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HIGHLIGHTED TOPIC | *The Role of Clock Genes in Cardiometabolic Disease*

How nuclear receptors tell time

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Teboul M, Gréchez-Cassiau A, Guillaumond F, Delaunay F. How nuclear receptors tell time. *J Appl Physiol* 107: 1965–1971, 2009. First published July 23, 2009; doi:10.1152/japplphysiol.00515.2009.—Most organisms adapt their behavior and physiology to the daily changes in their environment through internal (~24 h) circadian clocks. In mammals, this time-keeping system is organized hierarchically, with a master clock located in the suprachiasmatic nuclei of the hypothalamus that is reset by light, and that, in turn, coordinates the oscillation of local clocks found in all cells. Central and peripheral clocks control, in a highly tissue-specific manner, hundreds of target genes, resulting in the circadian regulation of most physiological processes. A great deal of knowledge has accumulated during the last decade regarding the molecular basis of mammalian circadian clocks. These studies have collectively demonstrated how a set of clock genes and their protein products interact together in complex feedback transcriptional/translational loops to generate 24-h oscillations at the molecular, cellular, and organism levels. In recent years, a number of nuclear receptors (NRs) have been implicated as important regulators of the mammalian clock mechanism. REV-ERB and retinoid-related orphan receptor NRs regulate directly the core feedback loop and increase its robustness. The glucocorticoid receptor mediates the synchronizing effect of glucocorticoid hormones on peripheral clocks. Other NR family members, including the orphan NR EAR2, peroxisome proliferator activated receptors-α/γ, estrogen receptor-α, and retinoic acid receptors, are also linked to the clockwork mechanism. These findings together establish nuclear hormone receptor signaling as an integral part of the circadian timing system.

circadian physiology; feedback loop; hormones

MOST LIVING ORGANISMS ADAPT their behavior and physiology to the daily environmental changes caused by the light-dark cycle. In mammals (~24 h), circadian rhythms regulating key biological processes, such as sleep, body temperature, hormone secretion, blood pressure, and metabolism, are driven by self-sustained endogenous clocks located in all cells of the body and organized as a hierarchical system (34). The circadian system is composed of a master clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus that is directly reset by light every day through the retino-hypothalamic tract and that, in turn, synchronizes peripheral clocks through poorly defined neurohormonal pathways. Although peripheral clocks display self-sustained oscillations at the single-cell level, like those from the SCN neurons, at the organ and systemic levels they require the SCN clock to keep a defined phase relationship (71). Notably, they can also be entrained by hormonal and metabolic cues independently of the SCN (5, 11). Physiological outputs of central and peripheral clocks are ultimately regulated through the rhythmic transcription of tissue-specific, clock-controlled gene networks (14). Pioneering studies in the *Drosophila* model system have established the genetic basis of circadian oscillations and proposed, following the identification and analysis of the first clock gene *Period*, that a feedback loop mechanism was the molecular basis for circadian timing (7, 29). Clock genes have since then been identified from cyanobacteria to mammals. Although the molecular components are not evolutionary conserved, they interact to form transcriptional/translational feedback loop-based oscillators in all species. In mammals, the core loop generating the rhythm is interlocked with multiple additional regulatory loops, many of which involve nuclear receptors (NRs).

THE MAMMALIAN MOLECULAR CLOCK

During the last 12 yr, a considerable knowledge has accumulated regarding the molecular makeup of mammalian circadian clocks. Forward genetics and biochemical approaches have collectively identified a set of so-called clock genes and demonstrated that they interact through complex positive and negative feedback loops to form a molecular clock generating ~24-h oscillations. Importantly, the molecular basis for circadian clocks is comparable between the SCN neurons and non-SCN neurons or peripheral cells. In this mechanism, the two basic helix-loop-helix-Per-ARNT-Sim transcriptional activators CLOCK (or NPAS2 in some extra SCN tissues) and BMAL1 heterodimerize and transactivate the *Per1*, *Per2*, *Cry1*, and *Cry2* clock genes through binding to E-box DNA

