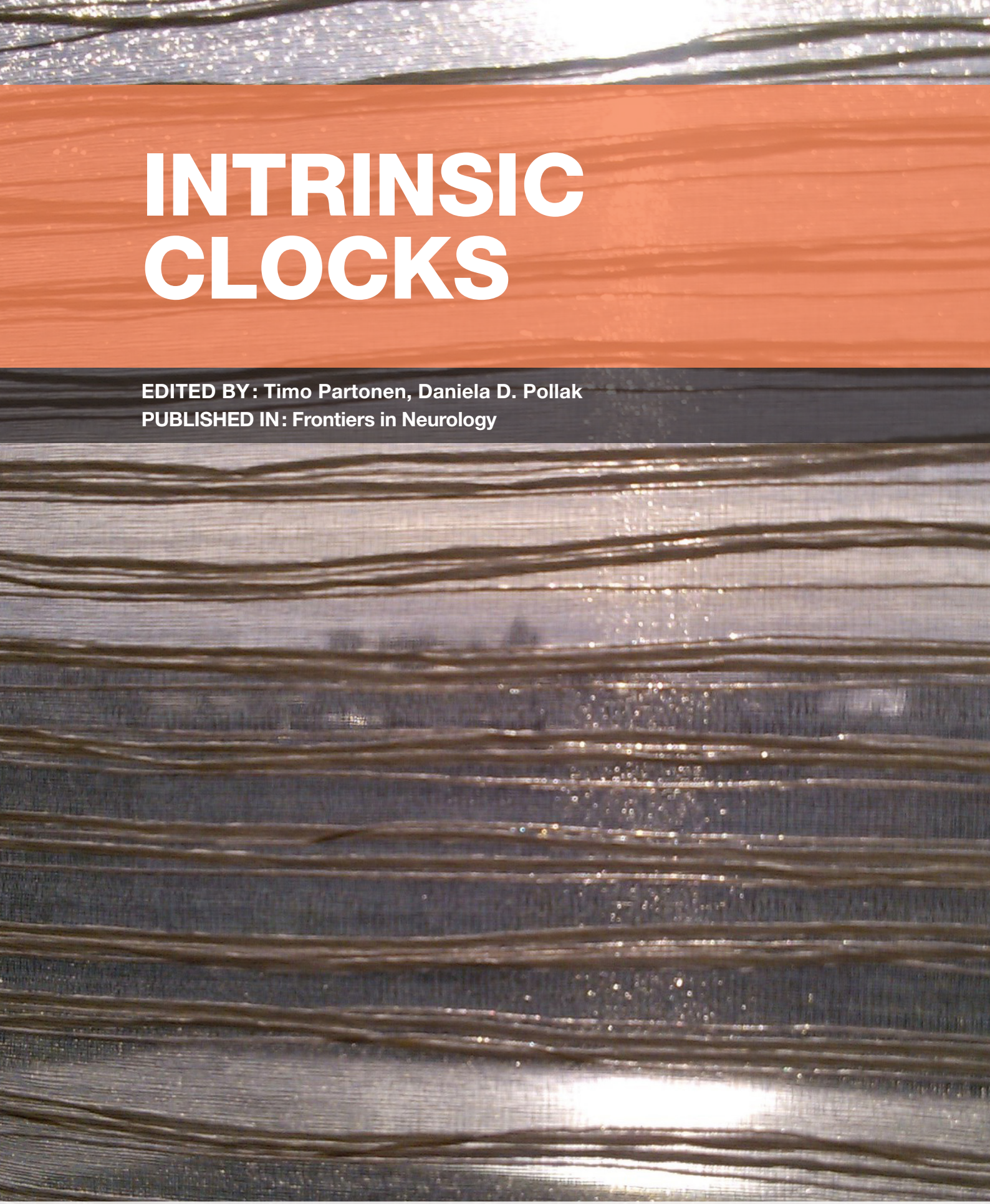

INTRINSIC CLOCKS

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INTRINSIC CLOCKS

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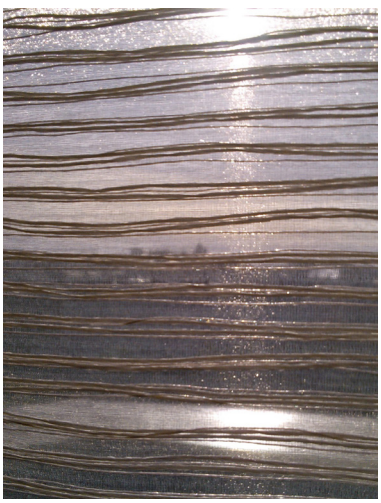

Light entrains the master intrinsic clock. Image: Timo Partonen.

"Intrinsic Clocks" presents an array of current research activities on intrinsic clocks and their contributions to biology and physiology. It elucidates the current models for the intrinsic clocks, their molecular components and key mechanisms as well as the key brain regions and animal models for their behavioral analysis.

It provides a timely view on how these clocks guide behavior, and how their disruption may cause depressive-like behavior and impairment in cognitive functions. Thereby, any specific method by which the mood-related functions of the intrinsic clocks might be influenced bears therapeutic potential and has clinical interest.

The importance of some of these mechanisms was highlighted by the 2017 award of the Nobel Prize in Physiology or Medicine to Jeffrey C. Hall, Michael Rosbash, and Michael W. Young for their discoveries of the genetic control of the daily biological rhythm. The key to the explanation was the discovery of transcription-translation feedback loops of the so-called "clock genes."

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5. Potential Therapeutics

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EDITORIAL
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Editorial: Intrinsic Clocks

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Keywords: circadian rhythm, depression, marine biology, mouse model, plasticity, seasonality, small-molecule, systems biology

Editorial on the Research Topic

Intrinsic Clocks

The existence of living organisms on our planet has been dependent on and co-evolved with the foreseeable variations in environmental conditions oscillating over recurring periods. All species have responded to these exogenous rhythms by developing endogenous clocks that allow for an approximate, but reliable estimation of the periodic changes and elicit corresponding adaptive processes.

The importance of these mechanisms for health and disease has been highlighted by the 2017 award of the Nobel Prize in Physiology or Medicine to Jeffrey C. Hall, Michael Rosbash, and Michael W. Young for their discoveries of the genetic control of the daily biological rhythm. They explained in molecular terms how the gene named as *period* contributed to the emergence (eclosion from the pupal case) rhythm of a population and to the locomotor activity of individual flies (*Drosophila melanogaster*). The key to the explanation was the discovery of transcription-translation feedback loops of the so-called "clock genes."

This research topic on *Intrinsic Clocks* which appeared earlier comprises a well-balanced collection of original research and review articles on endogenous rhythms from seasonal and monthly to daily and hourly oscillations in different experimental model systems with analytical approaches from systemic to cellular and molecular levels.

Serchov and Heumann in their review focus on the role of Ras, an enzyme which hydrolyzes guanosine triphosphate and dependent intracellular signaling cascades in the regulation of the circadian rhythm in mice. They elegantly summarize how Ras activity forms a molecular bridge between entrainment of the suprachiasmatic nucleus that is the master clock in the brain and synaptic plasticity in dependent brain regions, such as the hippocampus, and corresponding functions. The extensive study by Chiang et al. specifically investigated rhythmic alterations in the murine hippocampus. They characterized the protein phosphorylation using a mass spectrometry approach with which they provided large-scale quantitative analysis of the daily oscillation of hippocampal phosphorylation events over a range of biological pathways. The hippocampus is a key focus also in the review by Urs Albrecht. It features the role of circadian proteins in the control of adult hippocampal neurogenesis, reciprocally implicated in depression and antidepressant responses. He discusses neurobiological mechanisms implicated in the pathogenesis of mood disorders, such as monoaminergic neurotransmission and stress response by the hypothalamic–pituitary–adrenal axis. The hypothalamus and the pituitary are further involved in seasonal cycles as highlighted in the review by Lewis and Ebling who elaborate in detail on the role of tanycytes, pituitary radial glial cells, in the regulation of circannual clocks in hamsters. They provide evidence supporting their hypothesis that tanycytes serve as central organizers of seasonal rhythms in the adult hypothalamus. Raible et al. present in their review on marine animals the current insight in the cellular mechanisms in molecular detail the monthly or semi-monthly rhythms. They express their worry about light pollution and further review the relevance of circalunar rhythms to mammalian physiology

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and reproduction in specific. They speculate that these rhythms may be the remnant of evolutionary ancient clocks, which were uncoupled from a natural entrainment mechanism.

Bourguignon and Storch summarize recent findings of the cellular substrate and mechanism, which generate locomotor activity with periods of 2–6 h. Such rhythms are normally integrated with circadian rhythms, but often lack the period stability and expression robustness. They further review the concept of the dopaminergic ultradian oscillator and show that ultradian locomotor rhythms rely on cells in the brain using dopamine for transmission. Intriguingly, Monje et al. report in their study on interleukin-6 knockout mice that the ultradian locomotor rhythm was impaired under both light-entrained and free-running conditions, whereas the circadian period and the level of locomotor activity as well as the phase shift response to light exposure at night remained normal. During the day, *Cry1* and *Bhlhe41* expression levels were increased whereas those of *Nr1d2* were decreased in the hippocampus. Liu and Zhang first created mutants of cryptochrome circadian clock 1 (Cry1) protein at potential phosphorylation sites and conducted thereafter a screen in *Cry1*/*Cry2* double deficient cells. They targeted at identifying mutations that disrupted circadian rhythms. They found that these single amino acid substitutions changed not only the circadian period, but also repression activity, protein stability, or cellular localization of the protein. Concerning the circadian period, Narasimamurthy and Virshup elucidate in their review the molecular mechanisms that regulate an enigma of the clock. Unlike other chemical reactions, the output of the clock as measured with the period remains nearly constant with fluctuations in ambient temperature. This is called as temperature compensation. The key lies especially in the mechanism that controls the stability of period circadian clock 2 protein. Clock-enhancing small molecules have become of particular interest as candidate chronotherapeutics, since there is a close association of circadian amplitude dampening with progression of chronic diseases, especially that of mood disorders. Gloston et al. present in their review an update of the regulatory mechanisms of circadian amplitude and the current status of these small molecules of therapeutic interest. Millius and Ueda introduce the readers to study of biology which takes advantage of engineering and mathematical tools to model and test the behaviors of the intrinsic clocks. It has evolved through the development of both wet lab and *in silico* work. The goal here is to understand the clocks that are made up of a range of complex properties of cells, tissues, and organisms.

The cross-section of studies comprised in this research topic on *Intrinsic Clocks* highlights the vibrant scientific activity in the field of the investigation of endogenous biological rhythms and their relevance for physiology and pathology.

AUTHOR CONTRIBUTIONS

TP and DP planned and wrote the manuscript together.

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MINI REVIEW
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Molecular Mechanisms in Mood Regulation Involving the Circadian Clock

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The circadian system coordinates activities and functions in cells and tissues in order to optimize body functions in anticipation to daily changes in the environment. Disruption of the circadian system, due to irregular lifestyle such as rotating shift work, frequent travel across time-zones, or chronic stress, is correlated with several diseases such as obesity, cancer, and neurological disorders. Molecular mechanisms linking the circadian clock with neurological functions have been uncovered suggesting that disruption of the clock may be critically involved in the development of mood disorders. In this mini-review, I will summarize molecular mechanisms in which clock components play a central role for mood regulation. Such mechanisms have been identified in the monoaminergic system, the HPA axis, and neurogenesis.

Keywords: clock genes, depression, monoamines, glucocorticoids, neurogenesis

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A plethora of human genetic studies have identified polymorphisms in clock genes that associate with psychiatric disorders [reviewed in Ref. (1)]. This suggested that abnormalities in clock genes may be one of the causes for the development of mood disorders. At the cellular level, clock genes (*Bmal1*, *Clock*, *Per*, *Cry*, *Rev-erb*, and *Ror*) make up an autoregulatory transcriptional/translational feedback loop with a period of about 24 h (**Figure 1**, top gray circle) [reviewed in Ref. (2)]. These clock genes and their proteins not only self-promote their own temporally fluctuating transcription but they also regulate transcription of target genes (**Figure 1**) and/or modulate key molecular pathways *via* protein–protein interactions, such as the monoaminergic system, the HPA axis, or neurogenic pathways.

TRANSCRIPTIONAL REGULATION OF MONOAMINE SIGNALING BY CLOCK COMPONENTS

Neuroimaging studies in humans indicated that the monoaminergic system (dopamine, serotonin, and noradrenaline) was altered in subjects with mood disorders (3). This was further supported by optogenetic studies, in which control of neuronal activity of dopamine neurons in mice modulated mood, anxiety, and reward, confirming the importance of the monoaminergic system in mood-related behaviors (4, 5).

Interestingly, several studies described daily changes in dopamine, serotonin, and noradrenaline levels [reviewed in Ref. (6)]. Because these molecules modulate arousal, motivation, and reward, one would expect them to be targeted at the activity period of the day in order to avoid conflicts with sleep signals. Hence, monoaminergic signaling is likely to be regulated by the circadian clock, either directly or indirectly. In the last years, several investigations aimed at uncovering the role of circadian clock components in the direct transcriptional regulation of

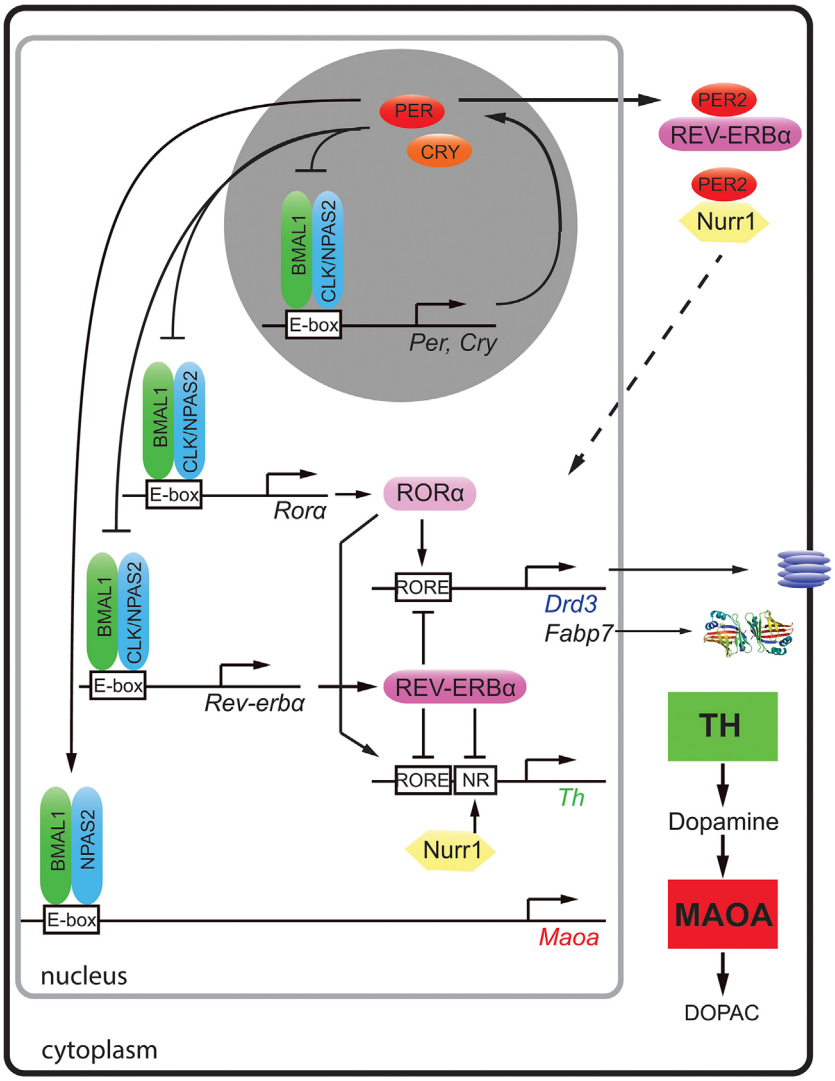

FIGURE 1 | Molecular regulation of clock and clock-controlled genes of the monoaminergic system and neurogenesis. The clock proteins BMAL1 (green), CLOCK (blue), and NPAS2 (blue) bind to E-box elements present in the promoters of clock genes (*Per*, *Cry*, *Rorα*, and *Rev-erbα*) and the clock-controlled gene for monoamine oxidase A (*Maoa*). PER (red) and cryptochrome (CRY, orange) proteins inhibit the action of BMAL1/CLOCK and BMAL1/NPAS2 heterodimers, respectively. The nuclear receptors [retinoic orphan receptor α (RORα, rose)] and REV-ERBα (purple) both bind to RORE elements of dopamine receptor 3 (*Drd3*), fatty acid binding protein 7 (*Fabp7*), and tyrosine hydroxylase (*Th*) in a competitive manner and activate or inhibit their expression, respectively. The nuclear receptor Nurr1 (yellow) regulates *Th via* its NR promoter element. *Via* protein–protein interactions, PER2 can modulate the actions of REV-ERBα and Nurr1 (hatched arrow). This regulation results in temporally regulated expression of the dopamine synthesizing (TH, green square) and degrading enzymes (MAOA, red square) leading to fluctuating levels of dopamine in the striatum.

elements important for monoaminergic signaling, such as the enzymes monoamine oxidase (MAO) and tyrosine hydroxylase (TH) both key enzymes for the degradation and synthesis of dopamine, respectively.

Dopamine degradation is under clock control. This was first suggested by the observation that the clock components BMAL1 and NPAS2 transcriptionally activated a luciferase reporter driven by the murine monoamine oxidase A (*Maoa*) promoter in a circadian fashion. This indicated that these two clock components directly regulated *Maoa* transcription (**Figure 1**). This notion was further strengthened by the observation that BMAL1 protein was recruited to the *Maoa* promoter in brain tissue (7). Interestingly, the regulation by BMAL1/NPAS2 was modulated by PER2 in a positive fashion, but not in the predicted negative manner (**Figure 1**). This lead to increased *Maoa* mRNA levels (7). This finding suggested potential tissue specific regulatory factors that turned PER2 into a positive regulator of BMAL1/NPAS2-driven transcriptional regulation in the striatum. As a consequence of lack of PER2, not only *Maoa* mRNA but also MAOA protein levels were decreased. Hence, dopamine degradation was reduced, and dopamine levels in the nucleus accumbens were increased. This was paralleled by

a depression-resistant-like phenotype and changes in neuronal activity in response to MAO inhibitors in mice (7). These findings strongly suggested that the degradation of monoamines was clock modulated. It is very likely that the described clock-mediated regulation of monoamines is relevant for humans, because single-nucleotide polymorphisms in *Per2*, *Bmal1*, and *Npas2* associated in an additive fashion with seasonal affective disorder or winter depression (8).

