations with highest levels at the beginning of the active period in both rats (72) and hamsters (73). Systemic and intracerebroventricular injections of 5-HT agonists like quipazine, a nonspecific 5-HT agonist, and 8-OH-DPAT, a 5-$HT_{1A/7}$ agonist, induced nonphotic-like phase shifts in Syrian hamsters and Wistar rats, i.e. with phase advances in response to daytime injections (17,74-76). A similar role of 5-HT and 5-HT agonists has been shown in the isolated SCN *in vitro* (77-79; for detailed review, see Ref. 80).

Strain and species differences appear to be important in the phase shifting effects induced by 5-HT stimulation. For example, quipazine injected at night has been observed to have photic-like effects in several strains of rats (81,82). This may be because of variable expression in the number or affinity of 5-HT receptor subtypes in the circadian system.

Local injections of 5,7-dihydroxytryptamine into the SCN region lead to specific damage of the 5-HT terminals in the SCN (14). This method has been used to demonstrate a role of the raphe-SCN serotonergic pathway in nonphotic phase shifting. With the exception of novelty-induced running in hamsters (83), these localized lesions impaired phase shifts in response to triazolam (14) and 8-OH-DPAT injections in Syrian hamsters (85), forced running on treadmills (67) and voluntary wheel-running (85) in mice, and timed hypocaloric feeding in rats exposed to a light-dark cycle (86).

A few studies have indicated that 5-HT fibers in the IGL from the dorsal raphe can play a role in 5-HT phase shifting, possibly by activating (or disinhibiting) geniculo-hypothalamic inputs to the SCN. Daytime microinjections of 8-OH-DPAT in the dorsal raphe (87) and those directly into the IGL (88) induced phase advances of the locomotor activity rhythm in hamsters. Systemic injections of 8-OH-DPAT can still induce phase advances in hamsters with chemical lesions of the 5-HT terminals in the IGL (89). These data thus suggest that brain 5-HT activation involves synergistic interactions between direct 5-HT inputs to the SCN and those (possibly NPYergic) from the IGL. Similar conclusions have been drawn from phase shifting effects of forced running in mice (67) and timed hypocaloric feeding in rats exposed to a light-dark cycle (68,86).

4.2.2. Geniculohypothalamic NPYergic pathway

In addition to photic inputs, the IGL is also known to mediate nonphotic cues to the SCN, as demonstrated by a variety of experimental approaches (Figure 2). Microinjections of NPY into the SCN region induced behavioral phase shifts according to a nonphotic phase response curve (90). The same pattern of phase shifting responses has been obtained in isolated SCN slices *in vitro* after NPY administration (91). Electrical stimulation of the geniculohypothalamic tract also induced phase shifts similar to those of NPY and nonphotic cues (62). Other arguments favoring a major role of the IGL in nonphotic phase shifting come from lesion studies. Lesions of the IGL impaired phase shifts resulting from novelty-induced wheel running in hamsters (92,93), forced running on treadmills in mice (67), triazolam injections in Syrian hamsters (89,94), injections of chlordiazepoxide, a benzodiazepine, in mice (66), and timed hypocaloric feeding in rats kept under a light-dark cycle (68). Furthermore, pretreatment with NPY antiserum markedly attenuated phase advances induced by novelty-induced wheel running in hamsters (95).

4.2.3. Other afferent pathways

The afferent pathway coming from the ventromedial hypothalamic nuclei (VMH) to the SCN (96,97) has been also involved in mediating the effects of timed calorie restriction (Figure 2). Such a role has been demonstrated by using chemical lesions of the VMH in calorie-restricted rats (98) and mice (99). Some of the VMH inputs to the SCN are thought to release excitatory amino acids (100). In addition to these direct projections to the SCN, indirect pathways from the VMH to the relay structures could also participate in the metabolic modulation of SCN function. These areas that receive projections from the VMH include the IGL (101), a key structure in nonphotic phase shifting as noted above.

The paraventricular thalamic nuclei (PVT) are another candidate structure in nonphotic phase shifting (Figure 2). Among various afferents, they receive projections from the VMH and possibly the IGL (102). Moreover, the PVT send direct fibers releasing excitatory amino acids to the SCN, as the VMH do (100). In contrast to the photic-like phase shifting effects of glutamate on *in vitro* SCN slices (see Ref. 52), *in vivo* injections of glutamate induce nonphotic-like phase shifts of the circadian rhythm of locomotor activity (103). To explain these unexpected results, it was initially proposed that *in vivo* treatment with glutamate may have stimulated efferent pathways that would, in turn, project back to the SCN and cause phase shifts (103). Alternatively, we suggest that this treatment could have stimulated glutamatergic afferent pathways other than the retinohypothalamic tract, for example, fibers conveying nonphotic cues from the VMH and/or the PVT to the SCN.