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response elements located in their promoter regions. PER and CRY proteins then associate and translocate to the nucleus to repress their own genes by inhibiting CLOCK/BMAL1-dependent transcriptional activity (55). The repression potential of the PER/CRY complex is dependent on the PER and CRY isoform present (43, 46). This core feedback loop is the subject of an extensive and increasingly complex posttranslational control through which the oscillations are critically sustained and adjusted to a period length of ~24 h (Fig. 1) (20). A major posttranslational regulation of the molecular clock is the phosphorylation of PER1 and PER2 by casein kinase isoforms (CKI)-ε and CKI-δ and subsequent recruitment of the ubiquitin ligase adapter F-box protein bTrCP, followed by proteasomal degradation (16, 57). Consistently, mutation of a CKI phosphorylation site within the human PER2 protein has been shown to cause a familial advance sleep-phase syndrome due to a reduced delay of PER2 nuclear entry (61). The stability of PER2 is also regulated by the phosphatase PP1 and casein kinase 2 (19, 35). Additional CKI substrates include CRY and BMAL1 proteins, but, while degradation of CRY1 and CRY2 appears to be regulated through the F-box protein FBXL3, that of BMAL1 is dependent on sumoylation (10, 15, 23, 58). Furthermore, the circadian function of BMAL1 requires its acetylation by its heterodimerizing partner CLOCK, a reaction that is reversed by the action of the NAD^+-dependent deacetylase SIRT1 (4, 25, 39).

The CLOCK/BMAL1/PER/CRY core feedback loop is interlocked, with additional transcriptional loops thought to stabilize and increase the robustness of the oscillations. These loops all involve transcriptional regulators, which are primary targets of the CLOCK/BMAL1 heterodimer and reciprocally regulate either positively or negatively specific clock and clock-controlled gene promoters through their cognate response elements. An important such modulatory loop that links the positive and negative limbs of the core loop involves the REV-ERB/retinoid-related orphan receptor (ROR) NR subfamily. Although their description is beyond the scope of this review, it is important to mention the feedback regulation of the core loop by the three transcriptional activators, D-element binding protein, hepatic leukemia factor, and thyrotrophic embryonic factor, together with the E4BP4 repressor, as well as by the two basic helix-loop-helix transcriptional repressors DEC1 and DEC2 (38, 42). These transcriptional feedback loops are probably the tip of the iceberg, as many more regulatory mechanisms are predicted by system biology approaches (64), while a number of transcription factors, including NRs, appear to be regulators of core clock gene or protein activity.

ROLE OF THE REV-ERB AND ROR NRs IN THE MAMMALIAN CLOCKWORK

Analysis of the circadian phenotype of *Per2* mutant mice revealed that PER2 was an upstream positive regulator of *Bmal1* gene expression (56, 72). This suggested that PER2, a known inhibitor of CLOCK/BMAL1 activity, regulated *Bmal1* through an unknown repressor. Analysis of the *Bmal1* proximal promoter identified the presence of two specific response elements for *Rev-erb-α* (NR1D1), a NR previously implicated in lipid metabolism and known to be expressed with a robust circadian rhythm in many organs, including the SCN (6, 48, 51, 62). Consistently with the hypothesis that *Rev-erb-α* would be a link between *Per2* and *Bmal1*, disruption of the *Rev-erb-α* gene in mice resulted in an increased of *Bmal1* gene expression (48). This result, together with the observation that CLOCK and BMAL1 regulate positively *Rev-erb-α* expression through multiple E-box response elements, led to a model in which REV-ERB-α links the negative and positive limbs of mammalian clocks (48, 63). *Rev-erb-α*$^{-/-}$ mice exhibited an exacerbated response to light resetting and an unstable period length of their circadian locomotor activity; however, they remained rhythmic (48). This subtle circadian behavioral phenotype suggested that some functional redundancy could occur via REV-ERB-β (NR1D2), a paralog of REV-ERB-α also ex-