A recent study showed that not only dopamine degradation but also dopamine synthesis is under clock influence. The mouse, rat, and human *Th* promoters were repressed by REV-ERBα, and they were activated by retinoic orphan receptor α (RORα) and nuclear receptor-related protein 1 (NURR1) (9). Chromatin immunoprecipitation experiments revealed that REV-ERBα and NURR1 were binding to the *Th* promoter in an antagonistic manner (9). In accordance with this mechanism (**Figure 1**), *Rev-erb*α knock-out mice displayed elevated *Th* mRNA and protein levels leading to increased dopamine amounts and firing rate in the striatum (9, 10). As a consequence, these animals showed less depression-like and anxiety-like behavior compared to wild-type animals (9). The temporal regulation of TH may be further modulated through protein–protein interactions. For example, PER2 has the potential to interact with both REV-ERBα and NURR1 proteins (11), which would allow temporal synchronization of the action of these two nuclear receptors (**Figure 1**, top right, hatched arrow). This is, however, a speculation and needs verification.

Interestingly, REV-ERBα and RORα were described to regulate the expression of the dopamine D3 receptor gene (*Drd3*) in an antagonistic manner (12) (**Figure 1**). This provided a molecular explanation why this receptor was expressed in a diurnal manner in the striatum (13). DRD3 inhibits adenylyl cyclase through inhibitory G-proteins [reviewed in Ref. (14)] and mutation of DRD3 in mice suggested an involvement of this receptor in mediating emotional behavior and depression in mice (15). A role of NPAS2 in the regulation of *Drd3* has also been suggested (16), although it is unclear how NPAS2 would regulate the *Drd3* promoter. Taken together, it appears that REV-ERBα and RORα synchronize dopamine production and the expression of DRD3 in the striatum probably to optimally restrict dopamine signaling in the striatum to a particular time window. This implies that the targeting of DRD3 and/or REV-ERBα/RORα by pharmacological agents may benefit from timed application. This would reduce dosage and diminish side effects such as weight gain, which is observed often in patients treated for mood disorders.

MOLECULAR REGULATION OF COMPONENTS OF THE HPA AXIS BY CLOCK PROTEINS

Epidemiological studies suggested that stressful life events play a role in the etiology of depression (17), and hypercortisolemia was observed in a subset of patients with depression [reviewed in Ref. (18)]. Furthermore, antidepressant treatment appeared to stabilize the function of the HPA axis *via* the serotonergic system (19), suggesting an involvement of the HPA axis and glucocorticoids in mood regulation [reviewed in Ref. (20)].

Conditional mutagenesis in mice of the glucocorticoid receptor (GR) in the nervous system provided evidence for the importance of GR signaling in emotional behavior (21). Overexpression of GR lead to depressive-like behavior, and these mice showed enhanced sensitization to cocaine (22), consistent with observations that GR may be a potential target to reduce cocaine abuse (23). Interestingly, GR bound to NURR1 thereby increasing the transcriptional potential of NURR1 to induce TH (24) (**Figure 1**). Hence, the amount of nuclear GR appeared to be important for this function. Although glucocorticoids displayed circadian rhythmicity [reviewed in Ref. (25)], GR expression was constant over 24 h in the liver, which applies most likely to the brain as well. However, GR nuclear localization appeared to be gated by REV-ERBα in the liver with nuclear GR levels high at zeitgeber time 20 (activity period of mice) (26). If this would apply to the brain, REV-ERBα would gate binding of GR to NURR1 for induction of the *Th* promoter (**Figure 2**). As illustrated above, mood-related behavior and dopamine levels were changed in *Rev-erb*$\alpha^{-/-}$ mice, and this may also involve GR, which regulates catechol-*O*-methyltransferase (26), an enzyme degrading the MAOA product 3,4-dihydroxyphenylacetic acid to homovanillic acid. Therefore, it is likely that the monoaminergic system and the glucocorticoid pathway are linked *via* GR.

The cryptochrome (CRY) proteins interact with GR in a ligand-dependent manner in mouse liver leading to rhythmic repression of GR activity (27). Additionally, the CRY proteins participate in glucocorticoid-dependent suppression of the HPA axis and the production of endogenous glucocorticoids (27). Mice

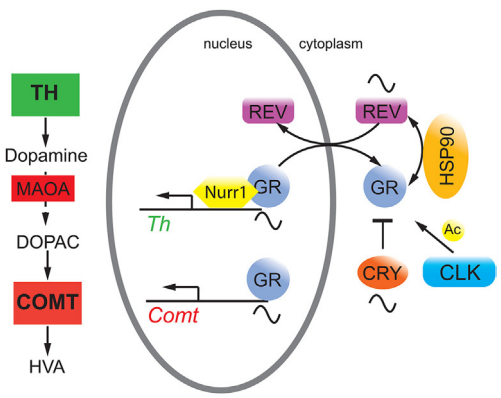

FIGURE 2 | Hypothetical model on the interaction of circadian clock proteins with the glucocorticoid receptor (GR). REV-ERBα (REV, purple) gates nuclear localization of the GR (gray) *via* an unknown mechanism probably involving heat shock protein 90 (HSP90, yellow). GR function is inhibited by cryptochrome (CRY, orange) proteins and is modulated by CLOCK (blue) *via* acetylation (Ac), although it is unclear whether this happens in the cytoplasm and/or the nucleus. GR regulates target genes such as *catechol-O-methyltransferase* (*Comt*) whose protein is an enzyme (COMT, red square) that degrades 3,4-dihydroxyphenylacetic acid (DOPAC) to homovanillic acid (HVA). GR may also interact with Nurr1 to modulate *tyrosine hydroxylase* (*Th*) expression thereby influencing dopamine production.

lacking *Cry1* showed depression-like behavior combined with reduced levels of dopamine in the striatum (28). This phenotype was most likely the result of the effects of CRY on both pathways illustrated in **Figures 1** and **2**. Furthermore, GR was acetylated by CLOCK, which lead to decreased sensitivity to glucocorticoids in the morning in humans and to an increased sensitivity at night when acetylation was reversed (29).

Recently, CHRONO, a protein that acts as a repressor in the circadian clock mechanism similar to CRY2 appeared to have the potential to interact with GR as well (30). Interestingly, *Chrono* mRNA was induced in the hypothalamus after stress stimulation whereas *Cry2* mRNA was not. This suggested that CHRONO may be a stress-inducible repressor of the circadian clock coupling the clock with the HPA axis (30). However, it is not known whether *Chrono* knock-out mice display alterations in mood-related behaviors.

TRANSCRIPTIONAL REGULATION OF NEUROGENESIS BY CLOCK PROTEINS

Adult neurogenesis is an important process to replace lost or dysfunctional neurons with new neurons produced from neuronal stem cells. Most of them are found in the subventricular zone lining the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus. Environmental stimuli, such as stress, physical activity, sleep deprivation, enriched living conditions, and jet-lag, can influence adult hippocampal neurogenesis in mammals (31–35). These environmental stimuli directly affect the circadian clock as well [reviewed in Ref. (36)], suggesting that the clock plays a mediator role between environmental change and neurogenesis. Animal studies showed that chronic stress and depression-inducing behavior reduced hippocampal neurogenesis while antidepressants enhanced it (37), suggesting a connection between neurogenesis and depressive behavior (38). Hence, change of the clock by environmental stimuli may affect neurogenesis, which in turn affects mood-related behaviors. Interestingly, neurogenesis varied over the day (39–42), and mutations in clock genes affected adult hippocampal neurogenesis (28, 43–46). The effect of the clock on this process was at least in part due to the control of the timing of cell-cycle entry and exit of quiescent neural progenitor cells (QNPs) (47). For example, absence of *Per2* abolished the gating of cell-cycle entrance of QNPs (43, 47), whereas lack of *Bmal1* resulted in constitutively high levels of proliferation and delayed cell-cycle exit (46, 47).

On the molecular level evidence of direct clock gene-mediated regulation of neurogenesis is scarce. The mechanism of *Clock*- and *Bmal1*-mediated neuronal differentiation appeared to be associated with the neurogenic transcription factor NeuroD1 (48), although a direct regulation of its promoter by clock genes was not shown. In contrast, the regulation of fatty acid binding protein 7 (*Fabp7*), also termed brain lipid-binding protein, by the clock component REV-ERBα has been elucidated (44). FABP7 facilitates the solubility of long-chain fatty acids and is implicated in cell growth and differentiation (49). It affects neuronal differentiation (50) and is a marker for neuronal progenitor cells (51, 52). The promoter of the *Fabp7* gene was directly suppressed by REV-ERBα, and this suppression was relieved by RORα, a positive competitor of REV-ERBα (**Figure 1**) (44). Mice lacking *Rev-erb*α displayed increased levels of FABP7, which was associated with alterations in mood-related behaviors, changes in hippocampus-dependent cognitive performance, and increased hippocampal neurogenesis (44).

Taken together, this overview illustrates multiple levels of molecular mood regulation with REV-ERBα (and PER2 as REV-ERBα modulator) being involved in all of the processes described; regulation of the monoaminergic system, the HPA axis, and neurogenesis.

In the future, a better understanding of the hypothetical molecular processes illustrated in **Figure 2** will be of great importance, because it is unknown whether CRY and CLOCK affect GR function in the nucleus or the cytoplasm. This would distinguish whether the influence of these two clock components is directly on transcription or on modulation of GR protein stability and transport, which would influence GR-mediated transcription in an indirect manner. Furthermore, the posttranslational regulation of REV-ERBα is poorly understood with the exception of its residues S55/S59, which are phosphorylated by GSK3β and may mediate cellular sensitivity to lithium (53). Time–of-day-dependent phosphorylation sites on REV-ERBα and GR (54) may contribute to the gated regulation of nuclear presence of these two receptors and hence on the regulation of metabolism and mood-related behaviors.

AUTHOR CONTRIBUTIONS

UA wrote the manuscript and prepared the figures.

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Ras Activity Tunes the Period and Modulates the Entrainment of the Suprachiasmatic Clock

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The small GTPase Ras is a universal eukaryotic cytoplasmic membrane-anchored protein, which regulates diverse downstream signal transduction pathways that play an important role in the proper functioning of neurons. Ras activity is a central regulator of structural and functional synaptic plasticity in the adult nervous system, where it channels neuronal responses to various extracellular cues allowing the organism to adapt to complex environmental stimuli. The suprachiasmatic nucleus (SCN) is the principle pacemaker of the circadian clock, and the circadian and photic regulation of Ras activity in the SCN is an important modulator of the clockwork. We have generated transgenic mouse expressing constitutively active V12-H-Ras selectively in neurons *via* a synapsin I promoter (synRas mice), which serves as a suitable model to study the role of neuronal Ras signaling. Modulation of Ras activity affects ERK1,2/CREB signaling and glycogen synthase kinase-3 beta expression in the SCN, which in turn modify the photoentrainment of the clock and the fine tuning the circadian period length. The main focus of this review is to offer an overview of the function of Ras signaling in the circadian rhythm and its potential role in learning and memory consolidation.

Keywords: Ras, circadian, glycogen synthase kinase-3 beta, synRas mice, ERK1/2

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INTRODUCTION

Most organisms living on earth exhibit circadian rhythm controlled by autonomous timekeeping circadian clock. The circadian oscillation of intracellular clock is driven by transcription/translation-based feedback/feedforward loops, composed of a set of clock genes, as well as kinases and phosphatases that regulate the localization and stability of the clock gene protein products. Positive regulatory elements are BMAL1 and CLOCK, which form heterodimer and regulate the rhythmic transcription of Period (Per1 and Per2) and Cryptochrome (Cry1 and Cry2) genes. The PER and CRY proteins interact and translocate to the nucleus, where they act as negative regulators inhibiting further transcriptional activation. In addition to the transcriptional regulation, posttranslational mechanisms, such as phosphorylation of core clock proteins, play an important role in the regulation of the circadian clock. The casein kinases and glycogen synthase kinase-3 beta (GSK3β) have a critical function in the control of circadian period length by phosphorylating several core clock proteins, regulating their degradation, protein stability, and nuclear translocation (1–5).

In mammals, the circadian master pacemaker is located in the suprachiasmatic nucleus (SCN) of the ventral hypothalamus (6, 7). The SCN synchronize numerous biochemical, physiological, and behavioral processes in the peripheral organs with an approximate 24 h periodicity. An important feature of circadian clockwork is the ability to be reset by light, thus, allowing animals to adjust their biological rhythms to changes in the length of daytime and nighttime (6).

Recently, we demonstrated that the circadian and photic activation of the small GTPase Ras is an important modulator of the clockwork in the SCN. Ras activity fine tunes the period length and modulates photoentrainment of the circadian clock (8). The main focus of this review is to offer an overview of the function of Ras signaling in the circadian rhythm and its potential role in learning and memory consolidation.

Ras SIGNALING AND synRas MOUSE MODEL

Ras is a universal eukaryotic intracellular membrane-anchored protein, which cycles between inactive GDP-bound and the signaling competent GTP-bound conformation. Several extracellular signals from multiple receptor types and intracellular second messengers converge onto the activation of Ras, including neurotrophin tyrosine kinase receptors, G-protein-coupled receptors, and local increase of intracellular Ca^{2+} concentration or Ca-calmodulin kinase II, resulting in the activation of NMDA receptors or voltage-gated Ca^{2+} channels (9, 10). Ras once activated transduces signals to several signaling pathways, including the major mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase (ERK) cascade and phosphatidylinositol-3 kinase/Akt pathway. Ras plays a central role as a regulator of structural and functional synaptic plasticity in the adult mammalian brain modulating neuronal architecture and synaptic connectivity and tuning synaptic efficacy (11–14).

In order to study the role of Ras and its specific downstream effectors, we have generated a transgenic mouse model, which expresses constitutively active V12-H-Ras selectively in neurons *via* synapsin I promoter (synRas mice) (15). The synRas mice have brain hypertrophy, which results from an increased cell size and changed morphology of the pyramidal neurons (14, 15). The constitutively activated Ras increases the dendritic length, complexity, and spine density leading to a change in synaptic connectivity in the synRas mice cortex (12–14, 16). The investigation of the signal transduction in the synRas neurons showed that the expression of the constitutively activated V12-H-Ras leads to drastic increase of Ras activity and corresponding elevation of the phosphorylation level of MAPK (ERK1,2) in the cortex and hippocampus. No such changes have been observed in PI(3)K/Akt activity in adult synRas mice (15). In addition, we found increased total expression level of GSK3β (17), which might be result of enhanced Ras–MAPK signaling and ETS-p300 transcriptional complex activation (18). Furthermore, specific increases of pCREB and brain-derived neurotrophic factor (BDNF) levels in the cortex of synRas mice during the developmental stages—postnatal day 7—have been described (19).

Ras SIGNALING AND PHOTOENTRAINMENT OF THE CIRCADIAN CLOCK IN SCN

The potential involvement of Ras signaling in the regulation of circadian clock has been proposed in numerous studies (8, 20–27). The small GTPase Ras appears to be the major effector of BDNF-mediated signaling and one of the main upstream regulators of ERK pathway resulting in elevated levels of CREB phosphorylation (19) (**Figure 1**). Indeed, the activation of MAPK pathway and particularly ERK1,2 and its coupling to the activation of transcription factors Elk-1 and CREB (28, 29) is an important molecular mechanism for photoentrainment of the SCN (**Figure 1**). *In vivo* studies have shown that inhibition of ERK1,2 in mouse SCN attenuates both the phase shifting effects of light (28, 30) and immediate early gene expression (31). BDNF and its receptor, TrkB, are also necessary for photic resetting. BDNF protein levels oscillate in the SCN with high levels at night, when photic stimulation and glutamate can reset the circadian clockwork (32). The inhibition of TrkB receptors blocks photic- and glutamate-induced clock resetting (33, 34).

Consistently, photic stimulation at early and late subjective night activates Ras in the SCN (8) and Ras activation correlates with the length of the light exposure (20), suggesting a direct involvement of Ras in the signaling pathways, coupling photic input to the SCN clock. The light stimuli induce glutamate release from the nerve terminals of the retino-hypothalamic tract, which results in activation of NMDA receptors with a subsequent influx of Ca^{2+} (35, 36), activating the Ca^{2+}-calmodulin kinase II that in turn stimulates Ras (9, 10) (**Figure 1**).

Direct evidences for the involvement of Ras in the molecular mechanisms that adjust the circadian clock to the light/dark cycle come from the synRas mice (8, 25). The enhanced Ras activation in the SCN of synRas mice leads to potentiation of the light-induced phase delays at early night and total inhibition of the light-induced phase advances at late night of spontaneous locomotor activity (8). The magnitude of Ras-regulated ERK phosphorylation correlates with the extent of the phase delays at early subjective night—with stronger ERK activation leading to larger phase delays in circadian behavior (20, 37). ERK1,2 phosphorylates p90 ribosomal S6 kinase, which in turn phosphorylates CREB, required for the photic resetting of the SCN (38, 39). In addition, ERK1,2/CREB pathway couples light to immediate early genes expression c-Fos, a robust marker of SCN activation by photic stimuli, and the induction of the clock gene PER1 (31, 40, 41). Therefore, the enhanced activation of the Ras/ERK1,2/CREB pathway in the SCN of synRas mice at early subjective night leads to increased phase delays and enhanced photic induction of c-Fos protein expression in the SCN. Though several reports have shown that the photic stimulation of ERK1,2/CREB phosphorylation is an essential event for the clock photoentrainment, the activation of this pathway is not sufficient to induce c-Fos expression and phase advance the clock of synRas mice at late subjective night (8, 37). Thus, the enhanced basal levels of activation of Ras/ERK in the SCN of synRas mice at early subjective night phase delay the circadian clock and compensate the photic-induced resetting in the late subjective night.