Another structure that has been shown to mediate nonphotic cues to the SCN is the pretectum that projects to both the SCN (104) and the IGL (70). Electrolytic pretectal or tectal lesions impaired the phase shifting properties of triazolam, but not those of novelty-induced running (70).

4.3. Neurochemical interactions

4.3.1. Interactions during the night

Besides direct effects on the SCN clock during the day, both 5-HT and NPY are thought to play a role in modulating the light-induced phase shifts during the night. On the one hand, 8-OH-DPAT injections decreased the magnitude of light-induced phase shifts in hamsters (105) and chemical lesions of the 5-HT terminals in the SCN region increased light-induced phase delays in mice (106). On the other hand, light-induced phase advances in hamsters were inhibited by microinjections of NPY in the SCN region (107) and enhanced by pretreatment with NPY antiserum (108). The effects of NPY on the circadian responses to light, however, were opposite to those expected from studies using electrolytic lesions of the IGL (see § 4.1.). The reasons for this discrepancy are not yet fully understood, but it may be due to the use of electrolytic lesions (for a detailed discussion on the drawbacks of electrolytic lesions of the IGL, see Ref. 69). Specific chemical lesions of the IGL (for example, ones that would deplete the NPY content) may provide results to better delineate the role of the IGL in photic and nonphotic modulation *in vivo.*

SCN slices maintained *in vitro* have been a model of choice for investigating neurochemical interactions

within the deafferented SCN (i.e., lacking regulatory inputs). During the subjective night, both phase advances and delays induced by glutamate administration in the SCN slice were prevented by application of NPY (91; for a detailed review, see Ref. 109). Serotonergic modulation of photic phase shifting during the night has been proposed to involve presynaptic 5-HT_{1B} receptors located on retinohypothalamic fibers. Due to presynaptic location of these receptors, 5-HT_{1B} receptor agonists applied to the SCN *in vitro* did not modify responses to glutamate (110).

4.3.2. Interactions during the day

As mentioned earlier (§ 3.1.), exposure to light during most of the subjective day (i.e., during the dead zone of the light phase response curve) has no phase shifting effect in rodents, when nonphotic cues can induce phase advances. Exposure to light during the subjective midday, however, inhibits the phase advancing properties of nonphotic factors. Nonphotic phase shifting caused by novelty-induced running was reduced when the induced wheel-running was followed by a 1 h light pulse (111). Similarly, the phase advancing effects of NPY infusion in the SCN region of hamsters and mice were markedly reduced by a subsequent light pulse (111,112). This photic inhibition, that appears to involve the IGL (88, 111), has also been demonstrated for 8-OH-DPAT injected either systemically (113) or in the IGL region of Syrian hamsters (88).

During daytime, combined applications of different neurotransmitters have revealed numerous neurochemical interactions within SCN slices maintained *in vitro*. In accordance with *in vivo* photic inhibition of nonphotic phase resetting, *in vitro* application of glutamate blocked SCN phase shifts to NPY (91) and 5-HT (114). Also, while treatment with PACAP phase advanced the SCN clock, co-application of NPY blocks this effect (115). Moreover, neurotransmitter systems involved in conveying nonphotic signals, namely NPY and 5-HT, interact *in vitro* to modulate the phase of the circadian clock. NPY antagonized 5-HT-induced phase advances during the subjective midday, while neither 5-HT nor 8-OH-DPAT altered NPY-induced phase advances during the late subjective day (116). 5-HT-induced phase advances were reduced by melatonin treatment, while the opposite effect did not occur, i.e. 5-HT agonists did not modify melatonin-induced phase advances (117).

Specific neuronal signaling pathways mediating photic and/or nonphotic cues and producing phase advances or delays within the SCN neurons will not be considered here (for review, see Ref. 118).