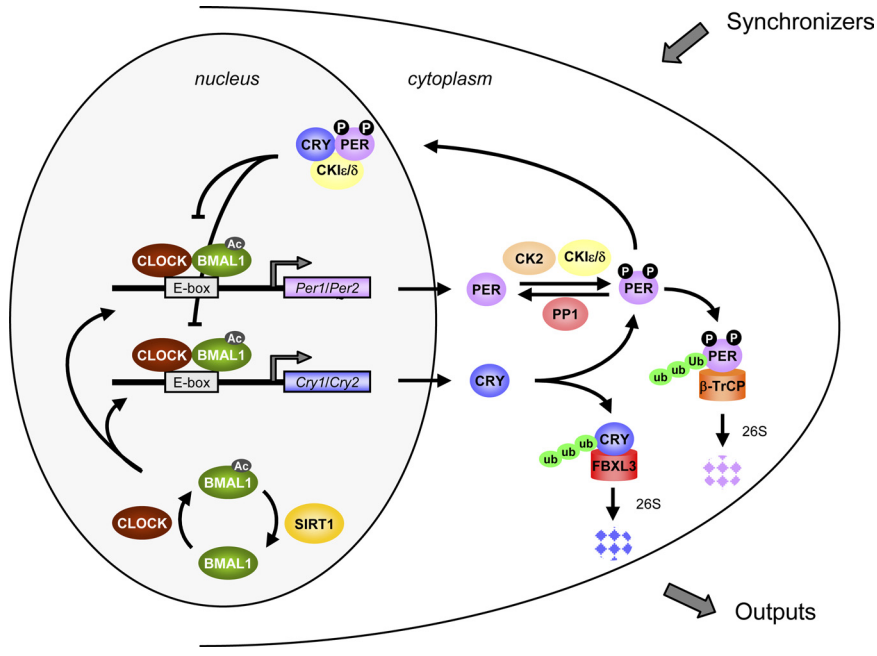

Fig. 1. The essential feedback loop governing mammalian circadian clocks. The model presented includes the core clock genes *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2*, which are essential for the generation of circadian oscillations. The stability of clock proteins is coordinately regulated by multiple posttranslational regulatory mechanisms to adjust the period length of the oscillations to 24 h. This molecular oscillator operating in both suprachiasmatic nuclei and non-suprachiasmatic nuclei neurons, as well as in peripheral cells, is reset by external or internal synchronizers and regulates downstream output processes.

pressed rhythmically in multiple tissues. By using small interfering RNA-mediated silencing of *Rev-erb-β* in *Rev-erb-α*$^{-/-}$ cells, Liu and colleagues (32) could demonstrate that both REV-ERBs were indeed redundant for driving the rhythmic transcription of *Bmal1*. This approach also showed that REV-ERBs control the rhythmic expression of the clock gene *Cry1*, as well as that of another NR, ROR-γ. However, cells lacking both REV-ERB-α and REV-ERB-β or expressing constitutive BMAL1 still displayed rhythmic *Per2* expression (32).

The REV-ERB response element, which consists of a consensus NR half-site motif flanked by a 6-bp AT-rich sequence $(A/T)_6$ PuGGTCA, is also bound by RORs, which, in contrast to REV-ERBs, activate transcription (18). A functional genomic strategy was used by Sato and colleagues (54) to discover that ROR-α (NR1F1) was rhythmically expressed in the SCN and activated *Bmal1* gene transcription. ROR-α also activates *Bmal1* transcription in peripheral tissues, and, in liver, this requires peroxisome proliferator activated receptor (PPAR)-γ coactivator-1α, which is a clock-controlled NR coactivator (33). As expected, the ROR-α-dependent transactivation of *Bmal1* was antagonized by REV-ERB-α. *Staggerer* mice, which expressed a nonfunctional truncated form of ROR-α, exhibited a decreased robustness of circadian rhythms. However, they showed a slightly shorter free-running period of the circadian locomotor activity rhythm like *Rev-erb-α*$^{-/-}$ mice. The paradoxically similar circadian behavioral phenotype in *Rev-erb-α*$^{-/-}$ and *Staggerer* mice may result from the fact that *Rev-erb-α* is negatively autoregulated and consequently positively regulated by ROR-α, unless the *Staggerer* mutation has other unknown effects (1, 52). In contrast, genetic ablation of ROR-β (NR1F2), a clock-controlled paralog of ROR-α expressed in the pineal gland, retina, and SCN, three essential structures of the mammalian circadian system, resulted in slightly longer free-running period of the rest-activity cycle (3). Interestingly many peripheral tissues rhythmically express ROR-γ (NR1F3), a third ROR-α paralog, which also can activate the *Bmal1* promoter (24). However, despite this biochemical evidence, loss of function experiments could not demonstrate a role for ROR-γ in the regulation of peripheral clocks, possibly because many peripheral cells constitutively express ROR-α (32). This does not exclude that ROR-γ plays a role in specific tissues devoid of the ROR-α protein.