Ras SIGNALING AND CIRCADIAN PERIOD LENGTH

Several reports demonstrated circadian oscillation of Ras activity in various brain regions and peripheral organs,

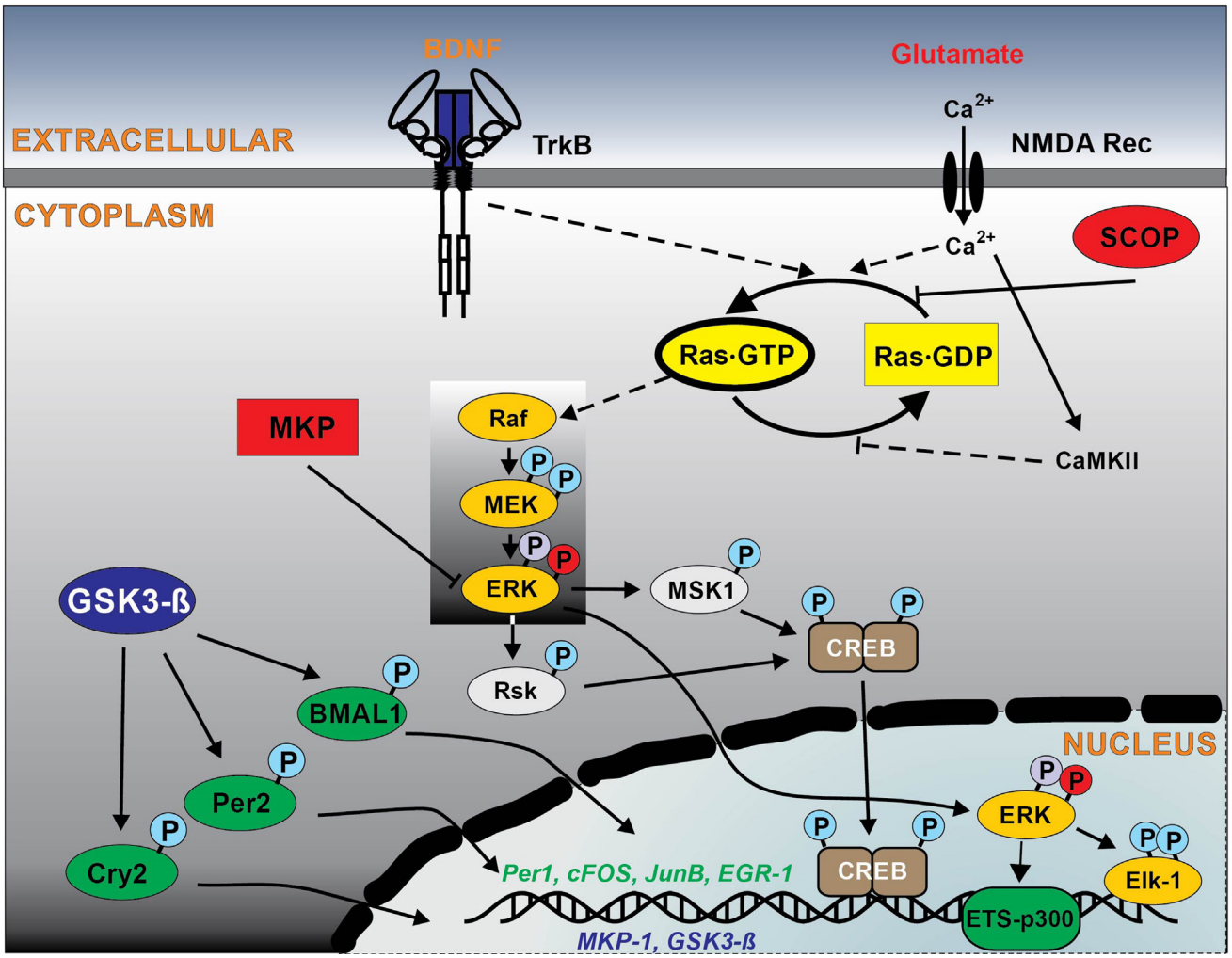

FIGURE 1 | Schematic outline of intracellular Ras signaling pathways in the suprachiasmatic nucleus (SCN) regulating circadian clockwork. Solid lines show the signal pathways observed in the SCN, and broken lines indicate hypothetical pathways observed in other neuronal systems. Glutamate/NMDA and brain-derived neurotrophic factor (BDNF)/TrkB are the major ligand–receptor systems within SCN involved in the light-induced phase shifting circadian clock. The light stimuli at night induce glutamate and BDNF release, which result in activation of NMDA receptors (with a subsequent influx of Ca^{2+}, activating the Ca^{2+}-calmodulin kinase II) and TrkB receptor that in turn stimulates Ras. Ras is also negatively regulated by the circadian protein SCN circadian oscillatory protein (SCOP). Ras activates ERK1,2 pathway, which couples to transcriptional factors CREB and Elk-1 phosphorylation, that regulate the transcription of the immediate early genes sFos, JunB, and EGR1, clock protein Per1, the regulator of ERK1,2 pathway MAPK phosphatase 1 (MKP-1). Enhanced Ras signaling *via* ERK1,2 also activates ETS-p300 transcriptional complex, which in turn regulates circadian clock proteins modulator glycogen synthase kinase-3 beta (GSK-3β). Other abbreviations are explained in manuscript. Please note: Ras downstream effector pathways other than RAF kinase, such as PI3 kinase and Ral/GDF have been omitted for reasons of simplicity and lack of specific information in the SCN.

including chick pineal gland (23), mouse hippocampus (22), liver (26), and SCN (8). Potential regulator of Ras activity in the brain is the SCN circadian oscillatory protein (SCOP) (42). The expression of SCOP reach peak levels during late subjective night to inhibit the Ras/ERK pathway by binding to the nucleotide-free state of Ras and preventing the binding of GTP (42, 43) (**Figure 1**). Furthermore, Ras is one of the main targets for neurotrophins (44). BDNF mRNA and protein levels show a circadian oscillation in different regions of the brain (32, 33, 45–48). Given that multiple extracellular signals such as growth factors and cytokines can stimulate Ras activation in a context-dependent manner, circadian oscillations of circulating humoral factors may lead to rhythmic Ras activation in the liver.

Numerous studies support a model that the circadian activation of ERK1,2 is regulated by the oscillating activation of Ras *via* the classical Ras–MAPK pathway, which is commonly involved in numerous intracellular events. The Ras-mediated regulation of ERK1,2 activity is conserved mechanism used in many clock-containing tissues, such as the mammalian SCN, as well as in other regions, like hippocampus, pineal gland, and liver (8, 22, 23, 26, 30). Indeed, the data from synRas mice show that the enhanced Ras activity increases ERK1,2 phosphorylation at early subjective night (8). However, the circadian regulation of

pERK1,2 levels was preserved, as result of the rhythmic oscillation of the endogenous Ras and the rhythmic expression of MAPK phosphatase 1 (MKP-1) (8, 20) (**Figure 1**). MKP-1 shows circadian oscillation with the peak time at night in mouse liver and the SCN (8, 26).

It has been recently shown that modulation of Ras activity affects the period length (τ) of circadian oscillation (8). Enhanced activation of Ras in the SCN of synRas mice results of shortening of τ, while *in vitro* inhibition of Ras activity lengthens the circadian oscillation of BMAL1 promoter-driven luciferase activity (8). By contrast, inducible overexpression of Ras in cancer cell lines disrupts the circadian clock enhances the circadian period, while Ras inhibition leads to a shortening of period length, as mathematically predicted by simulations of BMAL1-mediated transcription (24). However, the mechanism of Ras-mediated modulation of the circadian period length is not well investigated yet. Though, the fine tuning of the molecular clock might have a tumor suppressive role in Ras-driven lung cancer (49).

Within the regulation of τ, phosphorylation of core clock proteins plays an important role, as it determines their stability and degradation (1–3). GSK3β acts as one of the upstream kinases phosphorylating several clock proteins, such as CLOCK, BMAL1, PER 2, Rev-erbα, and CRY2 (1, 4, 5, 50–53) (**Figure 1**). Enhanced Ras activity results in high total protein expression and low levels of deactivating phosphorylation of GSK3β in the SCN (8), as well as increased GSK3β activity in the cortex of synRas mice (17). Moreover, the inhibition of Ras activity decreases protein expression and increases inhibitory phosphorylation level of GSK3β *in vitro* (8). Thus, the increased GSK3β activity leads to a shortening, whereas a decreased function leads to substantial lengthening, of the circadian period length (1, 51, 52). By contrast, other studies show that GSK3β inhibition shortens the circadian period *in vitro* (50), while its chronic activation lengthens the period of mice (54). Interestingly, Ras-mediated regulation of τ may also be a result of the direct influence of Ras on the MAPK pathway. Reduction of MAPK signaling by deletion of MSK1, a target kinase of ERK1,2, results in a lengthened period of circadian behavior (55). In addition, downregulation of ERK1,2 activity inhibits the rhythm and dampens the basal level of the expression of several clock genes (56). Taken together, these data suggest that changes in GTP-Ras levels influence τ *via* modulation of ERK1,2 and GSK3β activity.

ROLE OF Ras IN LEARNING AND MEMORY CONSOLIDATION

The circadian clocks regulate various neural functions, including cognitive performance. Several studies have demonstrated diurnal modulation of learning and memory in different paradigms, such as Morris water maze task (57), novel object recognition task (58), and fear-related tasks (59). Many investigators have linked Ras and ERK1/2 to learning and memory, since temporal modulation of ERK1/2 activation by Ras is known to play a critical role in several forms of neuroplasticity (60). Spatial and declarative memories are processed in the hippocampus (61), where Ras/MAPK pathway and the downstream CREB transcriptional pathway play an important role. Indeed, it is reported that Ras, ERK, and CREB activities show daily (basal) fluctuations in the mouse hippocampus (22). It has been recently shown that the consolidation of long-term recognition memory is a circadian-regulated process, mediated by the Ras-inhibitory protein SCOP (62). On the other hand, synRas mice showed impaired spatial short-term memory associated also with a reduced proliferation of newborn cells in the *dentate gyrus* of the hippocampus (63, 64) and decreased short-term recognition memory (65, 66). All these studies suggest that modulation of Ras activity is critical for memory performance, but a question remains as to whether and how circadian regulation of Ras is associated in this process.

Several reports suggest that a disordered circadian system is implicated in the etiology and symptomatology of many psychiatric disorders. Interestingly, the therapeutic action of lithium, an effective mood stabilizer for bipolar affective disorder, may be related to direct effects on the circadian clock *via* the inhibition of GSK-3β (51, 67). Although this enzyme has a number of functions that could potentially mediate the therapeutic effects of lithium (68), one possibility is *via* its function as a central regulator of the circadian clock. Consistently, it has been shown recently that activation of GSK-3β may link to the activity in the SCN neurons by regulating their persistent sodium currents (69). However, in order to understand how timing of action potentials is coupled to the pacemaker activity in the SCN, it still needs to be investigated how Ras signaling encodes electrical activity specifically in the SCN neurons.

CONCLUSION

The small GTPase Ras activity plays a role as a central regulator of structural and functional synaptic plasticity in the nervous system, where it mediates neuronal responses to various extracellular cues allowing the organism to adapt to complex environmental stimuli. Thus, the circadian and photic regulation of Ras activity

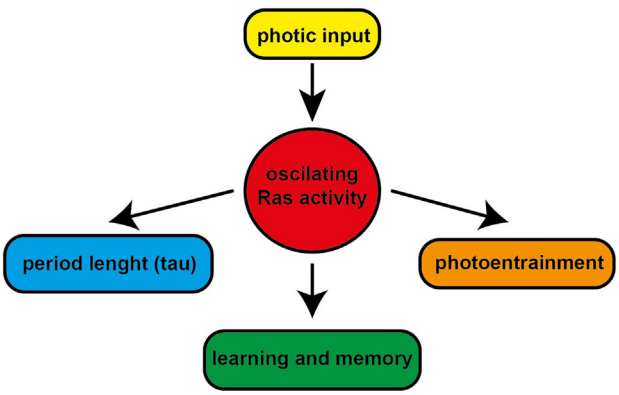

FIGURE 2 | The role of Ras in the regulation of circadian clockwork and learning and memory. The circadian and photic regulation of Ras activity in the suprachiasmatic nucleus modulates the light-induced phase resetting of the clock and fine tunes the circadian period length. The circadian modulation of Ras signaling might have potential role in learning and memory.

in the SCN is an important modulator of the clockwork influencing the light-induced phase resetting of the clock and fine tuning the circadian period length. Furthermore, the circadian modulation of Ras signaling might have potential role in memory consolidation and mood regulation (**Figure 2**). However, the involvement of Ras-controlled GSK-3β expression and its mechanism of regulation in the pathophysiology of bipolar disorder still remain to be investigated.

AUTHOR CONTRIBUTIONS

TS wrote the manuscript with assistance from RH.

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Phosphoproteome Profiling Reveals Circadian Clock Regulation of Posttranslational Modifications in the Murine Hippocampus

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The circadian clock is an endogenous oscillator that drives daily rhythms in physiology, behavior, and gene expression. The underlying mechanisms of circadian timekeeping are cell-autonomous and involve oscillatory expression of core clock genes that is driven by interconnecting transcription–translation feedback loops (TTFLs). Circadian clock TTFLs are further regulated by posttranslational modifications, in particular, phosphorylation. The hippocampus plays an important role in spatial memory and the conversion of short- to long-term memory. Several studies have reported the presence of a peripheral oscillator in the hippocampus and have highlighted the importance of circadian regulation in memory formation. Given the general importance of phosphorylation in circadian clock regulation, we performed global quantitative proteome and phosphoproteome analyses of the murine hippocampus across the circadian cycle, applying spiked-in labeled reference and high accuracy mass spectrometry (MS). Of the 3,052 proteins and 2,868 phosphosites on 1,368 proteins that were accurately quantified, 1.7% of proteins and 5.2% of phosphorylation events exhibited time-of-day-dependent expression profiles. The majority of circadian phosphopeptides displayed abrupt fluctuations at mid-to-late day without underlying rhythms of protein abundance. Bioinformatic analysis of cyclic phosphorylation events revealed their diverse distribution in different biological pathways, most notably, cytoskeletal organization and neuronal morphogenesis. This study provides the first large-scale, quantitative MS analysis of the circadian phosphoproteome and proteome of the murine hippocampus and highlights the significance of rhythmic regulation at the posttranslational level in this peripheral oscillator. In addition to providing molecular insights into the hippocampal circadian clock, our results will assist in the understanding of genetic factors that underlie rhythms-associated pathological states of the hippocampus.

Keywords: hippocampus, circadian rhythm, quantitative proteome and phosphoproteome analysis, phosphorylation, kinase–substrate relations

INTRODUCTION

Many behavioral and physiological processes exhibit daily fluctuations or circadian rhythms, which are governed by an intrinsic timekeeping mechanism. The circadian system ensures that the timing of these processes is optimal with respect to other ongoing internal events as well as to the external environment. In mammals, a central pacemaker that is situated in the suprachiasmatic nucleus (SCN) of the brain coordinates rhythms in peripheral tissues (1–3). Circadian timekeeping, be it central or peripheral, is a cell-autonomous phenomenon that is based on interconnecting transcription–translation feedback loops. To date, several large-scale proteomic studies of circadian regulation within the SCN (4, 5) as well as in peripheral tissues such as the heart (6) and liver (7, 8) have been conducted to understand clock-controlled mechanisms at the protein level. Chiang et al. (4) reported that temporal regulation of mitochondrial oxidative phosphorylation, as well as posttranscriptional regulation, plays an essential role in the SCN. Robles et al. (7) and Mauvoisin et al. (8) showed that metabolic and physiological functions in the liver are under circadian control at the transcriptional, posttranscriptional, and posttranslational levels.

In addition to the liver, several studies have reported the presence of circadian oscillations within the hippocampus, a region in the brain associated with the conversion of short- to long-term memory (9, 10). Schaaf et al. (11) and Wang et al. (12) found that the expression of brain-derived neurotrophic factor and Period2 (Per2), respectively, are circadian in the rodent hippocampus. Furthermore, genetic ablation of Per2 or Period1 (Per1) in mice leads to disruptions in hippocampal-dependent trace fear conditioning (12) and spatial memory performance (13). Given the pivotal role of cAMP-responsive element-binding protein (CREB) in memory processing (14–16), the observed rhythm in CREB phosphorylation in the hippocampus (13), but also in the SCN (14–16), provides a molecular mechanism by which the circadian clock may control the formation of long-term memories. Overall, the evidence of circadian rhythmicity within the hippocampus has provided an intriguing basis for understanding memory formation.

To understand how hippocampal function is impacted by the circadian clock, we sought to define the circadian proteome and phosphoproteome of the murine hippocampus, since phosphorylation is a major posttranslational modification (PTM) that regulates protein function. Using super-stable isotope labeling by amino acids in cell culture (super-SILAC) (17) and high accuracy mass spectrometry (MS), we identified in our unbiased screen a total of 4,953 unique proteins and 9,478 phosphorylation events. Out of those, there were 149 phosphorylation events that displayed circadian profiles of expression at the posttranslational level only. Bioinformatic analyses revealed that the circadian phosphoproteome peaked in the mid-to-late day and that those rhythmic phosphoproteins were preferably involved in diverse biological functions such as synaptic processes and cytoskeletal organization.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted at the University of Toronto at Mississauga and were approved by the local animal care committee in compliance with institutional guidelines and the Canadian Council on Animal Care. Male C57BL/6J mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA) were utilized for all experiments. Mice were group-housed in polycarbonate cages and given *ad libitum* access to rodent chow and water throughout the study.

Tissue Collection

Thirty male C57BL6/J mice, aged 8–12 weeks, were stably entrained for a minimum of 2 weeks to a 12-h light:12-h dark (LD) schedule (light intensity during the light phase was 200 lux) prior to transfer to complete darkness (DD) for two full cycles. Dark adaptation was achieved by placing cages into light-tight ventilated cabinets. On day 3 of DD, five mice were sacrificed at each time point corresponding to circadian time (CT) 2, 6, 10, 14, 18, and 22, where CT was defined by the Zeitgeber time of the previous LD schedule. Mice were killed by cervical dislocation and decapitated under dim red light, and eyes were covered with black electrical tape. Subsequently, whole hippocampal tissues were dissected, immediately flash-frozen in liquid nitrogen, and stored at −80°C until further processing.

Proteomic Analysis of Hippocampal Tissues Using Super-SILAC-Based Quantitative MS

To isotopically label murine cells, five cell lines, including Neuro-2a (neuroblastoma), AtT-20 (pituitary) acquired from ATCC (Manassas, VA, USA), mHypoE-N38, mHypoA-2/21 (CLU-181), and mHypoA-2/28 (CLU-188) (hypothalamus) acquired from Cedarlane Laboratories (Toronto, ON, Canada) were individually cultured in SILAC media at 37°C in a 5% CO_2 humidified incubator. For the SILAC media, customized DMEM by AthenaES (Baltimore, MD, USA) in which the natural lysine and arginine were replaced by heavy isotope-labeled amino acids, $^{13}C_6$ $^{15}N_4$ L-arginine (Arg 10) and $^{13}C_6$ $^{15}N_2$ L-lysine (Lys 8) was supplemented with 10% (v/v) dialyzed FBS (GIBCO-Invitrogen; Burlington, ON, Canada), 1 mM sodium pyruvate (GIBCO-Invitrogen), and 28 μg/mL gentamicin (GIBCO-Invitrogen). Complete (>98%) incorporation of the isotopically labeled amino acids into cellular proteins was achieved after at least 10 cell doublings in SILAC media.

