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FROM LIGHT TO GENES: MOVING THE HANDS OF THE CIRCADIAN CLOCK

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1. ABSTRACT

Mammalian circadian rhythms are generated by the hypothalamic suprachiasmatic nuclei and finely tuned to environmental periodicities by neurochemical responses to the light-dark cycle. Light reaches the clock through a direct retinohypothalamic tract, primarily through glutamatergic innervation, and its action is probably regulated by a variety of other neurotransmitters. A key second messenger in circadian photic entrainment is calcium, mobilized through membrane channels or intracellular reservoirs, which triggers the activation of several enzymes, including a calcium/calmodulin-dependent protein kinase and nitric oxide synthase. Other enzymes activated by light are mitogen-activated- and cGMP-dependent protein kinase; all of the above have been reported to be involved in the circadian responses to nocturnal light pulses. These mechanisms lead to expression of specific clock genes which eventually set the phase of the clock and of clock-controlled circadian rhythms.

2. INTRODUCTION

We come to Earth fully equipped with a brainwatch that helps us around to cope with the predictable temporal changes in our home: the day and the year. Although many tissues have been shown to possess autonomous oscillatory properties (1-3), the kings of mammalian endogenous clocks are still the hypothalamic suprachiasmatic nuclei (SCN). The lack of a functional SCN renders its bearer arrhythmic under constant conditions, and the transplantation of a foraneous clock recovers the cyclic *remembrance of things past* (4, 5). The SCN continue to work precisely under constant or even *in vitro* conditions, originating *circadian* (i.e., with a period of about 24 h) rhythms in free-running conditions; much work has been done in recent years to determine its precise neuroanatomy (e.g., 6-8) and molecular machinery (e.g., 9, 10). The purpose of this short review will be to present some of the mechanisms that make our mammalian circadian clock tick in tune with the environment, focusing on the neurochemical changes that make synchronization possible.

3. BEHAVIORAL EFFECTS OF LIGHT

A free-running clock is obviously useless in a non free-running planet. So, if the clock was left to itself and its *circadian* periodicities, life on Earth would have been very different (if there had been any life at all). Finding food or a mate, or being able to escape from predators, would have been a simple matter of chance, if it not were for two direct effects of the environment on circadian rhythms: entrainment and masking. When subjected either to short (i.e., in the order of minutes) light pulses or predictable photoperiods, animals adjust their brain watches and change their cyclic behavior accordingly. These changes are chronic and induce synchronization and, what is more interesting, they are temporally gated: animals respond differentially to light pulses given at different times of day. Typically, under constant dark conditions, early subjective night pulses (defining subjective night as the time when the animals behave as in a regular nocturnal situation) induce phase delays while late subjective night stimulation produces phase advances in behavioral rhythms controlled by the clock (11). It is not surprising that light pulses administered during the subjective day do not induce phase shifts, since it does not seem very reasonable for a nocturnal animal to be out there in plain daylight, while diurnal animals have plenty of light to chose from at that time.

Experimental photic stimulation provided one of the most powerful tools for studying the clock: the phase response curve (PRC), which graphs the change in a phase marker of the rhythm (for example, activity onset) depending on the time of stimulation. Aschoff (12) also showed continuous effects of light on circadian rhythmicity, which are dependent on light intensity and