ROR and REV-ERB NRs have long been considered as true orphan NRs. In particular, REV-ERBs lack the AF2 transactivation domain found in all ligand-activated NRs and have a ligand binding pocket that is too small to accommodate classical small lipophilic ligands. This view was recently challenged by structural and functional data, suggesting that the activity of both types of receptors is regulated by ligands. Cholesterol was, for instance, proposed to be a ROR-α ligand, while retinoids behave as inverse agonists for ROR-β and ROR-γ (28, 59). However, there is, to date, no evidence that these molecules regulate circadian clock function through RORs. More surprisingly was the finding that the ligand binding domain of both REV-ERB-α and REV-ERB-β reversibly associates with heme, an interaction that allows the recruitment of the corepressor, nuclear corepressor, and subsequent repression of *Bmal1* transcription (49, 70). Heme serves as a prosthetic group for a wide variety of proteins, such as hemoglobin, myoglobin, cytochrome b_5, as well as *P*-450 cytochromes. Interestingly, the heme bound E75 (NR1D3) protein, which is the *Drosophila* ortholog of vertebrate REV-ERBs, is a nitric oxide (NO) and carbon monoxide (CO) responsive protein (53). This finding was recently extended to mammalian REV-ERBs by structural studies showing that heme-bound REV-ERBs are redox and NO/CO sensors (36, 47). The REV-ERB-mediated transcriptional repression was further shown to be relieved upon treatment of cells with a chemical NO/donor. Expression of aminolevulinate syntase 1, the rate-limiting step in the heme synthesis, is regulated by the circadian clock, and heme is a component of the NO and CO-producing enzyme systems (27). Additionally, production of the redox cofactor NAD^+ is also under circadian regulation (40, 50). Thus the modulation of REV-ERBs by heme, diatomic gases, and the redox state provides an important novel feedback mechanism whereby the core clock mechanism can be regulated by the cellular metabolic status. Furthermore, this unanticipated mechanism of REV-ERB activity modulation is likely to play a significant role in the regulation of clock-controlled processes known to be regulated by NO, such as metabolism, blood pressure, and cell proliferation.

In analogy to core clock proteins, posttranslational control of REV-ERBs also involves more classical mechanisms, such as phosphorylation. REV-ERB-α, which is a short-lived protein, was recently shown to be stabilized upon phosphorylation by glycogen synthase kinase-3β (69). Interestingly, glycogen synthase kinase-3β-dependent phosphorylation of REV-EB-α is inhibited by lithium, a compound used to treat bipolar depression, a psychiatric disorder associated with altered circadian rhythms.

Collectively, these studies have established the ROR/Rev-erb/Bmal1 regulatory loop as an important functional component of SCN and peripheral clocks. However, in contrast to the SCN, the role of RORs remains unclear in the periphery, where REV-ERBs appear to be the main determinants of the high-amplitude oscillation of *Bmal1* transcription. The lack of a pronounced circadian phenotype in the absence of REV-ERBs or RORs suggests that their main role is to contribute to the robustness of the oscillation generated by the core loop and to drive the transcription of rhythmic outputs. Although the heme, redox, and gas modulation of REV-ERB activity has not yet been addressed in the context of the dynamic regulation of clock genes, it may further reinforce the role of REV-ERBs in the context of the metabolic regulation of central and peripheral clocks. Importantly, this unanticipated discovery is making REV-ERB receptors druggable NRs and will probably stimulate a novel research area aimed at evaluating how gas-releasing molecules could be used to target REV-ERBs in the treatment of disorders as diverse as depression and sleep disorders, diabetes, and cardiovascular disease.