5. MOLECULAR STUDIES

5.1. Effects of light on clock gene expression

Synchronization of the SCN clock to environmental cues has recently been associated with transcriptional mechanisms involving induction and inhibition of clock gene expression. The molecular core of the SCN clock is considered to involve self-sustaining transcriptional/translational feedback loops involving several "clock genes"(119). A negative loop encompasses the rhythmic transcription of three mammalian orthologs of the Drosophila *Period* (i.e., *Per1, Per2* and *Per3*) and two *Cryptochrome* genes (i.e., *Cry1* and *Cry2*). The rhythmic expression of these genes is driven by BMAL1/CLOCK heterodimers, a process inhibited by CRY1 and CRY2 (120-122). PER2 is considered to play a critical role in the molecular clockwork through indirect activation of *Bmal1* transcription (122). PER1 may regulate PER2 at a post-transcriptional step in the SCN (123,124) and play a role in peripheral oscillators (125). In contrast to the important role of *Per1* and *Per2* in the oscillating mechanism, *Per3* does not seem to be essential for the molecular core of the clock and may rather be involved in the regulation of circadian outputs (123,126).

It is now well established that light exposure induces expression of *Per1* and *Per2* genes during the night, a period during which light is also capable of resetting the SCN clock. An acute light-induced response of *Per1* occurs in the early and late night, while *Per2* expression is only strongly stimulated by light in the early night (127-130). Moreover, intracerebroventricular injections of antisense oligodeoxynucleotides to *Per1* and/or *Per2* inhibited light-induced phase delays in mice (131,132). Mice with a mutated *Per1* gene exhibited altered light-induced phase advances, while *Per2* mutant mice displayed impaired light-induced phase delays (133). These findings, therefore, indicate the likely participation of *Per1* and *Per2* at the end of the light-associated input pathway, although this view has been challenged (see Ref. 134). While the light-induced expression of *Per1* and *Per2* occurs in the ventrolateral SCN neurons, circadian expression of *Per* is not uniform within the SCN, suggesting a topographic compartmentalization of the circadian clock (135).

Another clock gene possibly involved in photic resetting is *Bmal1*. Expression of *Bmal1* may be modulated by light in the rat SCN (136) and *Bmal1-/-* (i.e., *Mop3-/-*) mice showed altered synchronization to a light-dark cycle (137).

5.2. Effects of nonphotic cues on clock gene expression

The role of *Per* genes in the SCN synchronization is also supported by the study of nonphotic cues. Nonphotic signals applied during the subjective day can act on the SCN both by inducing phase shifts (§ 3.2.) and by reducing *Per1* and *Per2* expression. These effects of nonphotic signals on clock gene expression include *in vivo* paradigms such as novelty-induced running in hamsters (138,139), injections of 5-HT agonists in hamsters (140) and intracerebroventricular injections of NPY in mice (112), as well as *in vitro* analysis of the effects of NPY treatment on SCN slices (141) or previous *in vivo* novelty-induced running (142).

In contrast to nonphotic cues associated with increased locomotor activity, a melatonin injection during the late subjective day inducing a subsequent phase advance of the circadian clock did not lead to acute changes in clock gene expression within the SCN (Poirel and Gauer,

unpublished data), suggesting that, at least for melatonin, mechanisms associated with phase shifting can occur at the post-transcriptional level.

Nonphotic phase shifting has been shown to be altered in mice heterozygous for *Clock*. Repeated novelty-induced running led unexpectedly to phase delays in these mutants compared to the phase advances observed in wild-type animals. Moreover, timed hypocaloric feeding induced a 1.5 h phase advance of the locomotor activity rhythm in wild-type mice, but not in *Clock*/+ mice (12). Since circadian expression of *Per* genes can be reduced in *Clock* mutant mice (143), the results for nonphotic phase shifting in *Clock* mutants can be interpreted as suggesting either an involvement of CLOCK in the integration of nonphotic cues or secondary effects due to deficits in CLOCK-controlled factors, such as *Per* genes.

5.3. Interactions between photic and nonphotic cues

Until now, only a few studies have examined the modulation of nonphotic signals on changes in clock gene expression associated with photic phase shifting. Because nonphotic cues have been shown to reduce light-induced phase shifts during the night (§ 3.3.), one could expect that nonphotic stimuli may also reduce light-induced expression of *Per* genes. In support of this, injection of a benzodiazepine, brotizolam, given before a light pulse late in the night greatly reduced light-induced phase advances and *Per1* and *Per2* expression in hamsters (144). Moreover, the light-induced increase in *Per1* and *Per2* expression in the SCN during the night was attenuated by subsequent treatment of NPY *in vitro* (145). Unlike previous behavioral results showing that 5-HT activation during the night reduced light-induced phase resetting, injection of MKC-242, a 5-HT_{1A} receptor agonist, potentiated the light-induced increase in *Per1* and *Per2* expression in mice (146).