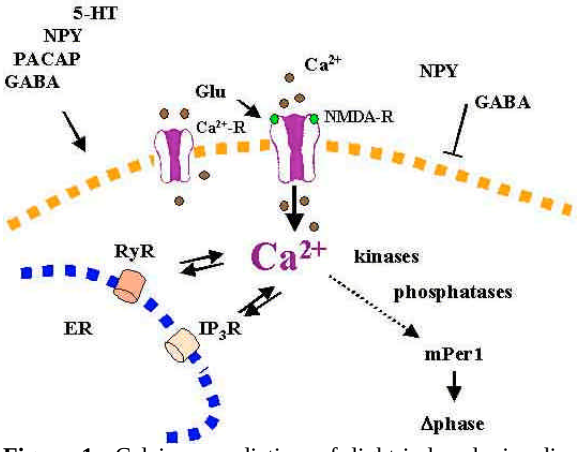

Figure 1. Calcium mediation of light-induced circadian entrainment. The retinohypothalamic tract releases glutamate onto SCN ventral neurons and interacts with several types of receptors, of which the NMDA type has been shown to mediate light effects. In addition, other types of Ca^{2+} channels are also present in the SCN and could contribute to ion influx through the cytoplasmic membrane. Calcium influx is agonized by several other neurotransmitters, including PACAP, 5-HT and, under particular conditions, GABA and NPY (which have also been reported to inhibit Ca^{2+} influx). The increase in calcium levels can also trigger the release of ions from intracellular stores by IP3 and ryanodine receptors. Several cytoplasmic mechanisms are activated in response to calcium, including enzymatic activities that eventually change clock gene expression and leads to phase shifts of the molecular clock.

receive the name of parametric entrainment, in contrast with the non-parametric entrainment exemplified by PRCs. For comparison reasons, phase changes are measured in circadian hours, defining one circadian hour as freerunning period/24 hours.

Photoperiodic entrainment is a predictable natural event and was therefore easily selected for its high adaptive value. However, more rapid changes in behavior are needed for unpredictable stimuli, including sudden changes in lighting conditions. For example, turning on the lights late at night in a hamster room will induce a gloomy silence devoid of wheel running and other noises. These direct effects of light override clock instructions and affect circadian rhythms directly, "masking" the circadian plans for that time of day (13). From an evolutionary perspective, masking collaborates with entrainment for a precise response to environmental stimuli, providing adequate times for different kinds of behaviors.

4. THE EYE AND NEUROTRANSMITTERS

As the 20th century writer Julio Cortázar stated, "time enters through the eyes"; indeed, the main input pathway to the SCN comes from the retina through a monosynaptic retinohypothalamic tract (RHT). The elusive photopigment responsible for circadian entrainment, known from studies with mutant animals not to reside within rods or cones (14, 15), has not been uncovered yet, although a good candidate has recently been identified as melanopsin, found in a subset of retinal ganglion cells (16). Cryptochromes, originally thought to be responsible for retinal photic entrainment, are probably more related to circadian rhythm generation, although the function of their retinal expression remains to be fully established (17).

Photic neurochemical input to the SCN comes from different sources. The main input comes from the RHT, which uses glutamate as a neurotransmitter, as well as aspartate, PACAP and substance P (18-23). A multisynaptic pathway, also originating in the retina, innervates the clock from the thalamic intergeniculate leaflet, using NPY and GABA as transmitters. Different glutamatergic receptors have been found in the SCN, including NMDA, AMPA and metabotropic types (18, 24). Glutatamergic stimulation induces light-like phase shifts of circadian rhythms, and blocking these receptors –most efforts have concentrated on NMDA- inhibit the effects of light pulses (25-29). Some NMDA receptor subunits exhibit circadian changes in the SCN (e.g., 30), suggesting that the temporal gating of photic effects on rhythms might include the very first step of entrainment: the reception of the RHT photic message.

Other neurotransmitters have been involved in SCN photic signal transduction pathways, including histamine (31), neurotensin (32), and other neuropeptides (33, 34)

5. SIGNAL TRANSDUCTION

5.1. Calcium

Calcium acts as a universal second messenger in a variety of cells. Circadian rhythmicity of many different types of physiological process is modulated by calcium levels (35). Recent reports have shown that calcium ions play an important role in the rhythmicity of mammalian biological clocks (figure 1).

Colwell (36) demonstrated differences in basal $[Ca^{2+}{}_i]$ in rats sacrificed during the light or the dark phase of their cycle, which persisted under constant darkness. However, these differences could not be found in early postnatal days. Moreover, those variations were abolished by presynaptic inhibition, suggesting an extracellular control of basal $[Ca^{2+}{}_i]$, although these extracellular signals could also help to move internal stores.