Hippocampal tissues were homogenized in 300 μL of lysis buffer (8 M urea, 50 mM Tris–HCl (pH 7.5)), 100 mM DTT, 4% (v/v) SDS, 1 mM sodium orthovanadate supplemented with proteinase inhibitor cocktail (Roche, Mississauga, ON, Canada) and phosphoSTOP phosphatase inhibitor cocktail (Roche) with a pellet pestle and sonicated three times with 10 s pulses each (>30 s) on ice between each pulse. Protein concentrations were determined using the Bio-Rad DC Protein Assay. Hippocampal lysates (1 mg) and super SILAC-labeled cell lysates (0.2 mg from each of Neuro-2a, AtT-20, mHypoE-N38, CLU-181, and CLU-188 cells) were mixed at a 1:1 weight ratio, and SDS in solution was removed by an overnight incubation at −20°C in five volumes of ice-cold precipitation buffer [acetone/ethanol/acetic acid (v/v/v) = 50/50/0.1]. The precipitated proteins were washed twice with ice-cold acetone, and the protein pellets were redissolved in

50 mM NH_4HCO_3 solution containing 8M urea. For in-solution trypsin digestion, 1.2 mg of proteins in each sample was reduced with 5 mM DTT (Sigma, St. Louis, MO, USA) at 60°C for 1 h and alkylated with 10 mM iodoacetamide (Sigma) in the dark (40 min at room temperature). Each sample was diluted in fivefold volume of 50 mM NH_4HCO_3 (pH 8.5) solution to reduce the urea concentration to <2 M and digested overnight with TPCK-treated trypsin (Worthington, Lakewood, NJ, USA) at an enzyme-to-protein ratio of 1:25 (w/w). For proteomic analysis, 0.1 mg of resulting peptides were fractionated through an in-house constructed strong cation exchange (SCX) column with five pH fractions (pH 4.0, 6.0, 8.0, 10.0, and 12.0) followed by desalting with in-house C18 desalting cartridges and dried in a speed-vac prior to LC-MS analysis. The remaining 1.1 mg of tryptic peptides were desalted by SepPak C18 cartridges (Waters, Mississauga, ON, Canada), dried, and SCX fractionated into four fractions (pH 4.0, 6.0, 8.0, and 12.0) prior to phosphoproteome enrichment.

Hippocampal Phosphoproteome Enrichment by Ti^{4+}-IMAC Chromatography

Ti^{4+}-IMAC beads preparation and the phosphopeptide enrichment procedure were performed as described previously (18). Samples were resuspended in 1 mL loading buffer (80% (v/v) acetonitrile (ACN) and 6% (v/v) TFA) with 300 µL of Ti^{4+}-IMAC bead slurry (10 mg beads in 1 mL of loading buffer) for 30 min at 4°C. After centrifugation (16,000 × *g*, 10 min), beads were washed with 200 µL of washing buffer 1 [50% (v/v) ACN, 6% (v/v) TFA, and 200 mM NaCl], followed by two washes with 200 µL of washing buffer 2 [30% (v/v) ACN and 0.1% (v/v) TFA]. Phosphopeptides were eluted from the beads using 200 µL of 10% (v/v) ammonia solution for 15 min and sonicated for another 15 min at 4°C. After centrifugation, the retrieved supernatants were collected, acidified with 10% (v/v) TFA, desalted with in-house C18 desalting cartridges, and dried in a speed-vac prior to LC-MS analysis.

LC-MS Analyses

All resulting peptide fractions were reconstituted in 20 µL of 0.1% (v/v) FA and 4 µL of each sample was analyzed by online reverse-phase LC-MS/MS platform consisting of an Eksigent NanoLC-Ultra 2D plus system (AB SCIEX) coupled with a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) *via* a nano-electrospray source. Peptide mixtures were separated by reverse phase chromatography using a home-packed ReproSil-Pur C18-AQ column (75 µm internal diameter × 15 cm, 1.9 µm, 200 Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany) in 2 h LC gradient of 2–80% buffer B [ACN in 0.1% (v/v) FA] at a flow rate of 300 nL/min. The Q Exactive instrument was operated in the data-dependent mode to simultaneously measure survey scan MS spectra (from *m/z* 400–2,000) in the Orbitrap analyzer at resolution $R = 70{,}000$. Up to the 12 most intense peaks with charge state ≥2 and above a signal threshold of 500 counts were selected for fragmentation in the ion trap *via* higher-energy collisional dissociation. System controlling and data collection were carried out by Xcalibur software version 2.2 (Thermo Scientific).

Mass-Spectrometry Database Search and Bioinformatic Analysis

Mass spectrometry data from the hippocampal proteome and phosphoproteome were analyzed and quantified with MaxQuant (version 1.3.0.5) using Andromeda as the search engine against the UniProt (release 2014_04) database restricted to Mouse (*Mus musculus*) taxonomy concatenated with decoy reversed sequences. The precursor ion mass tolerance was 6 ppm and fragment mass deviation was 0.5 Da for MS/MS spectra. The search included variable modifications of methionine oxidation, N-terminal acetylation, Ser/Thr/Tyr phosphorylation, and fixed modification of cysteine carbamidomethylation. Trypsin/P (cleavage after Lysine and Arginine, including Lysine–Proline and Arginine–Proline) was set as the cleavage specificity with two missed cleavages. The false discovery rate (FDR) cutoffs for peptide and protein identification were both set to 0.01 and the minimum peptide length was set to 7. Identification across different replicates and adjacent fractions were achieved by enabling match between runs option within a time window of 2 min. Default settings were used for all the other parameters in MaxQuant. The proteingroup file from hippocampal proteome and Phospho (STY) Sites file from the phosphoproteome were imported into Perseus (version 1.5.2.4) for the analysis. The raw proteomic and phosphoproteomic datasets (4,953 proteins and 9,478 phosphosites, respectively) were filtered to include only proteins/phosphosites with quantification values in a minimum of 15 of 30 MS measurements (or 30 independent hippocampal samples), resulting in a stringently quantified dataset of 3,052 proteins and 2,868 phosphosites, respectively. Hierarchical clustering analysis, using the median value of logarithmized values for the normalized L/H ratio of each protein and phosphopeptide profile, was performed after *z*-score normalization of the data within Euclidean distances.

To identify the subset of 24-h rhythmic proteins and phosphopeptides, JTK_CYCLE algorithm (19) was used on the hippocampal proteomic (3,052 proteins) or the phosphoproteomic (2,868 phosphorylation events) dataset under R language. Prior to JTK_CYCLE analysis of those two datasets, any missing values (i.e., not detected by MS) were replaced with 0 and the minimum values observed in each screen (4), respectively. Only if both replacement methods showed a significant profile [*p*-Values (ADJ.P) less than 0.05] were the corresponding proteins/phosphopeptides classified as displaying a circadian rhythm. To find the 8- or 12-h rhythmic proteins and phosphoproteins within the 3,052-protein and 2,868-phosphoprotein datasets, another JTK_CYCLE analysis was separately performed with period lengths set at 8- and 12-h.

Unsupervised clustering analysis (fuzzy *c*-means) of the temporal profiles of cycling phosphopeptides was performed using the Mfuzz package (20) in R. The gene ontology (GO) annotation and pathway enrichment analysis of circadian phosphoproteins were implemented using the DAVID (21). The ingenuity pathway analysis (IPA) software (version 7.5) was utilized to analyze the biological functions, protein–protein interactions, and signaling pathway annotations of the rhythmically expressed proteins and phosphoproteins. Motif analysis was performed using iceLogo

(22), scoring by percent difference with a significance threshold of 0.05 for a sequence window of 15 amino acids surrounding the phosphorylated residues on hippocampal circadian phosphopeptides. Protein interaction network analysis of the cycling phosphoproteome was performed with the STRING (23) using medium to high confidence (0.5–0.7) and with co-expression and experiments as active prediction methods. iGPS 1.0 (24) was used to predict possible site-specific kinase–substrate relations (ssKSRs) between putative protein kinases (PKs) and circadian hippocampal phosphopeptides using a high threshold.

The MS proteomics and phosphoproteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) *via* the PRIDE partner repository (25) with the dataset identifier PXD005668 and PXD005669, respectively.

RESULTS

Super-SILAC-Based Quantitative Proteomic and Phosphoproteomic Analysis of the Murine Hippocampus

To explore the circadian proteome and phosphoproteome of the murine hippocampus, we stably entrained male C57BL/6J mice to a LD cycle and transferred them to constant darkness (DD) for 2 days. On day 3 of DD, hippocampal tissues were harvested from five mice at each of six time points, spaced 4 h apart (**Figure 1A**), to yield five independent biological replicates for each time point (5 mice per CT, $n = 30$ total mice). The proteome and phosphoproteome dynamics in the murine hippocampus over a 24-h cycle were then investigated by the state-of-art super-SILAC-based proteomics quantification method (17). This method, which results in accurate quantification, uses isotopically labeled peptides from a combination of five different SILAC-labeled cell lines with high labeling efficiency to serve as internal standards for MS-based analysis. Our super-SILAC mix included proteins from Neuro-2a (neuroblastoma), AtT-20 (pituitary), mHypoE-N38, mHypoA-2/21 (CLU-181), and mHypoA-2/28 (CLU-188) (hypothalamus) cells. "Light" hippocampal protein lysates (600 µg) were mixed with "heavy" lysates from our super-SILAC mix (120 µg of each cell line) at a 1:1 weight ratio. Following protein precipitation and tryptic digestion, 0.1 mg of the tryptic peptide mixtures were separated into five fractions for proteomic analysis, whereas 1.2 mg of resulting peptides were used for phosphopeptide enrichment analysis by Ti^{4+}-IMAC chromatography (18). All fractions were analyzed by nanoLC-MS/MS on a Q-Exactive MS in a total of 300 runs.

Out of the 4,953 proteins and 9,478 phosphorylation events identified in our proteome and phosphoproteome analysis, there

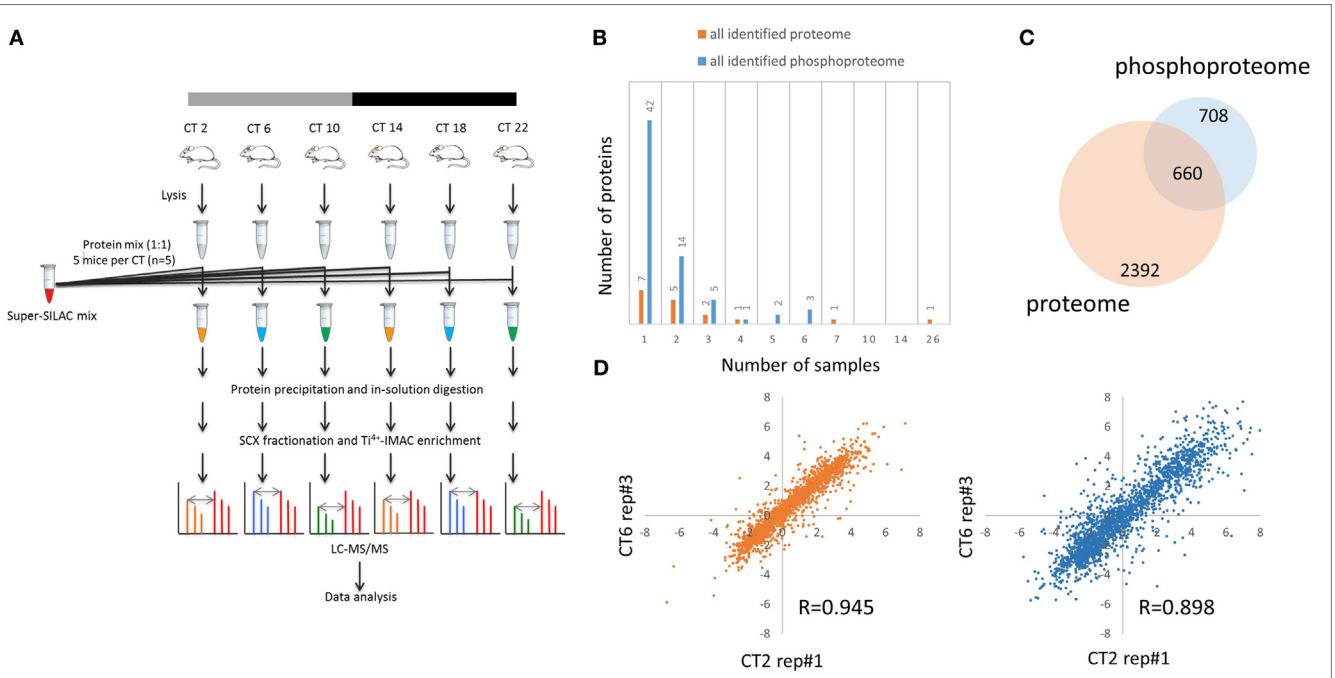


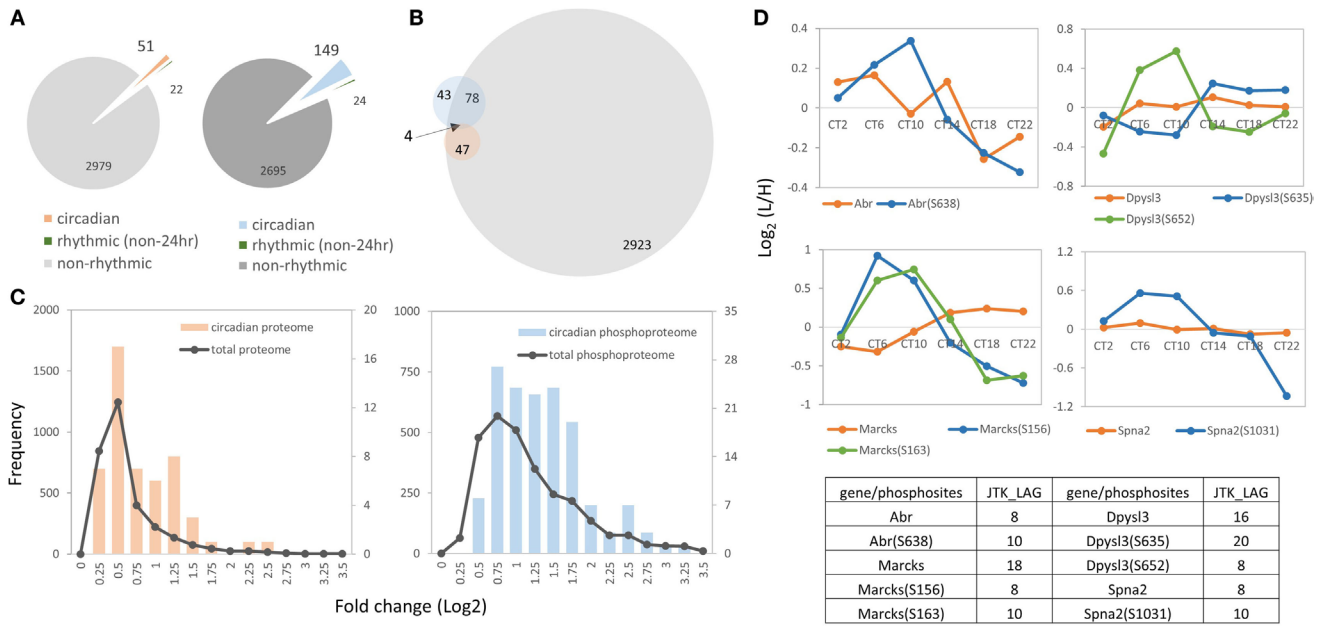
FIGURE 1 | Global proteomic and phosphoproteomic analysis of the murine hippocampus. **(A)** Schematic overview of super-SILAC-based quantification of the murine hippocampal proteome and phosphoproteome. Proteins extracted from hippocampal tissues of individual mice [$n = 5$ per circadian time (CT); 6 CT in total] were mixed with equal quantities of protein lysates from a super-SILAC mix (five isotopically labeled cells), digested with trypsin, and processed by SCX fractionation with Ti^{4+}-IMAC chromatography (phosphoproteome analysis) and without (proteome analysis). **(B)** Plot of 17 proteins and 67 phosphorylation events that were identified in the light (hippocampal tissues), but not heavy (super-SILAC mix), counterpart. *x*-axis indicates the number of hippocampal samples in which these proteins and phosphosites were detected. **(C)** Venn diagram indicates that 660 proteins were accurately quantified in both the proteome (orange) and phosphoproteome (blue) datasets, whereas 708 were found only in the phosphoproteome. **(D)** Representative scatter plots showing biological replicate measurement in proteomic and phosphoproteomic screens with a high degree of correlation among those 30 biological samples.

were 17 (0.3%) proteins and 67 (0.7%) phosphorylation events that were identified in the light samples that did not have a heavy counterpart (**Figure 1B**). Of these, only one protein (Ly-6/neurotoxin-like protein 1; Uniprot ID: Q9WVC2) was detected in more than half of the samples without a corresponding SILAC-labeled peak from the super-SILAC pooled standard. With a FDR of 1% at the peptide level, 6,204 of 9,478 phosphosites were classified as class I phosphorylation sites (localization probability score >0.75), with a distribution of 90.4% phosphoserine, 9.2% phosphothreonine, and 3.4% phosphotyrosine residues.

For further downstream bioinformatic analyses, we extracted from the raw dataset only the accurately quantified proteins and phosphorylation events. This resulted in more stringent, filtered datasets of 3,052 proteins (referred to as the hippocampal proteome, Table S1 in Supplementary Material) and 2,868 class I phosphorylation sites on 1,368 proteins (referred to as the hippocampal phosphoproteome, $p > 0.75$, Table S2 in Supplementary Material). Of the 1,368 phosphoproteins identified, 660 were accurately quantified in both the proteome and phosphoproteome datasets, whereas 708 were found only in the phosphoproteome, but not proteome, dataset (**Figure 1C**). Pairwise Pearson's correlation analysis of 30 independent MS measurements (Tables S3 and S4 in Supplementary Material) on both hippocampal proteome and phosphoproteome data showed good reproducibility of our results (**Figure 1D**), with an average Pearson *r* value of 0.943 and 0.895, respectively.