HORMONAL AND METABOLIC REGULATION OF CLOCK GENES BY NRs

The daily oscillation of glucocorticoid synthesis and release has been documented for decades and is now known to involve complex interactions between the hypothalamo-pituitary-adrenal axis, the SCN clock, the adrenal clock, and the autonomous nervous system (13). In mice, most of the corticosterone biosynthetic pathway is indeed under circadian regulation, and ACTH stimulation of corticosterone secretion is itself gated by the adrenal clock (44, 45). One role of this regulation is to

modulate rhythmically pathways controlling hepatic glucose metabolism. However, another important role that has emerged is that glucocorticoid signaling can reset peripheral clocks (5). This effect requires the glucocorticoid receptor (NR3C1), as it is blocked by the RU486 antagonist and does not impact the central clock, because SCN neurons do not express the glucocorticoid receptor. In addition, glucocorticoid signaling seems to interfere with other resetting pathways, as it inhibits the uncoupling between central and peripheral clocks caused by restricted feeding (see below) (30). The molecular mechanism underlying the glucocorticoid-dependent resetting of peripheral clocks is not precisely defined, but the induction of the clock gene *Per1* through a functional glucocorticoid response element within the *Per1* promoter is involved (67). This effect is likely to explain the negative regulation of *Rev-erb-α* by glucocorticoids (62). Interestingly, normal rhythmic expression of *Per1* in the forebrain was also recently shown to require the orphan NR EAR2 (NR2F6) (66). Interestingly, *Ear2*$^{-/-}$ mice entrained less efficiently to photic and feeding time cues. These results collectively suggest that *Per1*, a clock gene known to play a major role in the resetting of the SCN clock, may also be an important mediator of peripheral and non-SCN brain clock entrainment via multiple NR signaling pathways (2).

A link between estrogen signaling and the core feedback loop of circadian clocks is suggested by several independent lines of evidence. The clock protein PER2 suppresses 17β-estradiol-dependent transcriptional activation in tumor cells, physically interacts with estrogen receptor (ER)-α isoform (NR3A1) and triggers its degradation (22). Conversely, the *Per2* gene is estrogen inducible in ER-positive mammary gland tumor cells, through a direct mechanism. Consistently, in the uterus from PER2::Luciferase reporter mice, 17β-estradiol shortens the period of the circadian oscillations, possibly through the regulation of the *Per2* promoter (41). It is unknown whether ER-β (NR3A2) and the estrogen-related orphan receptor ERR-α (NR3B1) behaves as ER-α in other cellular systems where they are expressed, but both of them have been shown to be clock regulated, indicating that estrogen and circadian signaling are intimately linked (8, 26).

Restricted feeding to daytime is a very potent synchronizer of peripheral clocks, independent of the SCN in nocturnal rodents (11, 60). Although this has been confirmed independently by many groups, the molecular and physiological basis of this effect has remained elusive. However, accumulating evidence suggests that metabolic pathways not only are clock outputs, but also participate in the regulation of the core clock mechanism. For instance, PPAR-α (NR1C1), which is a critical metabolic sensor regulating fatty acid oxidation in liver, is both a clock-controlled and a positive regulator of *Bmal1* expression (9, 12, 31). Consistently with the hypothesis that PPAR-α is a component of the input pathway to peripheral clocks, fenofibrate, a synthetic PPAR agonist, could synchronize circadian oscillations in vitro (9). This finding was recently extended by the discovery that PPAR-γ (NR1C3), a paralog of PPAR-α, forms also a transcriptional loop with *Bmal1* in the vascular system and participates in the circadian regulation of blood pressure and heart rate (65). Interestingly, PPAR-α and PPAR-γ are also positive regulators of *Rev-erb-α* in liver and the adipose tissue, respectively, an effect that may further modulate their direct effect on the *Bmal1* gene promoter (17, 21). In the vasculature, all-trans retinoic acid was also shown to reset the clock through the inhibition of CLOCK/