The involvement of glutamate in the synapses that translate the photic stimulus carried by the optic nerve into the SCN has been extensively studied, and the Na^+/Ca^{2+} permeable receptor NMDA is necessarily involved in its function (25). Although no circadian experiments with calcium chelators were carried out, extracellular Ca^{2+} is thought to be the main source for triggering second messenger pathways. The Ca^{2+} influx associated with NMDA receptor activation is thought to produce brief, high concentration (> 100 μM) localized gradients of Ca^{2+} near open channels. Recently, a circadian oscillation in NMDA-induced Ca^{2+} transients of SCN rats

cells has been reported (37). These Ca^{2+} transients might play a role in the regulation of photic information reaching the SCN. Other messengers, such as 5-HT and GABA also increase $[Ca^{2+}_i]$ in adulthood, while NPY and GABA have been found to decrease basal $[Ca^{2+}_i]$ during development (38-40). The time-dependency of NMDA-induced Ca^{2+} currents is proposed as part of the circadian gating of photic entrainment: something must be going on at the receptor level that lets calcium in at some times but not at others. In our own experiments, however, circadian gating occurs downstream of this effect, since both 100 μM glutamate or NMDA exerted similar increased of intracellular Ca^{2+} levels as measured by FURA2-AM fluorometry (Ferreyra and Golombek, unpublished).

However, other Ca^{2+} membrane channels (such as voltage-gated ones), or Ca^{2+} organelles stores could play an important role in signal transduction, because metabotropic glutamate receptors and intracellular release also modulate $[Ca^{2+}_i]$ (41). $[Ca^{2+}_i]$ has also been shown to have ultradian cycles, apparently related to rhythms in organelle stores (38).

The endoplasmic reticulum neuronal network contains ryanodine and inositol (1,4,5)-trisphosphate (IP_3) receptors (RYRs and IP_3Rs, respectively), both of which are capable of regenerative Ca^{2+} release. There are three distinct isoforms of ryanodine receptors (RYR-1, RYR-2 and RYR-3), and the most abundant isoform in the brain is RYR-2. The SCN endoplasmic reticulum seems to play a role in phase delays, since the early subjective night (at circadian time – CT – 14, defining CT 12 as the time of locomotor activity onset) application of ryanodine agonists to SCN slices induces resetting of electrophysiological rhythms, and a ryanodine antagonist blocks light-induced phase delays at the same circadian time (42). This result is difficult to reconcile with the fact that another group found phase advances to ryanodine or caffeine applications at CT 7-9 correlating with the acrophase of the expression of the ryanodine receptor type II (43).

IP_3 receptors are also good candidates for Ca^{2+} mobilization in the SCN. Types I and III IP_3 receptors have been found to be expressed in the clock following a circadian fashion, peaking at CT 14 and CT 18, respectively (44). Moreover, pharmacological inhibition of IP_3R function was postulated to reduce phase shifts in the early night; however better control experiments are probably needed for this experiment (45). It is possible that the effects of chronic or acute stimulation with lithium, which modulates phase and period of the clock in several animal models (47, 48) are mediated by the IP3 system. Moreover, this could even be related with the effects of lithium on some circadian parameters of depressed patients (46) having to do with its modulatory properties of the inositol system, but at the moment this is purely speculative.

Many neurotransmitters that affect SCN cells, including glutamate, have been shown to induce changes in $[Ca^{2+}_i]$. The phase-resetting pituitary adenylate cyclase activating peptide (PACAP), a co-transmitter with glutamate in the RHT, induces changes in SCN calcium levels directly related to intracellular Ca^{2+} stores release (49). A note of caution should be taken in that PACAP's effects are dose-dependent and might have a complex modulation of glutamate effects (41, 50).

GABA's effects on SCN $[Ca^{2+}_i]$ are also complex, and depend on the time of administration and on the development stage of the animal. It has been shown that around embryonic day 20 GABA causes a depolarization in SCN postsynaptic neurons, which is followed by Ca^{2+} transients (39). It has also been reported that GABA might be a depolarizing signal during the night (51), probably reflecting changes in intracellular chloride concentrations and activity (52, 53).