Circadian Oscillations of the Murine Hippocampal Proteome and Phosphoproteome

To further identify proteins and phosphoproteins that showed a circadian pattern of abundance in our murine hippocampal dataset, JTK_CYCLE algorithm (19) was employed to identify rhythmic subsets of proteins and phosphorylation events with a period of 24 h. Ultradian patterns were identified by setting the period to 8 and 12 h. As shown in **Figure 2A**, 51 of 3,052 (1.7%) proteins (referred to as the circadian proteome, Table S5 in Supplementary Material) and 149 of 2,868 (5.2%) phosphorylation events on 125 proteins (referred to as the circadian phosphoproteome, Table S6 in Supplementary Material) exhibited a 24-h rhythm of abundance ($p < 0.05$, JTK_CYCLE), whereas less than 1% of hippocampal proteins (22/3,052, 0.7%) and phosphorylation events (24/2,868, 0.8%) exhibited ultradian oscillations with periods of 8 or 12 h. Notably, for the majority of circadian phosphopeptides, the corresponding proteins were non-rhythmic (97 phosphorylation sites on 78 proteins, 97/149, 65.1%); only 4 genes (6 phosphosites, 6/149, 4.0%) showed a



gene/phosphosites	JTK_LAG	gene/phosphosites	JTK_LAG
Abr	8	Dpysl3	16
Abr(S638)	10	Dpysl3(S635)	20
Marcks	18	Dpysl3(S652)	8
Marcks(S156)	8	Spna2	8
Marcks(S163)	10	Spna2(S1031)	10

FIGURE 2 | Overview of the hippocampal circadian proteome and phosphoproteome. (A) The left pie chart indicates the proportions of proteins that exhibit a circadian (orange), ultradian (8 or 12 h, green) or non-rhythmic (gray) temporal profile of abundance. The right pie chart illustrates the proportion of the phosphoproteome that exhibits a circadian (blue), ultradian (green), or non-rhythmic (dark-gray) pattern of phosphorylation (JTK_CYCLE algorithm, $p < 0.05$). **(B)** Venn diagram indicates that only four genes showed a circadian expression profile at both the protein (orange) and phosphorylation level (blue), whereas the majority of circadian phosphorylation events (97 phosphosites on 78 phosphoproteins) were non-rhythmic in terms of protein abundance (gray, hippocampal proteome). **(C)** Abundance profiles of the circadian phosphoproteome (blue) showed a higher magnitude of fluctuation than the circadian proteome (orange). Both datasets displayed a higher fold change than the total quantified dataset (gray). *x*-Axis indicates fold changes (log2 normalized ratios) of the proteome (left) and phosphoproteome (right) in a 24-h cycle. *y*-axis indicates the number of proteins or phosphopeptides from the total proteome/phosphoproteome (left *y*-axis) or from the circadian proteome/phosphoproteome (right *y*-axis). **(D)** Four proteins (*Abr, Dpysl3, Marcks*, and *Spna2*) exhibited a circadian profile at both the protein (orange) and phosphorylation (green and blue) level.

circadian expression profile at both the protein and phosphorylation level (**Figure 2B**).

Next, we analyzed the magnitude of the fluctuations in the circadian proteome and phosphoproteome by calculating the fold change of those cyclic subsets with the logarithmic normalized expression ratios across six CT. The mean logarithmic fold change of the circadian proteome is 0.73, whereas it is 1.26 for the circadian phosphoproteome (**Figure 2C**). The abundance profiles of both datasets displayed a higher fold change than the total quantified datasets (i.e., total proteome and total phosphoproteome) (**Figure 2C**). In cases where both the phosphorylation event and the protein abundance fluctuated in a circadian fashion, the amplitude of the phosphorylation rhythm exceeded the amplitude of the protein rhythm by approximately 40%. For example, *Abr, Dpysl3, Marcks*, and *Spna2* exhibited circadian rhythms both in their phosphorylation status and in total protein abundance, but the magnitude of the oscillation was greater for the former than it was for the latter (**Figure 2D**).

Collectively, our data reveal that a substantial portion of the hippocampal proteome exhibits significantly greater time-of-day oscillations at the level of phosphorylation than at the level of protein abundance, suggesting that posttranslational mechanisms play a prominent role in shaping the functions of proteins in the hippocampus.

Phase and Site-Specific Motif Enriched Analysis of the Circadian Hippocampal Proteome and Phosphoproteome

To further characterize the time-of-day-dependent proteome and phosphoproteome of the murine hippocampus, we analyzed the data by hierarchical clustering using the *z*-score normalization of the median value of normalized logarithmic expression ratios across a 24-h cycle. As shown in **Figure 3A**, peak times in the oscillations of the circadian proteome were evenly distributed across the 24-h cycle, whereas within the circadian phosphoproteome, the peak in abundance was largely confined to the mid-to-late day time points (CT6 and CT10). As shown in **Figure 3C**, unsupervised clustering analysis (fuzzy *c*-means) of the temporal profiles of cycling phosphopeptides revealed that approximately 70% of the temporal phosphorylation patterns had a marked increase in expression starting at CT2 and peaking by mid-to-late day (CT6 to CT10) and a gradual decline to trough levels in the

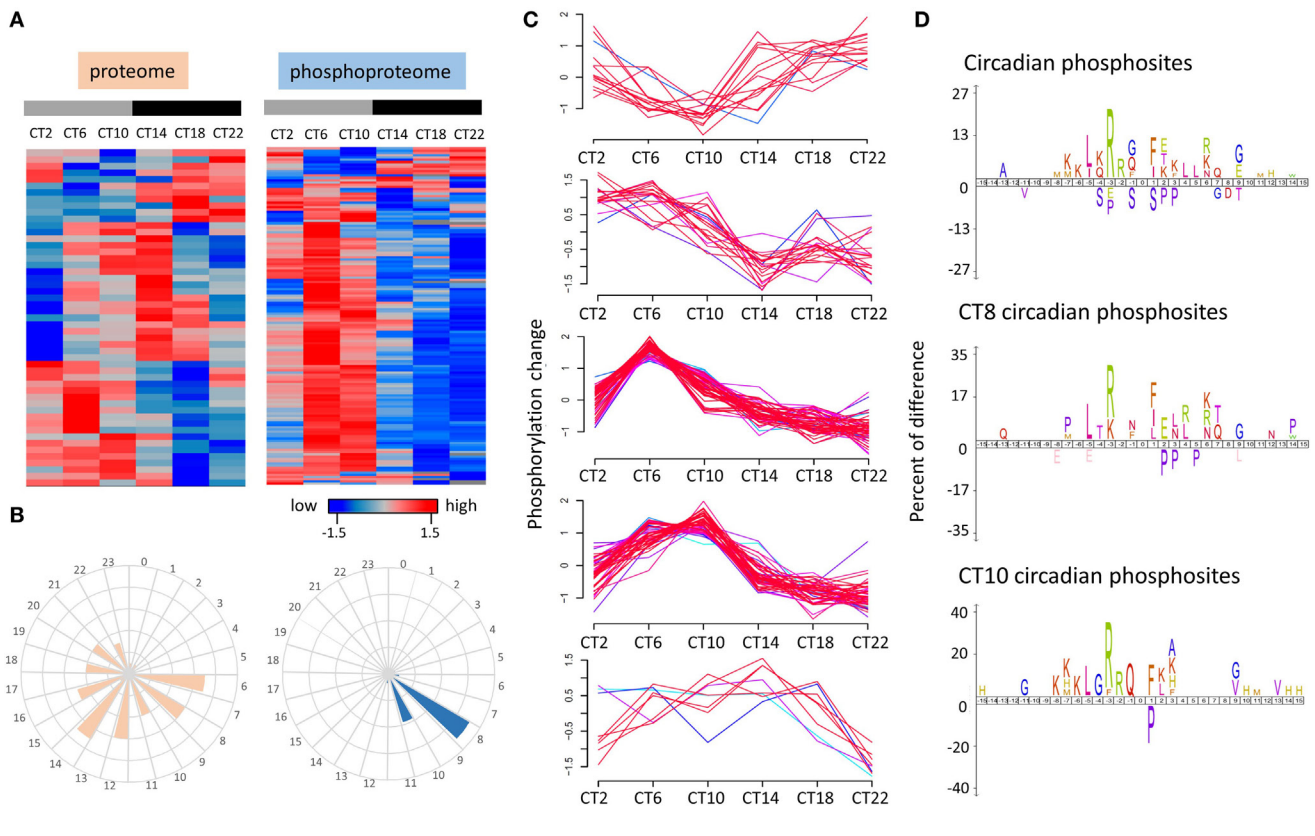


FIGURE 3 | Time-of-day profile of the circadian proteome and phosphoproteome over a 24 h cycle. **(A)** Hierarchical clustering of the circadian proteome (left) and circadian phosphoproteome (right) in the hippocampus. **(B)** Frequency distribution of abundance phases shows that the circadian proteome is evenly distributed across the 24-h cycle, whereas the circadian phosphoproteome peaks in the mid-to-late day. **(C)** Temporal expression profile of five phosphorylation patterns that are under circadian control in the hippocampus. **(D)** Sequence logos analysis (iceLogo) of the total circadian phosphoproteome (top) as well as the subsets that peak specifically at CT8 (middle) and CT10 (bottom).

late night (CT22). By plotting the frequency of oscillation phases of the circadian proteome and phosphoproteome, **Figure 3B** revealed that the phases of cycling proteins were distributed from CT6 to CT22, whereas the circadian phosphoproteins mainly peaked at CT8 (71/149 phosphosites, 47.7%) and CT10 (38/149 phosphosites, 25.5%).

Enrichment of amino acids surrounding the phosphorylated residues can be useful in revealing the broad classes of kinases that might control circadian phospho-oscillations in the hippocampus. To identify potential patterns in the enriched amino acids surrounding the phosphorylated residues of those cycling phosphoproteins, we employed iceLogo analysis (22) on the circadian phosphoproteome as well as on the peaking phosphoproteome at CT8 and CT10. Relative to all identified phosphorylation events in the murine hippocampus, circadian phosphoproteins were significantly overrepresented ($p < 0.05$) with basophilic-containing motifs, including arginine and lysine at the −2 to −7 positions, as well as hydrophobic amino acids at the −5 position (**Figure 3D**). Closer examination of the consensus of the cycling phosphoproteome that peaked at CT8 found a greater preference toward leucine and arginine/lysine residues at the −5 and −3 positions (**Figure 3D**). Phosphopeptides that peaked at CT10 exhibited an enriched motif composition that was similar to the circadian phosphoproteome with only modest differences at the C-terminal region (**Figure 3D**).

Differential Distribution of the Circadian Proteome and Phosphoproteome in the Murine Hippocampus

To gain insight into the subcellular locations of, and biological processes associated with, the rhythmic phosphoproteins identified in our study, we performed GO enrichment analyses by using the bioinformatics resources available *via* the Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.8, https://david.ncifcrf.gov/). Three available GO categories were utilized to classify the biological processes, cellular components, and molecular functions of phosphoproteins that were overrepresented in our dataset (**Figures 4A–C**). Relative to the accurately quantified hippocampal phosphoproteome, the circadian phosphoproteome was significantly enriched for

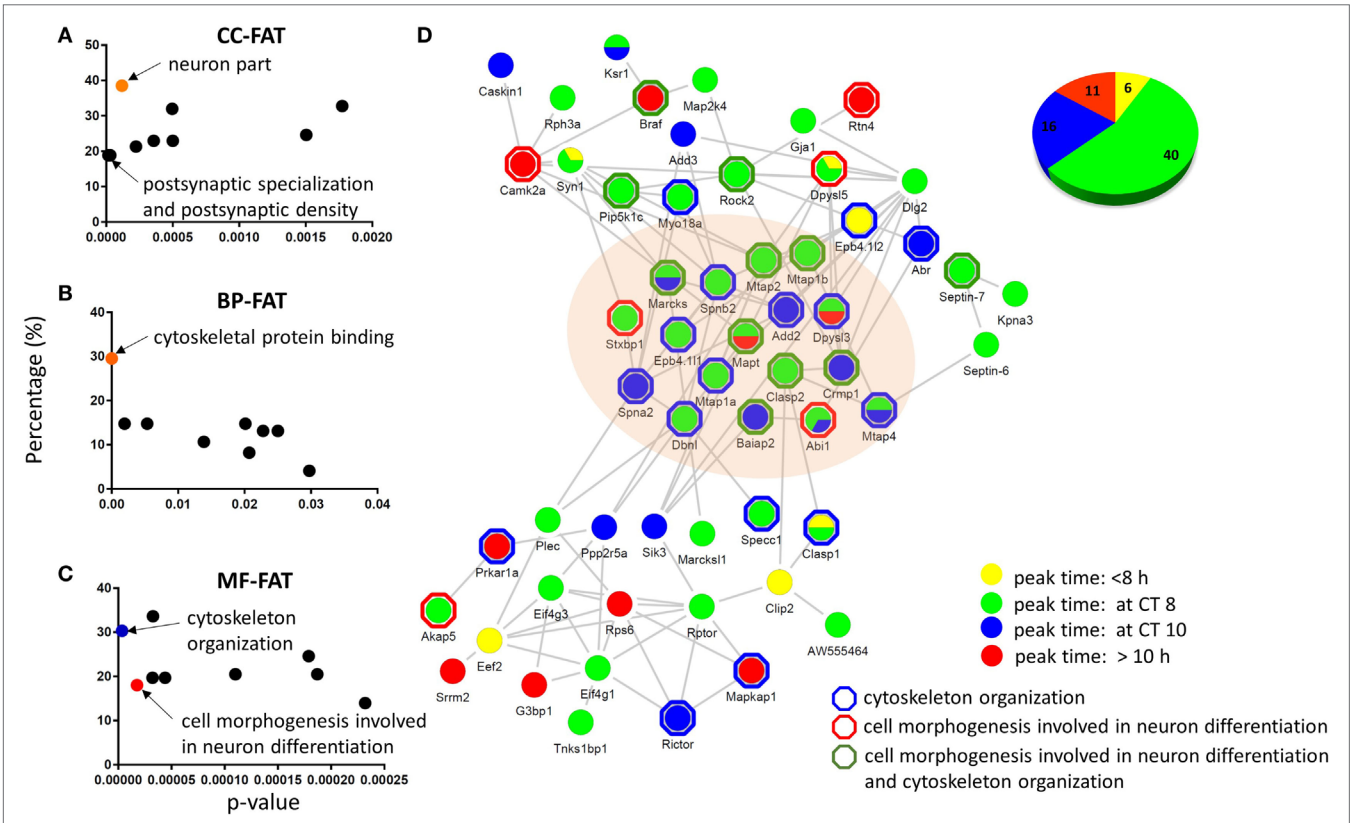


FIGURE 4 | Functional analysis and physical interaction of the circadian phosphoproteome. Graphical distribution of overrepresented gene ontology (GO) terms of the circadian phosphoproteome against the accurately quantified phosphoproteome dataset in **(A)** cellular component, **(B)** molecular function, and **(C)** biological process by DAVID. **(D)** The largest physical interaction network of the circadian phosphoproteome in the STRING database is comprised of proteins that are described by the GO terms cell morphogenesis involved in neuron differentiation and cytoskeleton organization. Each node represents a protein that is colored according to its peak phase (yellow: between CT0 and CT8, green: at CT8, blue: at CT10, and red: between CT10 and CT24) at the phosphorylation level. Proteins outlined by a hexagon are enriched in cytoskeleton organization (blue), cell morphogenesis involved in neuron differentiation (red), or both (green) by DAVID BP-FAT analysis (Fisher's exact test, $p < 0.05$). Inset (upper right): the pie chart indicates the proportion of phosphopeptides that belong to the four different peak phase categories.

GO-FAT cellular components that were classified as postsynaptic specialization and neuron part (Fisher's exact test, $p < 0.05$) (**Figure 4A**), whereas cytoskeletal protein binding was the most highly enriched category in the GO-FAT molecular functions analysis (**Figure 4B**). Additionally, several metabolic pathways including cytoskeleton organization, cell morphogenesis involved in neuron differentiation, and neuron projection morphogenesis were significantly enriched in this dataset based on GO-FAT biological process analysis (**Figure 4C**).

To delve further into the potential biological relevance of the cycling phosphoproteome within the hippocampus, we constructed functional protein–protein interaction networks of the circadian phosphoproteome in the STRING database (**Figure 4D**). Out of 149 circadian phosphorylation events on 125 proteins, 57 proteins (73 phosphopeptides, 73/149 = 49.0%) exhibited a high degree of connectivity in a functional protein network (**Figure 4D**). This network included a relatively large number of proteins where the phosphorylation events occurred at CT8 (54.8%) and CT10 (21.9%). Notably, many of these phosphorylation events that peaked at CT8 and CT10 occurred on proteins that were classified by the GO terms cytoskeletal protein binding and/or cell morphogenesis involved in neuron differentiation.