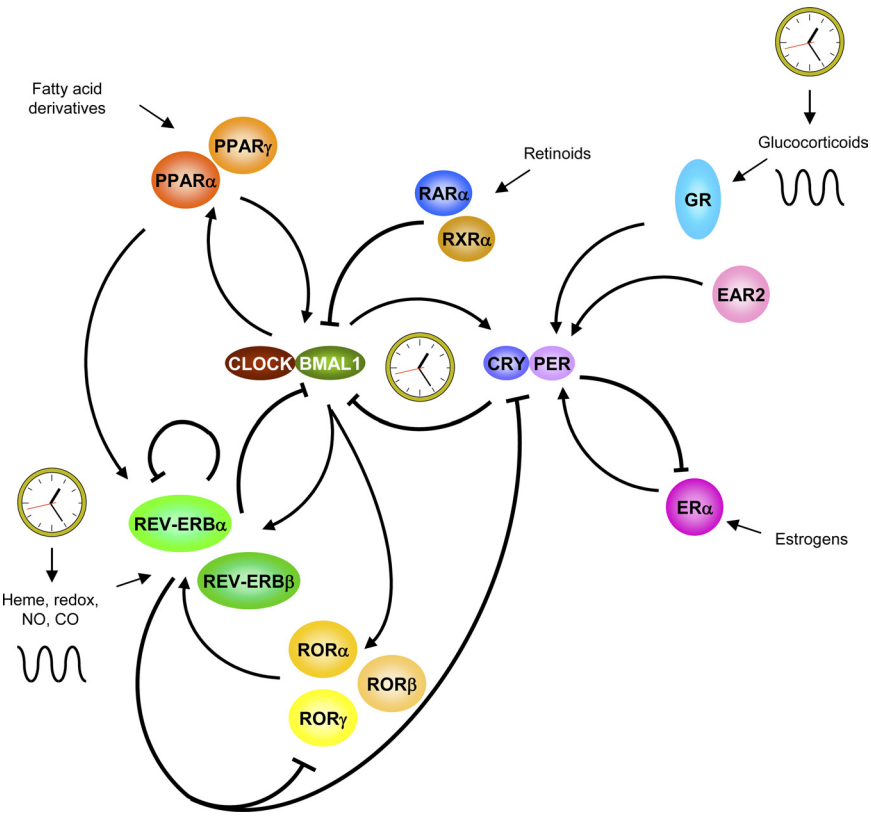

Fig. 2. A network of nuclear receptor signaling pathways regulates the core feedback loop of central and peripheral mammalian circadian clocks. Many of these nuclear receptor-dependent pathways are themselves regulated by clock genes and clock-regulated cues, such as glucocorticoids and heme. ER, estrogen receptor; GR, glucocorticoid receptor; PPAR, peroxisome proliferator activated receptor; ROR, retinoid-related orphan receptor; RAR, retinoic acid receptor, RXR, retinoid acid X receptor, NO, nitric oxide; CO, carbon monoxide.

BMAL1 transcriptional activity by the retinoic acid receptor-α (NR1B1) and retinoid acid X receptor-α (NR2B1) (37). This newly identified link between the PPAR signaling pathway and the essential clock gene *Bmal1* raised the important issue of the potential detrimental of beneficial effect of the drugs targeting NRs of the PPAR family on the clock system in specific organs or tissues. Indeed, one might hypothesise that PPAR ligands, such the thiazolidinediones, may perturb the clock mechanism, not only in the adipose tissue, but also in the cardiovascular system. Conversely, a better understanding of the cross talk between the clock and PPAR pathways may extend the therapeutic potential of PPAR synthetic ligands toward the treatment of circadian-related disorders.

SUMMARY AND CONCLUSION

A comprehensive framework is now available for understanding how circadian oscillations are generated, sustained, and entrained from the molecular to the organism level in mammals. While no NR taken independently seems to be an essential clock gene, accumulating evidence suggests that NR signaling is a pivotal interface between the molecular clock and physiology (Fig. 2). Indeed, we know now, as reviewed here, that at least 25% of the 48 mammalian NRs play a direct regulatory role in the core clock mechanism, while 50% exhibit a tissue-specific circadian expression profile (68). Importantly, many of the NRs that impinge the core feedback loop are metabolic sensors and cross talk. This suggests that the primary role of NR signaling in the circadian system is to coordinate metabolic inputs to modulate the core feedback loop. As a consequence of this tight and complex integration of the clock and NR signaling, many pathophysiological processes in which NRs are involved, and consequently therapies targeting NRs to treat these disorders, are likely to impact the circadian system. Reciprocally, with the continuous development of NR ligands or activators with increased specificity and tissue-selective modulatory activity, as well as the discovery gas sensor NRs, it may be possible to improve the treatment of circadian-related pathologies, such as sleep, mood, and metabolic disorders. These strategies may, for instance, target the resetting pathways of SCN or peripheral clocks or modulate clock-controlled outputs.

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