GABA effects on calcium are also modulated by the action of neuropeptide Y (NPY), a classical nonphotic input to the clock. During embryonic stages NPY blocks GABA-induced increases in $[Ca^{2+}_i]$, an effect that persists long after the peptide is removed (40). NPY also blocks glutamate-induced $[Ca^{2+}_i]$ increases in the SCN, causing a long-term depression of this synaptic activity (39).

As for downstream mechanisms related to $[Ca^{2+}_i]$, it has recently been shown that extracellular calcium influx is necessary for depolarization-induced *mouse Period1* (*mPer1*, one of the most studied clock genes, see below) expression in cerebellar cells (54). This effect was blocked by nifedipine and CaM-kinase inhibitors. It is most plausible that the same applies to SCN neurons, although it remains to be established.

5.2. Kinases and enzymes

Recent experiments from several laboratories are beginning to outline a model of the signaling pathways required for photic entrainment. However, the nature of the circadian gating of light effects, as well as the precise reason for the delay and advance responses, are not well understood. Here we shall review our and other results concerning mainly the mechanisms responsible for phase advances in response to light pulses in the late subjective night.

In our hands, glutamate release from the RHT and calcium influx in SCN cell suspensions are not temporally regulated (unpublished results), suggesting that the circadian gating of photic responses occurs in the intracellular pathway leading to changes in the hands of the clock. Our current working model includes specific transduction steps for achieving photic phase advances, which can be summarized as follows (19) (see figure 2):

- Calcium induces the activity of the Ca^{2+} /calmodulin dependent protein kinase II (CaMKII) in the SCN. It has also been shown that CaMKII is capable of phosphorylating the transcription factor CREB, which is also activated in response to light pulses (55-59). Although the levels of this kinase do not change throughout the day, its activity exhibits diurnal and

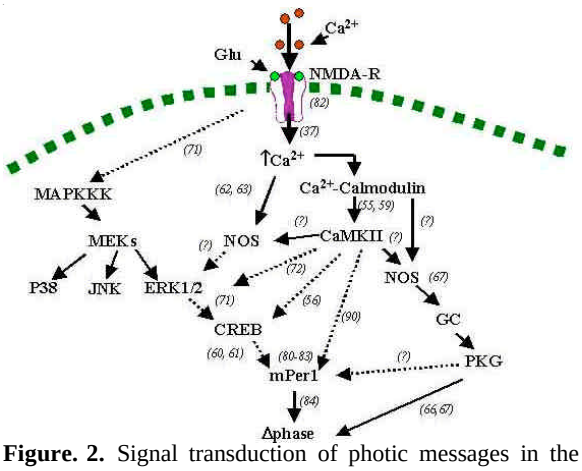

Figure. 2. Signal transduction of photic messages in the mammalian suprachiasmatic nuclei. NMDA-mediated calcium influx activates NOS and CaM kinase activity, which in turn modulates other kinases (such as PKG or MAP kinases) and eventually activate transcription factors leading to clock gene induction and entrainment. Numbers along the arrows correspond to citations in the references section. Question marks indicate mechanisms that are known in other tissues but remain to be established in the SCN.

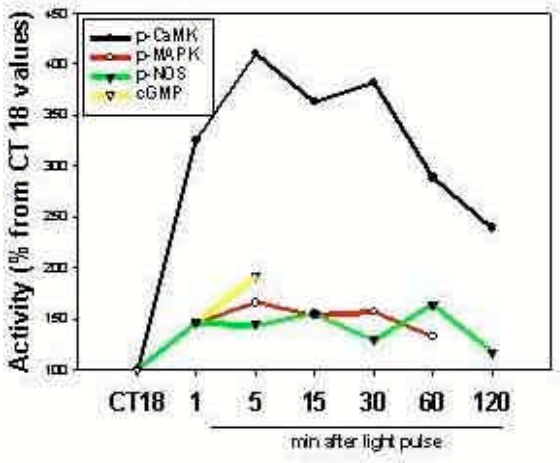

Figure 3. Time course for enzymatic activation and/or phosphorylation in the hamster SCN following a light pulse at CT (circadian time) 18 (defining CT 12 as the time for activity onset). At this time, light stimulation induces phase advances of the clock and overt behavioral rhythms. Shortly after 5-min pulses, NOS and MAPK are phosphorylated, and cGMP levels increase by about 50 %. A much larger increase (i.e., 4-fold) is found for CaMK phosphorylation after the light pulse. All of these changes return to dark control conditions within two hours after the pulse.