Next, we investigated the relationship between the circadian proteome and circadian phosphoproteome by constructing a direct protein interaction network using STRING, in an attempt to understand the underlying mechanisms for circadian post-translational regulation in the hippocampus. The top functions within the largest protein interaction network that was constructed (**Figure 5**) were cellular assembly and organization, and cellular function and maintenance. This network included a large number of proteins that are involved in several known canonical pathways by IPA, including mTOR signaling ($p = 3.02E{-}05$),

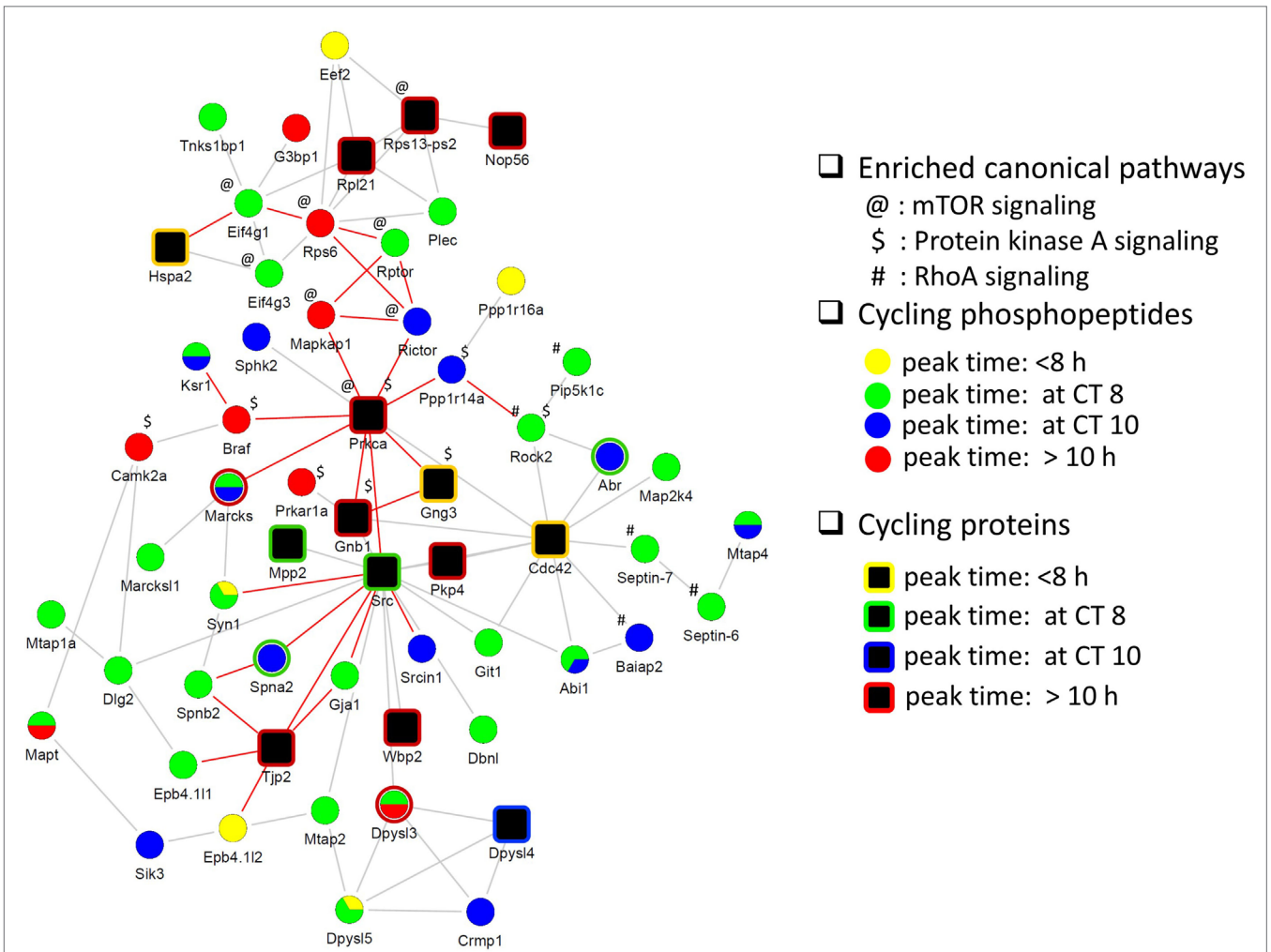


FIGURE 5 | Protein interaction network of cyclic hippocampal proteins and phosphoproteins. Network indicating direct physical interactions between the circadian proteome (node symbol: square) and the circadian phosphoproteome (node symbol: circle). Phases of peak abundance are denoted by different colors (yellow: between CT0 and CT8, green: at CT8, blue: at CT10, and red: between CT10 and CT24). Symbols indicate proteins and/or phosphorylation events that are involved in mTOR signaling (@), protein kinase A signaling ($), and RhoA signaling (#) pathways. Proteins connected by edges (red lines) are enriched in cellular assembly and organization, cellular function, and maintenance process.

protein kinase A (PKA) signaling ($p = 6.76E{-}05$), and RhoA signaling ($p = 4.27E{-}04$). Prior studies have shown that phosphorylation of eIF4E, 4EBP1, rpS6, Akt, and ERK1/2 (components of the mTOR pathway) are rhythmic in the murine hippocampus and disrupting their diurnal oscillations impairs memory consolidation (26). Similar effects on memory consolidation were observed upon inhibition of PKA activity in the rat hippocampus (27).

Our collective data suggest that phosphorylation events within the hippocampus, particularly those associated with the cytoskeleton and neuronal differentiation, are under circadian regulation, peaking in the mid-to-late day. Furthermore, some of these phosphorylation events are associated with PKs that are known to be clock-controlled and/or that regulate the entrainment of the clock by light (28, 29).

Kinase Responses and Predicted Kinase Regulators of Cycling Phosphopeptides in the Hippocampus

Identification of phosphorylation sites with their cognate PKs is important in understanding signal transduction within complex biological systems. In order to identify putative PKs underlying the circadian phosphoproteome, we utilized iGPS [GPS algorithm with the interaction filter, 1.0 (24)] to find kinase-specific phosphorylation sites at a high stringency level. In our systematic elucidation of ssKSRs from a circadian phosphoproteomic dataset of 149 phosphorylation events, 662 potential ssKSRs were identified among 190 PKs and 40 phosphosites (in 34 proteins), yielding a coverage rate of 26.8% (40/149). As shown in **Figure 6A**, top-ranking PK groups that were predicted to phosphorylate those sites belong to the AGC, CMGC, CAMK, STE, and TKL PK groups. Downstream Yates' chi-squared test showed that, when compared to the 2,868 accurately quantified phosphoproteome, a significantly higher proportion of circadian phosphorylation events ($p < 0.05$) were predicted to be modified by 27 PKs that belong to the AKT (v-Akt murine thymoma viral oncogene), CAMK2 (Ca^{2+}/calmodulin PK II), CAMKL (Ca^{2+}/calmodulin PK like), or STE20 kinase families (**Figure 6B**).

DISCUSSION

Major advancements in mass spectrometric methodologies coupled with phosphopeptide enrichment strategies have allowed us to obtain an unbiased view of phosphorylation dynamics in a systematic manner (18, 30, 31). In this study, we utilize the super-SILAC-based quantitative proteomics approach as well as phophoproteomics technology to gain a first look into the circadian phosphoproteome of the murine hippocampus. Out of 3,052 proteins and 2,868 phosphopeptides that were stringently quantified, 51 (1.7%) proteins and 149 (5.2%) phosphosites exhibited a circadian expression profile. Compared to the recently published proteome studies of the SCN (4) and liver (7, 8), our proteomic and phosphoproteomic screens failed to detect any core clock proteins, likely due to their significantly lower abundance relative to the many cytoplasmic proteins, which were detected. Furthermore, although the percentage of detected

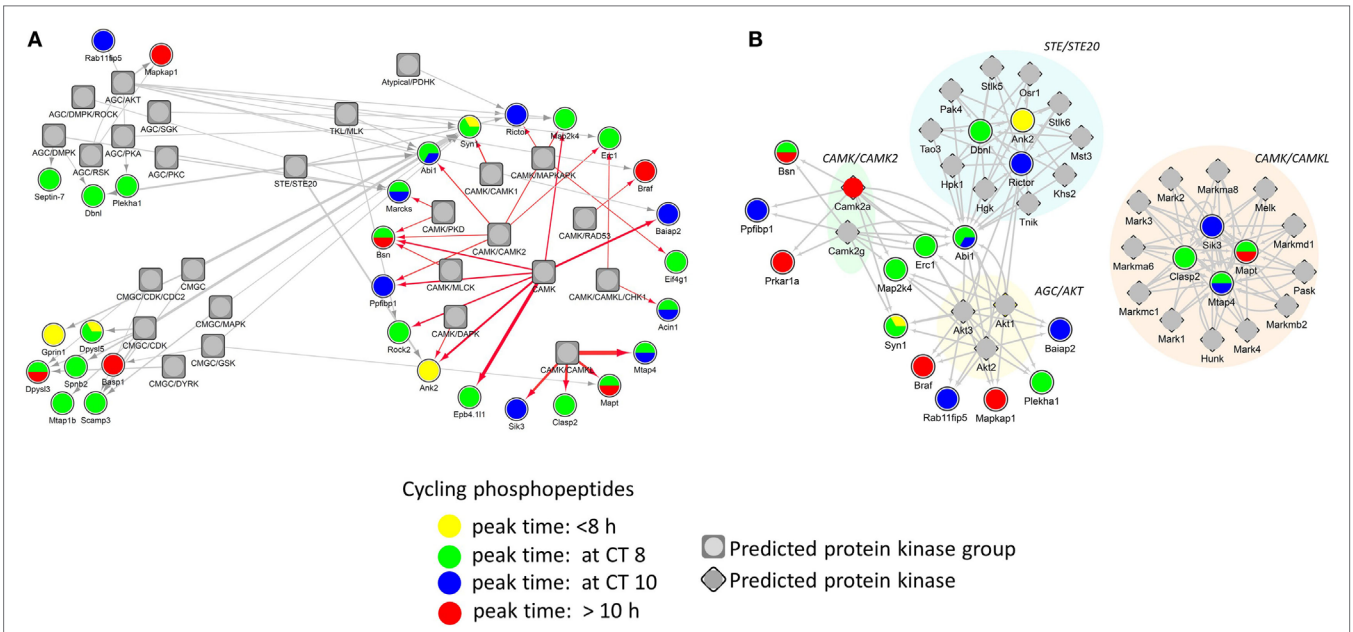


FIGURE 6 | Protein phosphorylation networks of the circadian phosphoproteome with putatively active protein kinases (PKs). Protein interaction networks of potential site-specific kinase–substrate relations between the circadian phosphoproteome and putative PKs by iGPS. **(A)** Top five PK groups (symbol: square) preferentially modify more phosphorylation sites on the circadian phosphoproteome. **(B)** Twenty-seven PKs (symbol: diamond) significantly modify more phosphorylation sites on the circadian phosphoproteome than the hippocampal phosphoproteome (Yates' chi-squared test, $p < 0.05$). Each edge (line) in the network represents a circadian phosphorylation event that is mediated by the specified kinase. Phases of peak abundance of the circadian phosphoproteome (node symbol: circle) are denoted by different colors (yellow: between CT0 and CT8, green: at CT8, blue: at CT10, and red: between CT10 and CT24).

circadian phosphoproteins in the hippocampus was similar to the percentage of the SCN or hepatic proteome that was rhythmic based on three previous studies (2.2% in the SCN, Chiang et al.; 6.0% in the liver, Robles et al.; 4.8% in the liver, Mauvoisin et al.), the percentage of rhythmic hippocampal proteins was markedly lower.

Protein phosphorylation and dephophorylation are highly controlled biochemical processes that respond to various intracellular and extracellular stimuli. Phosphorylation status modulates protein functions, which in turn regulate crucial biological processes and development. Wang et al. (32) showed that phosphosites on nuclear proteins in the liver were bimodally distributed at peak times in the middle of the day and the night. Robles et al. (33) discovered that phosphorylation cycles in the liver were much greater in amplitude than the fluctuations in protein abundance and markedly differed in phase when compared to the cycling proteome. In our present study, we noticed that the circadian phosphorylation events that occurred in the murine hippocampus peaked primarily at mid-to-late day (CT8 to CT10). Furthermore, the mean fold-change of the hippocampal circadian phosphoproteome was much greater than that of the hippocampal circadian proteome. The relatively high amplitude of phosphorylation rhythms is particularly noteworthy given that the hippocampus lacks intrinsic circadian rhythmicity at the level of *Per1* gene expression when cultured *ex vivo* (34). This suggests that hippocampal rhythms are either posttranscriptional or post-translational in nature or require ongoing signals from the SCN to maintain them.

Downstream consensus motif enrichment analysis indicated that circadian hippocampal phosphopeptides, regardless of their peak phase, were good substrates for CAMK2 (R-X-X-S/T) (35) and PKD (L/I-X-R-X-X-S/T) (36), whereas those phosphopeptides that peaked specifically at CT10 possessed a favorable kinase–substrate relations with PKA (R-R-X-S/T-Y, where Y tends to be a hydrophobic residue) (37). Saraf et al. found that phosphorylation of eIF4E (Ser209), 4EBP1 (Thr37/Thr46), ERK1/2 (Thr202/Tyr204), and Akt (Ser473) in the hippocampus peaked in the mid-day to activate translation initiation and promote memory consolidation (26). Abolishing diurnal oscillations in phosphorylation of the aforementioned proteins in the hippocampus leads to a reduction in contextual memory (26). Our bioinformatics analysis also pointed to the possibility that several putative PKs belonging to the AKT, CAMK (CAMK2 and CAMKL), and STE (STE20) PK families that may play a prominent role in shaping the landscape of circadian phosphorylation events in the hippocampus. STE20 kinases are best known as members of the MAPK cascade. Eckel-Mahan et al. (38) found that daytime rhythms of MAPK activity in the hippocampus are accompanied by parallel oscillations in cAMP levels and Ras activity. In light of these previous findings, our results suggest that PKs from the CAMK and CAMKL families contribute to the circadian rhythms of protein phosphorylation within the hippocampus.

Notably, our GO enrichment analysis revealed that proteins that are categorized under postsynaptic specialization and postsynaptic density, as well as cell morphogenesis involved in neuron differentiation and cytoskeleton organization, exhibited time-of-day dependent fluctuations in their phosphorylation status. Along these lines, rats experience a rapid increase in dendritic spine density of CA1 pyramidal neurons shortly after entering the dark phase and their awake state, an effect that is mediated by various kinase pathways including MAPK/ERK, PKA, and PKC (39). Moreover, there is evidence that the sleep–wake cycle, which is coordinated by the circadian timing system, is linked to structural plasticity within the hippocampus and memory processes (40). The observed peak in hippocampal protein phosphorylation at CT8–10 suggests that the circadian timing system may be mediating anticipatory changes in proteins that are implicated in synapse function or cytoskeletal organization, in preparation for the structural changes that occur in the hippocampus shortly after wake onset.

Finally, our functional interaction network analysis of the circadian proteome and phosphoproteome indicates that signaling by mTOR, PKA, and RhoA in the hippocampus is under circadian regulation. Interestingly, PRKCA and CDC42, both of which fluctuate at the level of protein expression, appear to be major hubs that connect to rhythmically phosphorylated proteins implicated in PKA and mTOR signaling (in the case of PRKCA), and RhoA signaling (in the case of CDC42) (**Figure 5**). PRKCA has previously been implicated in photic entrainment of the SCN through posttranslational regulation of PER2 stability and nucleocytoplasmic trafficking (41). CDC42 belongs to the Rho family and is critical for postsynaptic structural plasticity of CA1 pyramidal neurons (42). Our study revealed that CDC42 protein levels peaked at CT6, preceding the peak in cyclic phosphorylation events that are linked to RhoA signaling by at least 2 h. The collective data from our study strongly suggest that rhythmic PTM is an important mechanism by which the circadian clock exerts temporal control of hippocampal function.

CONCLUSION

Ours is the first study that investigates the circadian control of the global phosphoproteome of the murine hippocampus. Approximately 5% of detected phosphorylation events within the hippocampus oscillate in a circadian fashion and reach their peak in the mid-to-late day. In addition to this synchronicity in their peak phase, many of these phosphoproteins were associated with fundamental neuronal processes including neuronal structure. Our bioinformatics analysis also revealed putative ssKSRs within the hippocampus, thereby providing a better understanding of the mechanisms that underlie circadian regulation of hippocampal function.

AUTHOR CONTRIBUTIONS

C-KC, H-YC, and DF designed the experiments. C-KC, BX, NM, and WS performed the experiments. JD and HZ provided the Ti^{4+}-IMAC beads. C-KC, BX, KC, JM, ZN, H-YC, and DF analyzed the data. C-KC, BX, JM, H-YC, and DF wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fneur.2017.00110/full#supplementary-material.

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An Overview of Monthly Rhythms and Clocks

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Organisms have evolved to cope with geophysical cycles of different period lengths. In this review, we focus on the adaptations of animals to the lunar cycle, specifically, on the occurrence of biological rhythms with monthly (circalunar) or semi-monthly (circasemilunar) period lengths. Systematic experimental investigation, starting in the early twentieth century, has allowed scientists to distinguish between mythological belief and scientific facts concerning the influence of the lunar cycle on animals. These studies revealed that marine animals of various taxa exhibit circalunar or circasemilunar reproductive rhythms. Some of these rely on endogenous oscillators (circalunar or circasemilunar clocks), whereas others are directly driven by external cues, such as the changes in nocturnal illuminance. We review current insight in the molecular and cellular mechanisms involved in circalunar rhythms, focusing on recent work in corals, annelid worms, midges, and fishes. In several of these model systems, the transcript levels of some core circadian clock genes are affected by both light and endogenous circalunar oscillations. How these and other molecular changes relate to the changes in physiology or behavior over the lunar cycle remains to be determined. We further review the possible relevance of circalunar rhythms for terrestrial species, with a particular focus on mammalian reproduction. Studies on circalunar rhythms of conception or birth rates extend to humans, where the lunar cycle was suggested to also affect sleep and mental health. While these reports remain controversial, factors like the increase in "light pollution" by artificial light might contribute to discrepancies between studies. We finally discuss the existence of circalunar oscillations in mammalian physiology. We speculate that these oscillations could be the remnant of ancient circalunar oscillators that were secondarily uncoupled from a natural entrainment mechanism, but still maintained relevance for structuring the timing of reproduction or physiology. The analysis and comparison of circalunar rhythms and clocks are currently challenging due to the heterogeneity of samples concerning species diversity, environmental conditions, and chronobiological conditions. We suggest that future research will benefit from the development of standardized experimental paradigms, and common principles for recording and reporting environmental conditions, especially light spectra and intensities.

Keywords: circalunar, circadian, moon, light, sleep, mood, marine, reproduction

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THE OCCURRENCE OF CIRCALUNAR RHYTHMS AND CLOCKS

Physiological processes and behaviors often occur at specific times. Similar to human societies that follow not only the pace of the watch but also that of the calendar, many organisms structure their behavior and physiology not only by the regular cycles generated by the changes of sun (daily and seasonal timing) but also the cycles of the moon (monthly timing). Moreover, different

timing regimes can also be used in combination, for instance, to synchronizing reproduction to a particular season of the year, particular day(s) of the month and specific hours during these days.

Generally, periodic organismal processes (biological rhythms) can be orchestrated in two different ways (**Figure 1A**). On the one hand, they may be generated directly by changes in the regular external cues. Such a setting allows a given rhythm to adjust rapidly to any sudden changes in the external cues, but in turn makes the rhythm inherently sensitive to disturbance. On the other hand, organisms can possess internal timing systems of the respective period length (so-called biological clocks) that are adjusted to the external cues, but able to continue to run independently, thereby making the biological rhythm more robust against short-term disturbances. The evolution of such biological clocks has likely been favored by the extreme stability of geophysical cycles and the advantages organisms have when they can not only react to regular changes in the environment but also anticipate these changes and prepare accordingly.

Whereas biological rhythms have been observed over centuries, molecular details have so far best been worked out for the biological rhythms and clocks running on a 24-h cycle, reflecting the day and night cycle. Over recent years, progress has also been made in the molecular understanding of seasonal rhythms. Both rhythms reflect the natural cycles of the sun. This review focuses on rhythms and clocks of period lengths provided by the moon. These run with around 29.5 days (circalunar rhythms/clocks) or 14.75 days (circasemilunar rhythms/clocks) (see **Figure 1A**). The moon also generates rhythms with shorter period length of 12.4 and 24.8 h, so-called circatidal and circalunidian rhythms, respectively (1–5). We do not discuss these rhythms in our review, because they cover a time scale that is very different from the monthly and semi-monthly rhythms, and are thus likely to be functionally distinct.