Other studies have recently investigated the combined effects of light and NPY (112), as well as light and novelty-induced running (139) on the expression of *Per1* and *Per2* during the daytime. Both studies showed that subsequent light attenuated the nonphotic reduction in *Per* expression. Unfortunately, none of these experiments included a group of animals only exposed to daytime light. As mentioned earlier (§ 3.3.and 4.3.), light exposure during the daytime markedly attenuates the nonphotic phase shifting effects. In view of the lack of phase resetting effect of light during the (subjective) day in rodents, expression of *Per1* and *Per2* is commonly supposed not to be sensitive to light during that period. Current experiments in our laboratory, however, suggest that prolonged light exposure during the subjective day increases *Per* expression in mice, raising the possibility that light-induced potentiation of clock amplitude during the day may, to some extent, buffer the circadian system against the phase resetting effects of nonphotic cues and their reducing effects on *Per* expression (Challet and Pévet, unpublished data).

Schedules of restricted feeding (i.e., supplying food for a limited period during the day) have been used to study clock gene expression in the SCN and peripheral organs. In keeping with behavioral data showing that the outputs of the SCN are not entrained by restricted feeding (24, 25), this paradigm applied under a light-dark cycle synchronized clock genes in peripheral organs and some brain areas including cerebral cortex and hippocampus, but did not affect the expression of clock genes in the SCN (147-150). Current work, however, suggests that a timed hypocaloric diet that modifies the photic synchronization of the SCN (42-44) also alters clock gene expression within the SCN. This indicates that repetitive nonphotic (metabolic) signals presented, in addition to a light-dark cycle, affect the molecular clock of the SCN (Graff and Challet, unpublished data).

6. SUMMARY AND CONCLUDING REMARKS

Synchronization of the mammalian circadian timing system is accomplished mainly via daily resetting of the phase of the clock by photic information. The circadian clock, however, is also sensitive to phase shifting effects of various temporal and homeostatic (i.e., nonphotic) cues. In spite of opposite shape of their respective phase-response curves (Figure 1), the combination of photic and nonphotic signals does not necessarily result in a sum of their phase shifting effects. Rather, their combination leads to a complex interplay, both cues being able to modulate each other to varying degrees. The whole picture of circadian synchronization is further complicated by the direct and immediate effects of light (i.e., masking) on overt rhythms. Photic cues are primarily conveyed to the SCN via direct glutamatergic projections from the retina, and secondarily via indirect pathways including the IGL. Transmission of nonphotic cues to the SCN is thought to involve at least two well characterized tracts, a 5-HT one from the raphe and a NPYergic one from the IGL, as well as less studied pathways including glutamatergic fibers from the VMH (Figure 2). The SCN mechanisms underlying the integration of photic and nonphotic cues involve multiple pre and postsynaptic neurochemical interactions.

Synchronization of the circadian clock is associated with changes in clock gene expression within SCN neurons. These changes are thought to reset the transcriptional-translational feedback loops of the clock. To date, light-induced changes in clock gene expression concern almost exclusively changes in *Per1* and *Per2* expression. Current information indicates that integration of photic and nonphotic cues by the SCN is also translated into acute changes in *Per1* and *Per2* expression. Despite considerable progress in understanding the molecular mechanisms of circadian oscillation, a number of fundamental questions remain concerning the mechanism of synchronization. In particular, the cellular and molecular substrates allowing the SCN clock to integrate synchronizing cues need to be identified. It seems unlikely that only the levels of *Per1* and *Per2* expression at a given time of the circadian cycle account for the amplitude and direction of phase shifts. It is likely that additive, yet uncharacterized, mechanisms occur at the post-transcriptional level.

Further work using transgenic technologies coupled with more traditional approaches (e.g., *in vivo* physiology

or *in vitro* electrophysiology) is therefore needed to try to unravel how the mammalian circadian clock is synchronized to different, sometimes conflicting, temporal and homeostatic cues.

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Send correspondence to: Dr Etienne Challet, Laboratory of Neurobiology of Rhythms, CNRS UMR7518, University Louis Pasteur, 12 rue de l'université, F-67000 Strasbourg, France, Tel: +33 (3) 90 24 05 08, Fax : +33 (3) 90 24 05 28, E-mail: challet@neurochem.u-strasbg.fr