circadian changes peaking during the day, measured both by radioactive methods and by the presence of the activated phospho-enzyme (Agostino et al.,unpublished). Light pulses administered during the night increase the activity of CaMKII, both for delays and advances (Agostino et al., unpublished) (figure 3);- a recent report examined in more detail light-induced CREB activation, showing two independent phosphorylation sites, at Ser 133 and Ser 142, both of which would be involved in photic induction of Fos, *mPer* and phase shifts (60). Moreover, clock genes such as *mper* 1 and 2 have CRE sites in their promoters which contribute to their activation (61).

- the increase in $[Ca^{2+}_i]$ also triggers the activation of nitric oxide synthase (NOS) (62), with a higher nocturnal activity but constant expression levels (63). Light pulses activate NOS, maybe through CaMKII-induced phosphorylation (Agostino et al., unpublished) (figure 3). Indeed, inhibition of NOS blocks light-induced phase advances, while NO donors increase the effects of light on circadian phase (62, 64);
- another gaseous messenger that could participate in the phase-shifting pathway of photic stimuli is carbon monoxide (CO), synthesized by the enzyme heme oxygenase (HO). HO is strongly expressed in the SCN (65) and undergoes circadian variations in its levels, with nocturnal peaks (Rubio et al., unpublished)
- both NO and CO are capable of activating the soluble form of guanylate cyclase (GC), inducing circadian changes in both cGMP levels and cGMP-dependent protein kinase (PKG) activity (67). PKG isoform type II is expressed in the SCN and also exhibits circadian changes in its levels, peaking during the day (67). Pharmacological inhibition of PKG selectively blocks light-induced phase advances, offering an interesting substrate for looking into the circadian gating of delays and advances of the clock (66, 67).

Indeed, this pathway does not lead us to all circadian responses. For example, at some point, other mechanisms must be triggered in order to induce phase delays of the clock. Results from Ding et al (42) suggest that $[Ca^{2+}_i]$ mobilization through activation of ryanodine receptors, which also cycle in the SCN (43), leads to circadian phase delays, although the exact downstream mechanisms being triggered are not known. As for advances, we are currently investigating possible PKG substrates that might lead to eventual changes in clock gene expression (more on that later).

As for the gaseous neurotransmitters, it is also tempting to speculate that they might additionally function as retrograde messengers, maybe as a reverberating signal for glutamate release from the SCN. Another putative function for NO and CO is related to coupling between SCN cells. Indeed, since most SCN individual cells are mini-oscillators (68) there should be a mechanism that make them sound like a harmonic orchestra so that the clock can modulate body rhythms properly. Although GABA, also present in most, if not all, SCN cells, has been proposed as a coupling factor (as well as other mechanisms such as interneuronal or glial-neuronal junctions, e.g., [69]), it is tempting to speculate that gas messengers might be fast and accurate coupling agents. Other neurotransmitters such as gastrin-releasing peptide could also mediate intra-SCN information (70). Moreover, all of the above are putative candidates for conveying the photic

message from the retinorecipient portion of the clock to the dorsal SCN.

When following light induced biochemical changes in the SCN, it is obvious that CaMKII and nNOS are active already 1 min after the light pulse, and remain active for about 2 hours, declining to basal values afterwards. However, NOS activity is only increased by about 50% (and is multiplied in the pathway, since SCN cGMP levels double 5 minutes after the light stimulus), while CaMKII activity is increased 4-fold (figure 3).