Circalunar and circasemilunar rhythms are widespread among organisms, especially in the context of reproductive cycles of marine animals. This fact was likely already noted by fishermen in antiquity, due to the practical implication that the size of the (edible) gonads of local sea urchins varied over the lunar month (6). The notion became a piece of cultural memory through its generalization in Aristotle's work (De partibus animalium IV, 5), and its further tradition by classical authors [see Ref. (6) for the historical reception of the concept]. In the 1920s, the British zoologist Harold Munro Fox put the classical statements to systematic scientific tests, confirming the observation of lunar phase-dependent gonad changes in the Egyptian sea urchin *Diadema setosus*, while dismissing the concept for several other species (6, 7). Fox and other researchers (6–8) also started to compile published evidence for circalunar and circasemilunar rhythms in other marine species, a list that has steadily grown over the course of subsequent decades (2). **Figure 1B** provides some of the well-established examples for circalunar reproductive cycles in marine animals: the seasonal spawning of tropical corals such as *Acropora* during full moon nights (9), the reproduction of the annelid worm *Platynereis* during the waxing moon (10, 11), the precise emergence of the midge *Clunio* at neap tides (12), the lunar cycles of gonad growth in the sea urchin *D. setosus* (6, 7), as well as the circalunar spawning of several fish species (13), such as the goldlined spinefoot (*Siganus guttatus*) in tropical reefs (14), the California grunion (*Leuresthes tenuis*) (15), or the mummichog (*Fundulus heteroclitus*) (16). Besides its impact on reproductive cycles, the lunar cycle also affects the behavior of marine animals. For instance, during the Arctic winter, massive waves of diel vertical migration of the zooplankton are linked to the lunar cycle, reflecting the importance of moonlight as the predominant light stimulus in that period (17). While our review focuses on the animal kingdom, it should be noted that circalunar or circasemilunar reproductive rhythms also exist in species of other eukaryotic kingdoms, such as the brown alga *Dictyota dichotoma* (kingdom Chromalveolata) (18) or the Peruvian apple cactus *Cereus peruvianus* (kingdom Archaeplastida) (19, 20).

The aforementioned distinction between externally regulated rhythms and clock-mediated rhythms is also relevant for the discussion on the occurrence of circalunar rhythms. On the one hand, a reliable, monthly fluctuating environmental stimulus—such as the light stimulus of the full moon, or the mechanical stimulus of the spring/neap tides—can directly cause variation in animal physiology, pigmentation, or behavior, or trigger subsequent hormonal changes. In each of these cases, the stimulus directly translates into an observable biological rhythm (schematized in **Figure 1C** as "Stimulus-controlled"). On the other hand, the respective stimulus can also act to entrain a circalunar timing mechanism (a circalunar clock, also referred to as "circalunar oscillator" in this review). This clock then drives the observed circalunar rhythm (**Figure 1C**, "Clock-controlled"). A classical experimental approach in chronobiology that distinguishes between these two possibilities is the omission of the stimulus after an initial "entrainment" phase (**Figure 1C**, "Entrainment") [see, e.g., Ref. (21)]. Whereas a circalunar rhythm produced by direct impact will not persist under such conditions, a clock-mediated circalunar rhythm will be able to persist. Currently, nomenclature for such omission experiments differs [e.g., "free-running full moon" (22); "constant new moon" (23)].

APPROACHES TO UNRAVEL THE MOLECULAR AND CELLULAR MECHANISMS OF CIRCALUNAR RHYTHMS AND CLOCKS IN MARINE SYSTEMS

Even though circalunar and circasemilunar rhythms and clocks are widespread, and common in the marine environment, researchers have only recently started to tackle the underlying molecular and cellular changes and mechanisms. Most of the molecular data focus so far on the analysis of known circadian clock genes, putative photoreceptors, as well as transcriptomic studies over the course of circalunar rhythms (see **Table 1**). It lies in the nature of these approaches that most of the results are still on the correlative level. Here, we provide an overview of a selection of recent molecular approaches and try to derive more general conclusions from these studies.

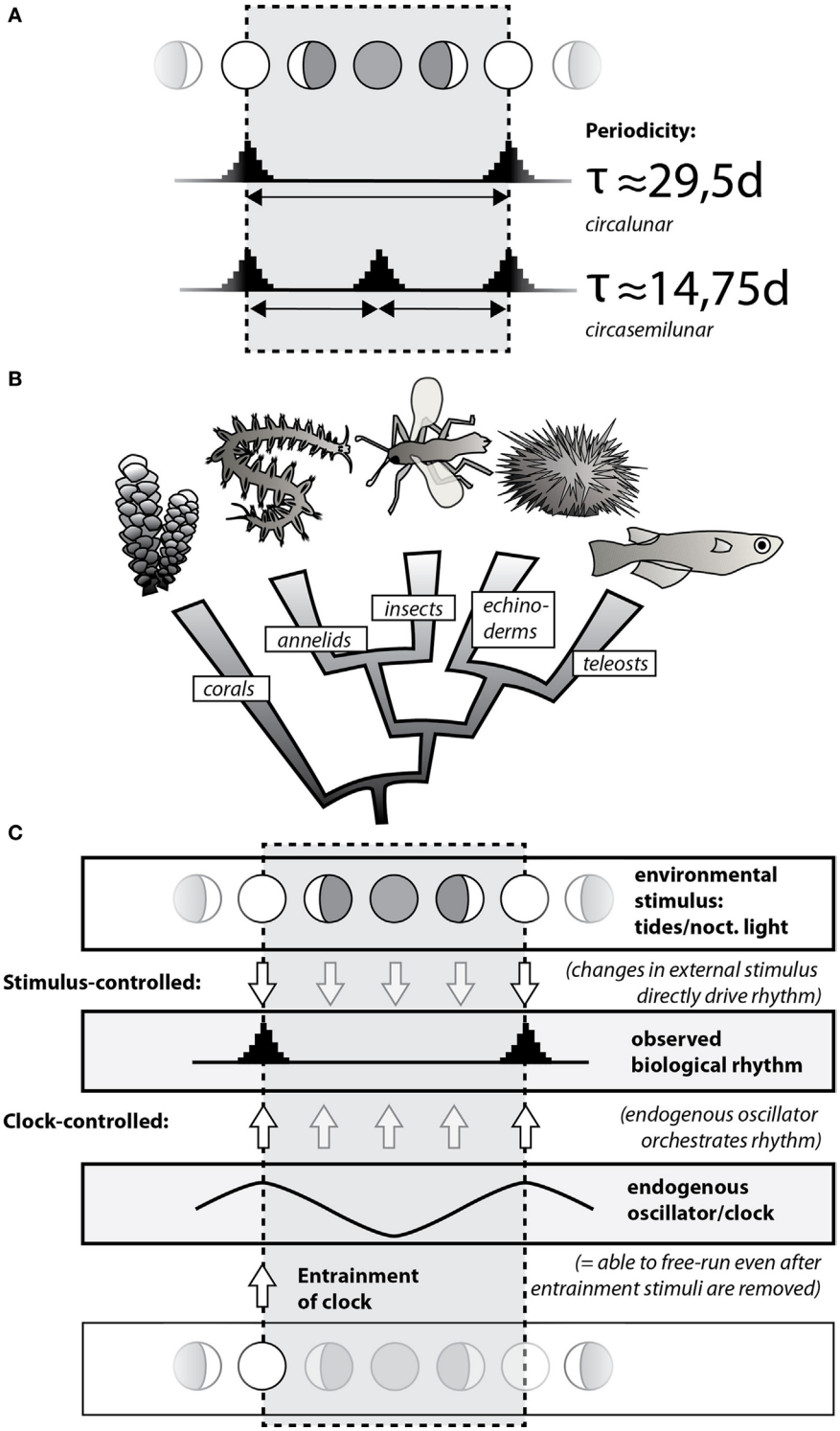

FIGURE 1 | **Circalunar and circasemilunar rhythms and clocks/oscillators are widely present in the animal kingdom**. **(A)** Common biological rhythms linked to the moon cycle can be classified into circalunar and circasemilunar rhythms based on their periodicity, reflecting the re-occurrence of specific events/states once or twice, respectively, during the lunar month. Note that these events/states can be matched with any of the lunar phases, with the example showing synchrony with the full/new moon. **(B)** Circalunar/circasemilunar rhythms are found in a broad range of animals, as demonstrated by the phylogenetic position of individual animal groups in which reproductive cycles have been linked to the lunar phase (see text). **(C)** Biological rhythms either reflect direct response of an organism to changes in the respective environmental stimulus, such as nocturnal light (top; "Stimulus-controlled"); or they are driven by endogenous clocks that are entrained/set by a particular state of the environmental stimulus (bottom; "Clock-controlled"). As the environmental stimulus is not required for an endogenous clock to continue, a clock-mediated biological rhythm also "free-runs" if the environmental stimulus is experimentally removed.

TABLE 1 | Overview on gene differences in the context of the lunar cycle.

Species	Genes analyzed for being affected by nocturnal light or circalunar clock	Analytical method(s)	Reference
Acropora millipora (coral)	cry1, cry2 expression at noon vs. midnight during new moon and full moon, protein location in tissue (note that coral cry1 and cry2 are not equivalent to bilaterian cry1/cry2)	qPCR	Levy et al. (24)
A. millipora (coral)	cry1, cry2, clk, cycle, tim, eya expression 2 sampling regimes: – every 4 h during new and full moon – midnight on 4 moon phases and 4 different lunar light regimes (normal lunar cycles, constant new moon, constant full moon)	qPCR	Brady et al. (23)
A. millipora (coral)	Transcriptome from various diel and lunar timepoints	Quantitative RNAseq	Kaniewska et al. (25)
Acropora gemmifera (coral)	Transcriptome from two diel and four lunar timepoints	Quantitative RNAseq	Oldach et al. (26)
Favia fragum (coral)	*cry1*, *cry2*, *clk*, *cycle* expression at various diel and lunar timepoints	qPCR	Hoadley et al. (27)
Platynereis dumerilii (annelid worm)	*clock*, *bmal (cycle)*, *tr-cry*, *L-cry*, *period*, *pdp1*, *vrille*	qPCR	Zantke et al. (22), Tessmar-Raible et al. (28)
Clunio marinus (dipteran insect)	Genomic loci that contain the genetic differences causing differences in monthly timing	QTL mapping/ genome sequencing	Kaiser et al. (29)
Siganus guttatus (fish)	*cry1*, *cry3*, *per4* transcripts at noon during 5 different lunar phases (natural lunar light, constant new moon, two different coverage regimes during different phases of the night) Cry3 protein localization in brain	qPCR	Fukushiro et al. (30), Toda et al. (31)

Characterization of Molecular and Behavioral Impacts of Circalunar Rhythms and Clocks

The Relationship of Circadian and Circalunar Rhythms

As mentioned earlier, circalunar timing mechanisms rarely exist in isolation, but are coordinated with other timing mechanisms, such as daily (circadian) timing. Therefore, several studies have investigated if the expression of known circadian clock genes is affected either by nocturnal light or by the phase of a circalunar clock (**Table 1**).

In this context, the genes encoding members of the Cryptochrome (Cry) family have received particular attention. Crys are flavoproteins involved in cellular signaling, which are anciently related to photolyases, UV-responsive DNA repair enzymes (32). Molecular phylogenetic analyses show that Crys form multiple, evolutionarily conserved subgroups (32–34). Several of these subgroups are of interest for circadian clock research: members of the d-Cry/Cry1/Lcry family can function as photoreceptors (activated by short-wavelength light) in insect and annelid circadian clocks (22, 33, 35, 36), whereas members of the distinct v-Cry/Cry2/tr-Cry family function as transcriptional repressors in the circadian transcription/translational core loop [reviewed in Ref. (37)]. Members of a family called "plant-type Cryptochromes" also exist in animals and diatoms. In plants, members of this family have been shown to function as photoreceptors for the plant circadian clock [reviewed in Ref. (38)]. Their role outside of the plant phylum is currently unknown (34).

Cryptochrome genes have been investigated in several coral species that display lunar reproductive cycles. In the coral *Acropora millepora*, three types of Crys were identified (24): *Ami*-Cry1 has closest homology to tr-Crys, *Ami*-Cry2 is positioned in the group of the 6-4 photolyases, while the third molecule, *Ami*-CryDash, is related to another ancient group of Crys that has been dubbed "cry-*Drosophila*, *Arabidopsis*, *Synechocystis*, *Homo*" (Cry-DASH) due to its broad evolutionary conservation (32). Different studies investigating the mRNA expression levels of *cry1* and *cry2* and their possible modulation in lunar reproductive cycles arrived at different results: the first study by Levy et al. showed that both *cry1* and *cry2* are induced by sunlight (with no reproducible transcript changes without light). In addition, when animals were sampled during natural full moon nights, *cry2* transcript levels were significantly higher than during new moon nights (24), while *cry1* levels did not show a difference. These results contrast with more recent research by Brady and co-workers in the same coral (23). While these researchers also describe changes in transcript levels for the *cry2* gene over the lunar light cycle, *cry2* showed elevated expression levels at midnight during new moon—and not full moon—nights. Furthermore, in their study, also *cry1* levels showed fluctuations, with elevated expression levels at midnight during the first-quarter moon (23). This study also tested transcript oscillations under constant nocturnal light and lack of nocturnal light over the course of an entire lunar cycle. These experiments assessed if the transcriptional changes are under the control of an endogenous oscillator or only under direct light control. Interestingly, midnight *cry1* and *cry2* transcript levels still showed differences at different phases of the lunar cycle independent of illumination, consistent with the idea that corals also possess a circalunar clock.

Finally, changes in gene expression in *A. millepora* over the lunar month have also been assessed using a transcriptomic approach (25) (also see below). In this study, *cry1* transcript levels were highest at midnight during full moon nights (25). The cause

of these differences is currently unclear and could range from variants in the environmental conditions or different subspecies to higher variation in the transcript changes than previously anticipated. Transcript levels of *cry1* and *cry2* orthologs have also been analyzed in a different coral species, *Favia fragum*. Both genes exhibit light-controlled daily oscillations and also transcript level differences between different moon phases (27). The correlation between the moon phase and the transcript level is, however, not fully clear, since no full lunar cycle was analyzed. Taken together, despite several discrepancies, these results suggest that *cryptochromes* are interesting genes for studying the effect of the lunar cycle on corals, and possibly allowing conclusions on the impact of the lunar cycle on circadian biology of these animals. However, as corals branch off the animal tree at a very basal position, one restriction at this point is that it is still unclear which of the investigated Crys are functionally relevant for circadian control in corals.

The assignment of Crys to circadian functions might be less problematic in other taxa, where Crys have also been investigated, given the clearer functional subgroup position. In the golden rabbit fish, *S. guttatus*, mRNA levels of two *tr-cry* homologs—*SgCry1* and *SgCry3*—fluctuate with the lunar cycle in the brain, but not the ovary (30). Whereas *SgCry1* levels are controlled by light, *SgCry3* levels continue to exhibit a monthly periodicity even in the absence of nocturnal light cues, providing strong evidence that this gene is under the control of an endogenous monthly clock (31).

The bristle worm *Platynereis dumerilii* possesses a complete set of animal Cry/photolyase genes, with one ortholog for each distinct subfamily (34). Of those, *tr-Cry* and *L-Cry* have been investigated with respect to nocturnal light cycles and the circalunar clock of the worm (22). When tested in S2 tissue culture cells, *Platynereis* tr-Cry functions as a transcriptional repressor, but not a light receptor, consistent with a conserved function of this molecule in transcriptional circadian control. The transcripts of *tr-cry* show a clear circadian rhythmicity (both during circadian light–dark and dark–dark conditions). Under nocturnal light conditions that are sufficient to reset the circalunar clock of these animals, the oscillations of *tr-cry* are abolished, indicating that nocturnal light stimuli can affect circadian clock gene expression. Conversely, there is no significant effect of the lunar clock itself on the transcript levels of this gene (22, 28). Also, *Platynereis L-cry*, which functions as a light receptor when tested in S2 cells, shows fluctuations in transcript levels between day and night. These, however, do not appear to follow a regular circadian pattern (22). Both nocturnal light and the circalunar clock appear to impact on the expression of this gene. Due to the irregularity of *L-cry* regulation, however, these changes are difficult to quantify reliably [Ref. (22, 28); Zantke and Tessmar-Raible, unpublished observations].

Besides *cry* genes, also other circadian clock gene homologs have been studied in these animals. In the bristle worm *Platynereis*, transcript levels of the core circadian clock genes, *pdp1*, *period*, and *clock*, exhibit clear changes depending on the worm's endogenous circalunar clock: compared to samples taken during new moon phase, levels are significantly elevated during the full moon phase, even in the absence of nocturnal light ("free-running full moon") (22). Interestingly, a circalunar regulation that persists under free-running conditions has also been observed for transcript levels of *per4* in the diencephalon of the reef fish *S. guttatus*. In the brain samples that were taken at different timed during the lunar cycle, this gene had its lowest expression around the first quarter of the moon, even if the fish were shielded from light during the night (31). Finally, free-running regulation was also observed for several coral genes, like the presumptive circadian clock genes *Ami-cycle*, *Ami-clock*, and *Ami-tim* (23).

Taken together, it appears that both natural and experimental changes in nocturnal illumination, as well as endogenously running circalunar clocks impact on the transcript levels of circadian clock gene homologs in marine organisms as diverse as corals, annelid worm, and fish. A major task for the future will be to work out if and how these transcript changes impact on the circadian rhythm of the respective model species. Interestingly, at least in the bristle worm *P. dumerilii*, the circalunar clock has also been shown to impact on circadian rhythms of locomotor activity, suggesting the possibility that the observed transcript regulations might be linked to these activity changes (22). Such behavioral changes might be due to hormonal fluctuations, as it has been shown that in several species with lunar controlled reproductive cycles, hormones, and hormonal receptors change with the lunar light cycle. In vertebrates, the melatonin pathway is one of the hormone pathways affected by the lunar cycle. For instance, moonlight changes the abundance of *aanat1* (the precursor of the synthesis enzyme AANAT) in the eye of the goldlined spinefoot *S. guttatus* (39). Moreover, at least two of the melatonin receptors in the mudskipper, *Boleophthalmus pectinirostris* fluctuate with semilunar periodicity, in phase with the *aanat2* gene in the pineal of that species (40). Such results provide interesting entry points for further research into the question how nocturnal light modulates circadian biology of animals. In turn, another conceptually interesting question is if the circadian clock components themselves are involved in the generation of circalunar or circasemilunar rhythms. Pharmacological interference experiments in the bristle worm *P. dumerilii* suggest that circadian clock gene oscillations are not required to maintain circalunar rhythms in this species (22). But this does not exclude a role for the circadian clock in entraining the circalunar clock (also see discussion below). Moreover, mass spawnings of marine animals are often not only synchronized to particular days but also particular hours of the day—sometimes with extreme precision (8, 9, 15). Such cases would predict that circadian and circalunar clocks are likely to converge at least on the level of regulating mating behavior or gamete release. Research into the interaction of circadian and circalunar clocks may therefore reveal interesting insight into the coordination between distinct timing mechanisms.