5.3. MAPping the clock with other kinases

A few years ago, Obrietan et al. (71) demonstrated that light pulses administered during the night activate Erk2, a member of the MAP kinase (MAPK) family, in the rat SCN. More recently (72), they showed that in vivo inhibition of MAPKs blocks the circadian response to light. The upstream and downstream pathways related to these kinases is not known. We have extended those results to other members of the MAPK family in the hamster, including Erk1/2, p38 and JNK (Pizzio et al., unpublished). The three of them exhibit diurnal and circadian changes in their SCN activity, as measured by the increase in their phosphorylated form during the day. Moreover, Erk1/2 and JNK (but not p38) respond to nocturnal light pulses (figure 3). These changes might be triggered by a differential effect of light on MAPK kinases and/or MAPK phosphatases, an issue currently under investigation. Moreover, the putative upstream pathways and substrates for these enzymes are not well understood, although a recent report suggests that some MAPKs are capable of phosphorylating CREB (73), which would then appear to be a common step in the photic entrainment pathway. Indeed, pharmacological inhibition of MAPKs blocks light-induced phase shifts, and there are also reports suggesting that MAPKs might be regulated by CaM kinase II (72), indicating a complex network of signal transduction pathways in the SCN (figure 2).

6. AND THEN THERE WERE GENES

The core molecular mechanism of the circadian clock is proposed to be composed by a transcription/translation feedback loop with specific actors that regulate each other's levels throughout the day. In mammals, mutations in the *period 1* and *2* (*per* 1 and 2), *cryptochrome 1* and *2* (*cry 1* and 2), *clock* and *BMAL1* genes alter rhythms or entrainment (and, in some cases abolish circadian rhythms altogether) (74-76). Data support a model in which *period* and *cryptochrome* expression are triggered by the activity of a CLOCK/BMAL dimer, which is in turn inhibited by CRY and promoted by PER2 (77). This cycle is autorregulated so that it generates close to 24 h rhythms; the presence of other promoters that respond to these clock genes ensures that this rhythmicity is transmitted downstream (78). As for the differential function for the various *per* and *cry* genes, Daan et al. (79) have recently proposed that *per1/cry1* and *per2/cry2* are elements of different oscillators interacting within the clock, responding to light-induced phase advances and delays, respectively.

However, this molecular loop needs an input pathway to keep it in tune with the outside world. Clock gene expression responds to light, both at the transcriptional and post-translational levels. Both *mPer1* and *mPer 2* exhibit a strong and differential response to light pulses or NMDA application (80-83). This is supported by the fact that *mPer1* homozygous mutant mice do not response to light pulses in the phase advance region of the PRC, while *mPer2* mutants are not able to respond to phase-delaying responses (84). Not all experimental results are in accordance with this differential role of *per1* and *per2* in advances and delays, respectively. Antisense inhibition of *mPer1* blocks light or glutamate-induced phase delays in the mouse (85), while according to the previous model, it should not have affected phase delays, but only advances. Moreover, we have found that pharmacological inhibition of PKG (which is involved in the phase advance pathway) blocks light-induced *mPer2* in the hamster SCN (Ferreyra et al., unpublished), a gene that should be only involved with delays, as proposed by Daan et al. (79). Another fact which needs to be reconciled with this model is that *per* is induced by light in cry1/cry2 mutant mice (86), so the relative contribution of the *cry* genes to photic responses remains to be fully established.

The signal transduction pathway leading to clock gene expression and modulation has not been completely mapped. Indeed, some of the candidate transcription factors proposed to be involved in circadian entrainment must, at some point, communicate with these genes (figure 4). So far, there is some evidence that relates the cAMP-CREB pathway to *per* expression (61, 87). Also, MAP kinases have been shown to interact with both *per* expression in fibroblasts (88) and *BMAL* expression in avian tissue (89) and, as stated, we have found evidence relating PKG activity to light induced *mper* expression in the SCN (Ferreyra et al., unpublished). The CaM kinase pathway has also been reported to lead to *per* expression in the SCN (90), thus relating Ca^{2+}influx to clock gene expression.

As for post-translational modifications of clock genes, it has been shown that the casein kinase Iε phosphorylates *per* genes, and a mutation in this enzyme (spontaneously occurring in the tau mutant hamster) changes both circadian period and responses to light (10). *In vivo* phosphorylation of SCN proteins has been difficult to assess, although data in liver clock genes suggests that this kind of post-translational modifications is crucial for normal clock functioning (3). Interfering with kinases and phosphatases does indeed affect the phase of overt rhythmicity. In particular, one of the aforementioned kinases, CKIε, is subject to dephosphorylation by calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase. Pharmacological inhibition of calcineurin results in nonphotic phase shifts of hamster circadian rhythms (91). It is interesting that calcineurin serves as an interface between neuronal and immune mechanisms; indeed, it is blocked by immunesuppressants such as cyclosporin A, suggesting a close tie between circadian and immune humoral factors (Marpegan et al., unpublished).

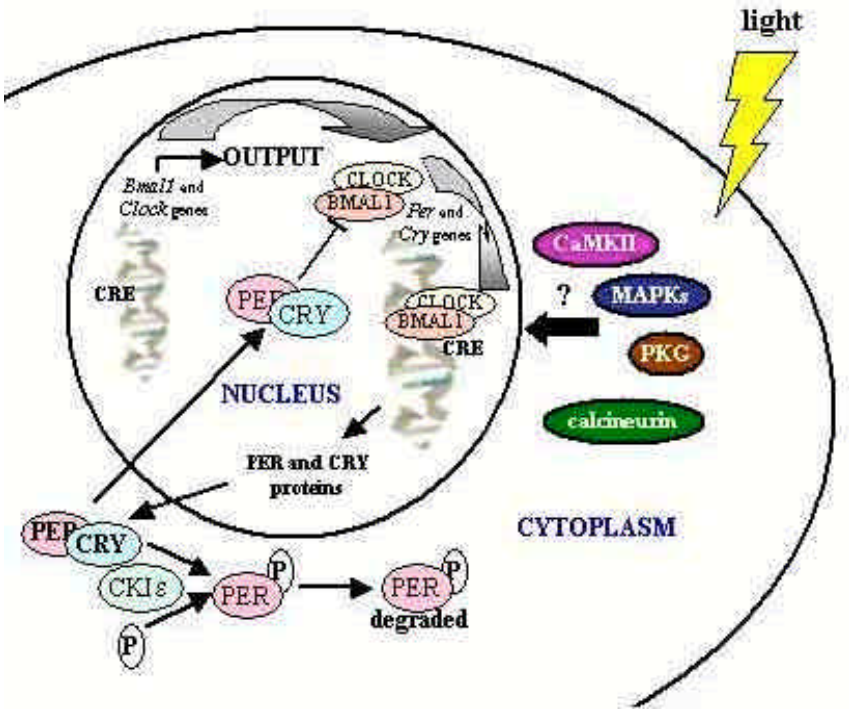

Figure 4. Signal transduction pathways in the SCN lead to changes in the expression and activity of clock genes. Kinases and phosphatases modify the activity of transcription factors (such as CREB) and/or clock genes. Clock genes and their products exhibit cyclic changes by means of feedback mechanisms: the CLOCK-BMAL dimer activates E-box sequences in several promoter regions, including those of the *per1* and *cry* genes, whose products in turn inhibit transcriptional activity of CLOCK-BMAL. PER1 also suffers posttranslational modification, being phosphorylated by casein kinase I epsilon (CKIε) and subsequently degraded. One of the putative ways of interaction with the aforementioned signal transduction pathways is through light-induced phosphorylation of transcription factors which might bind to clock gene promoters.

7. PERSPECTIVE: LIGHT TALKING TO THE CLOCK

So why is it important to understand these signal transduction pathways? On one hand, they might give us tools for treatment of "circadian" diseases, such as phase delay or advance of human sleep-wake cycle. Indeed, familial advanced sleep-phase disorder (FASPD) has been recently traced to a phosphorylation site mutation in the human *per2* gene (92). But also, we might predict that sooner or later the neurochemists, digging into the intracellular transduction pathway tunnels, will be able find a shortcut and to shake hands with the molecular biologists working out the orchestration of clock genes (93). Being able to disguise as light and enter the circadian signaling ways might give us better clues as to how does the clock really works and how has it been able to keep in track with the universe for the last few billion years. And, as ancient druids knew quite well, "*time and tide waiteth for no one*".

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