Omics Approaches to Identify Fluctuations Correlated with the Lunar Cycle

Whereas the aforementioned work investigated specific effects of the lunar cycle on circadian clock components, several researchers have tried to complement these experiments with broader approaches that also explore possible rhythmicity in the expression of other genes. High-throughput transcriptome profiling has become an attractive technology for this research. Again, work on corals has already spearheaded this direction

(**Table 1**). Quantitative RNA sequencing was performed on two *Acropora* species over the course of the lunar cycle. Samples taken from *Acropora millipora* at three different times during the day on new moon vs. full moon days revealed that 2% (midnight) to 6% (noon) of *Acropora* genes fluctuate between the two lunar conditions. Based on functional annotation of the encoded proteins, the regulated genes cover a variety of different biological processes, including cell communication, cell differentiation, and cell proliferation (25).

In the second study, *Acropora gemmifera* branches were sampled at four different moon phases and during two different times of the day (noon and midnight). Two sets of regulated transcript types were identified from the quantitative RNA sequencing: one set (55 isogroups) showed diurnal expression patterns that fluctuated over the course of the lunar cycle, whereas the second set (273 isogroups) exhibited differential expression over the lunar cycle when noon and midnight sampling timepoints were combined (26). These two gene sets were largely non-overlapping, resulting in an overall detected change of transcripts over the lunar cycle of about 0.6% [Ref. (28); Vince, personal communication concerning which EST dataset was exactly used for the mapping of the reads]. When considering these numbers, it should be noted, however, that in the second study, sequencing reads were mapped across species (i.e., *A. gemmifera* RNAseq reads onto an *A. millipora* transcriptome). It is thus likely that the real number of regulated transcripts is higher, since genes with lower sequence conservation would not map reliably.

More such studies, especially also under free-running conditions, will be needed to understand the impact of nocturnal light and the circalunar clock on the transcriptome of animals. One challenge that is already emerging from the data reviewed above is that experimental design, data acquisition, and analysis methods differ between studies, making comparisons between individual experiments difficult. A general trend in all of the reviewed studies is that both nocturnal light and free-running circalunar clocks impact on the transcript level of specific genes. The extent of this phenomenon, as well as the potential conservation of such regulated transcripts, remains to be analyzed in the future. Also, the functional meaning of such transcript changes is currently unclear.

Forward Genetic Approaches to Identify Molecules That Can Modulate Circalunar Timing

The third approach to identify molecular mechanisms involved in circalunar timing is to investigate factors that modulate this timing mechanism in natural populations. This approach draws on the idea that within the population of a given species, individual differences in timing exist. In humans, and with reference to daily timekeeping, such natural variants are called chronotypes, with the extremes of "larks" (early chronotypes) and "owls" (late chronotypes) (41). Individual timing differences, however, are neither restricted to humans nor to differences in the circadian clock. One very attractive model system is the non-biting marine midge *Clunio marinus*. This species possesses chronotypes with respect to both daily and monthly timing mechanisms, thereby allowing individual populations to time their emergence precisely to the local neap and spring tides (42). Importantly, these timing differences were shown to be genetically inherited (43). Combining rigorous genetic mapping of these differences with a high-resolution genome for this insect, as well as re-sequencing of distinct chronotypes, has recently allowed the identification of several candidate genes modulating circalunar (and circadian) timing in distinct *C. marinus* timing strains (29). Interestingly, the current analysis in the midge is consistent with the idea that circadian and circalunar timing mechanisms are distinct, as none of the core circadian clock genes is involved in circalunar timing variation (29). It is, however, currently still unclear if the gene loci responsible for the circalunar "chronotypes" are relevant for the entrainment pathway, the circalunar clock, or the output pathway. Hence, it can at presence not be excluded that there is an overlap between some components relevant for circadian and circalunar timing in this insect. The hope is that functional experiments in the midge will help to unravel by which mechanism any of the current candidate loci really contributes to the fine tuning of circalunar timing.

The Quest for the Moon Light Sensors

Another central question concerns the identity of the light receptor(s) that allow organisms with light-driven circalunar rhythmicity to perceive dim nocturnal light, and thereby endow these species either with the ability to directly react to nocturnal light or—in species where circalunar clocks exist—entrain these clocks to the light stimulus.

Due to their light-responsive properties, Crys—that we have discussed above in the framework of the circadian clock—have also received significant attention in this context. The aforementioned study by Levy in the coral *A. millepora* was the first to propose a member of the Cryptochrome family (Cry2) as a possible moon light sensor that could impact on the mass spawning of the coral around full moon (24), and thereby nurtured further interest in this protein group in other studies of animals displaying circalunar rhythms, including the research into reef fish discussed earlier (30).

While such studies suggest a possible function of Cry molecules—albeit of distinct subgroups—as nocturnal light receptors, it is important to emphasize that the speculation on the function of these molecules currently still relies on correlation between the regulation of transcript levels and environmental light. A functional requirement for moon light reception has neither been demonstrated for the reef fish nor for any of the corals. It is also unclear if upregulation of the respective genes is correlated with enhanced light receptive function, i.e., if the mRNA regulation translates into levels of functional protein. Of note, for the coral, the same authors have recently suggested another class of photoreceptors—a melanopsin—as possible light receptor relevant for gamete release, also based on RNA expression data (25).

Opsins have also been suggested to play a role in moonlight sensation in other models. A peculiar example is the Somalian cavefish *Phreatichthys andruzzii*. This species inhabits the dark phreatic layers beneath the desert and has evolved in isolation from surface populations for an estimated time of 3 million years (44). Whereas the species has lost its eyes, as well as many of its photoreceptor genes (45, 46), several Opsins have remained fully

functional (47). Together with observations that fish swim up to the surface of oasis fountains during moonlit nights, this has led to the speculation that these Opsins could be involved in moonlight reception [Ref. (47); Bertolucci, personal communication].

Given these different proposals, a key task for the future will be the functional test of individual light receptors in suitable model species. Here, an attractive model species in which the relevance of Opsins and/or Cryptochrome family members for lunar light reception is being tested is the marine bristle worm *P. dumerilii*. A classical study tested by tissue ablation if the worm's eyes are required for circalunar entrainment, concluding that the adult eyes are dispensable for that purpose (48). More recently, transgenesis and genome mutagenesis have allowed the generation of knockout strains for specific genes (49). A mutant strain has already been generated for the *L-cry* ortholog of the bristle worm, which is currently being used to test the contribution of this gene to circalunar entrainment (49). Interestingly, previous analyses on the properties of the light suitable for the entrainment of the worm's circalunar clock suggests that nocturnal light of different wavelengths is suitable as entraining stimulus (11). This may indicate the involvement of more than one photoreceptor in this process.

Besides the search for moon light sensors, another interesting aspect is the actual mechanism by which organisms distinguish moonlight from other light. One specific question is if there are mechanistic parallels, or even deeper evolutionary links, between the detection of nocturnal light stimuli (relevant for circalunar rhythms or circalunar clock entrainment) and the detection of long vs. short photoperiods (relevant for seasonal rhythms and the entrainment of circannual clocks). Photoperiodism is a widespread phenomenon, occurring in phyla ranging from rotifers and annelids to birds and mammals, helping these animals to anticipate the change of seasons and to adjust their physiology and behavior accordingly [reviewed in Ref. (50, 51)]. There are interesting commonalities between the detection of long photoperiod and moonlight: (i) in both cases, the relevant light stimulus is weaker than the sunlight that animals are exposed to during the day: the setting or rising sun causes less than 1% of the illuminance of the sun at noon. For moonlight, this difference in light intensity is even around five to six orders of magnitude (see **Figure 2**). (ii) Even though the intensity of the relevant light stimulus is therefore very small, in both cases, the actual time point of the stimulus with respect to the 24-h cycle is highly relevant for its interpretation.

For photoperiodic light detection, these considerations have led to the proposal of a "coincidence model," whereby the circadian clock of an organism allows it to set a certain time window of sensitivity, in which the presence vs. absence of light—even if weak—can be correctly interpreted as indication of long- vs. short-day length (18, 55). Interestingly, molecular analyses in mammals have revealed a gene regulatory system that matches this coincidence model. In the sheep pars tuberalis, transcript levels of the transcription factor Eyes absent 3 (Eya3) are controlled both by a circadian signal (that licenses *eya3* transcription 12 h after night fall) and by the acute levels of melatonin (that lead to a suppression of *eya3* transcription during darkness). As melatonin levels are suppressed by light, the combination of these regulatory mechanisms leads to a specific upregulation of *eya3* only under short photoperiod (56). Melatonin-proficient mice appear to possess a similar ability to induce *eya3*, suggesting that this mechanism could be evolutionarily conserved (57).

Given the very low intensity of moonlight, the coincidence model is also one plausible model how a moonlight stimulus could be detected by animals and distinguished from daylight. Like in the case of the photoperiod, the lunar cycle leads to periodic changes not only in the intensity but also the time of nocturnal light (as moon rise and moon set move with respect to the circadian cycle). These features could allow an animal to detect a change in lunar phase by a switch in light state during a sensitive nocturnal period. Experimental data in the midge *Clunio* are compatible with such a model (58); likewise, in *Platynereis*, the relevant stimulus for circalunar synchronization appears to be that the animals obtain a switch from a "light on" state to a "light off" state; notably, this could even be a switch between a long-day photoperiod to a short-day photoperiod, providing a direct parallel to photoperiodic responses in other animals (11). To which extent such mechanistic parallels might also be reflected in molecular similarities is still far from clear. It is interesting, however, that in one of the aforementioned studies on coral gene expression, Brady et al. also observed that levels of a gene with similarity to the *eyes absent* family changed when compared between full moon and new moon nights (23). Moreover, the mentioned influence of the lunar cycle on melatonin signaling in fish provides another interesting molecular link that might help to delineate similarities and differences between moonlight reception and photoperiodic mechanisms.

RELEVANCE FOR TERRESTRIAL SPECIES

As outlined earlier, the presence of circalunar rhythms and clocks across a broad spectrum of marine species (see **Figure 1**) is consistent with the idea that the respective timing mechanisms already predate the major diversifications of animals and the conquest of land. This would imply that also the ancestors of land-living animals likely possessed similar mechanisms. If so, is there evidence for any remnants of these mechanisms in land-living animals, including mammals? In keeping with the distinction between direct environmental impact and clock-mediated processes that we referred to above, we will focus here on two different aspects: the impact of nocturnal light or gravitational cycles on the physiology of terrestrial animals, and the evidence for internal clocks with a monthly period.

Influence of the Moon on Reproductive Timing of Terrestrial Animals

Given the strong role that the moon plays in popular belief and human mythology, scientists have generally remained critical toward reports of direct lunar impact on humans or other terrestrial animals. Moreover, light pollution caused by the process of industrialization/electrification is a factor that is likely to obscure natural responses to moonlight or even disturb the respective rhythms (see the more extended discussion on this topic below).

Nonetheless, a series of scientific studies has produced evidence for the existence of circalunar or circasemilunar rhythms

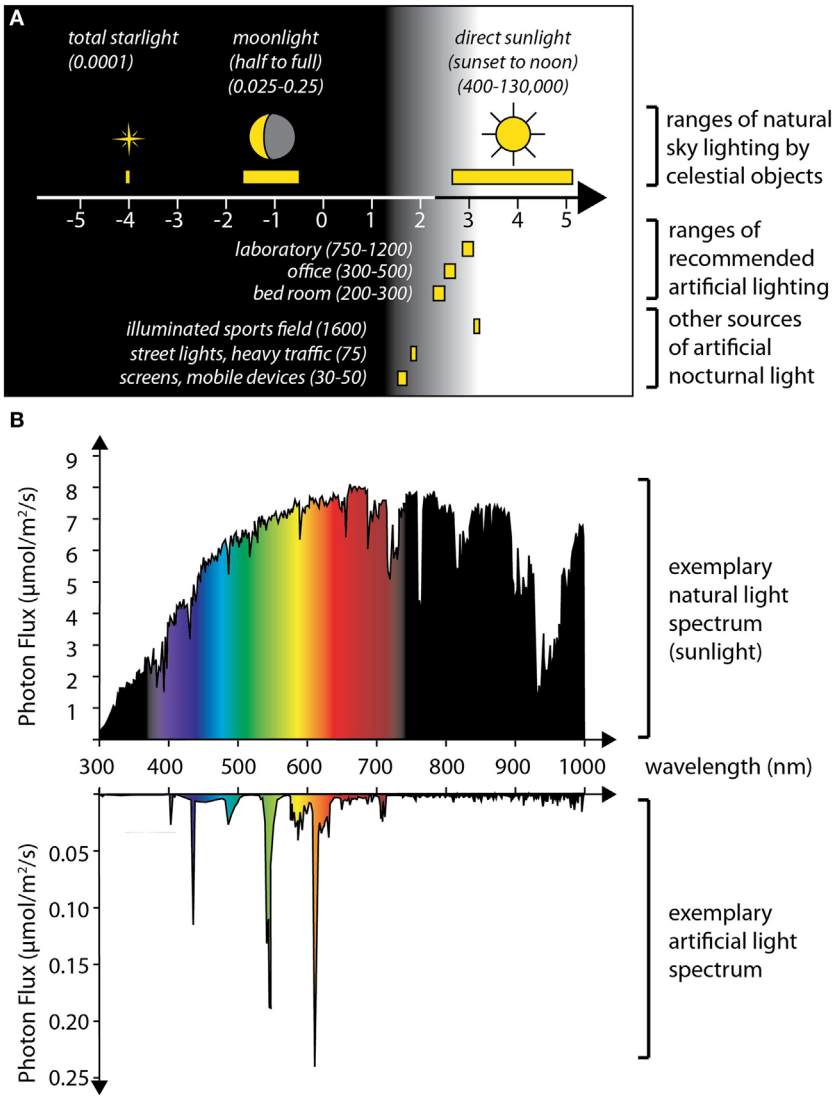

FIGURE 2 | Levels and spectra of artificial light compared to natural light sources. **(A)** The graph summarizes published values on the illuminance caused by celestial bodies (up) (top), and of various sources of artificial light (bottom), expressed in lux, plotted on a logarithmic scale. All displayed artificial light sources cause intensities that exceed maximal moon light intensities (approximately 0.25 lx at a clear full moon night) by at least two orders of magnitude. This indicates that artificial light is highly likely to interfere with any natural response to moonlight. **(B)** In addition to light intensities, artificial lights also have various distinct spectra. Top: Photon flux (expressed as micromoles per square meter per second photons) across the light spectrum (in nanometers), measured for sunlight (on noon of a summer's day, Vienna, Austria); bottom: spectrum of a Philips compact fluorescent lamp (14 W); depending on the specific light receptors affected, the effect of artificial light at night can be aggravated or reduced by changing its spectral composition. Data in panel **(A)** compiled from Ref. (52, 53) and an online version of the Handbook published by the Illuminating Engineering Society (https://www.archtoolbox.com/materials-systems/electrical/recommended-lighting-levels-in-buildings.html); spectra in panel **(B)** schematized based on published values (54).

also in terrestrial species. These affect diverse animal phyla and various aspects of animal life, ranging from reproduction to communication or behavior related to preying or protection from predators [reviewed in Ref. (59)]. Here, we focus primarily on reproductive rhythms, as these allow to consider differences and similarities to the reproductive rhythms introduced in the above sections.

One case where reproductive timing appears to be linked to the lunar cycle is the Serengeti wildebeest, a grazer that migrates each year in herds of enormous size across the Serengeti. The calves of the Wildebeest are typically born in a very narrow, 3-week period around January or February, months before the mass migration in May/June (60). One likely advantage for the synchronized reproduction is that it reduces predation risk. Using birth dates as well as embryo sizes, conception dates in this animal have been systematically estimated. Even though exact estimates are not possible (60, 61), it is remarkable that the estimated dates—which vary from year to year on the solar calendar—consistently fall into a time window in April to May that is determined by two consecutive full moons (60). It thus appears that—on top of a

seasonal signal—the fine tuning of conceptions is linked to the lunar phase. Of note, in the equatorial region, the waning and waxing moon probably provides a more robust light cue than the small differences in solar timing. Hence, other terrestrial animals in the equatorial zone that display narrow reproductive peaks may be interesting species to search for lunar reproductive mechanisms.

Another example of a mammal where the time window for conception appears to be correlated with the moon phase is the European badger. Also in this species, lunar timing is superimposed on a seasonal breeding cycle. The animals typically mate in February to March, soon after the females have given birth to the previous litter. During lactation, the embryos are in a diapause state, before seasonal cues (photoperiod and temperature) lead to implantation around the end of December (62, 63). Dixon and colleagues performed a systematic, long-term video surveillance study on badger behavior during the mating season and also compiled published records from over 100 years of natural history literature on badger behavior (64). The study then investigated the exact dates at which animals copulated and also tracked stereotypic behavior associated with mating, such as the increase in territorial behavior, as evidenced by squat marking (in both sexes) and raised-leg urination (in males). For all of these behaviors, the authors observed a significant correlation to the moon phase, with a peak around the new moon phase.

The third, less pronounced, but surprising case linking the moon phase to mammalian reproduction has been reported for domesticated cattle. In a systematic, 3-year study on over 400 Holstein cows raised on a Japanese farm, Yonezawa and colleagues recently reported a significant influence of the moon phase on spontaneous delivery dates, with deliveries peaking shortly before the full moon, while being minimal around new moon (65). As the animals were artificially inseminated, and the insemination dates were accurately recorded for each cow, the authors were able to show that the observed pattern was not generated by a fluctuation of conception frequencies. Rather, they could relate the observed pattern to deviations between the expected and actual delivery dates. Specifically, there was a significant effect of the moon phase in delaying (new moon) or accelerating (full moon to waning gibbous phase) the actual delivery for up to 2 days (65). The resulting changes are less than 1% in gestation length (average: 284 days), but the study strongly suggests that in a well-controlled system (low genetic variation, reduced artificial light sources), physiologically relevant effects of moonlight can be determined. Given that cattle have been domesticated for around 10,000 years (66), it is possible that this effect represents just a remnant of a more pronounced trait that might have been more relevant in the wild.

When comparing these examples with the aforementioned reproductive cycles in many marine animals, two aspects are interesting to note: (i) in terms of reproductive strategies, mammals are characterized by internal fertilization and typically an independence of the tides. This strategy represents an obvious contrast to the marine broadcast spawners and species reproducing within the tidal zone, where precise synchronization of mating time between sexes is essential for maintaining reproductive success. Otherwise, germ products would be quickly diluted in the water or the substrate/niche required for egg deposition would be unavailable. By contrast, internally fertilizing animals can uncouple copulation from fertilization. This happens, for instance, in numerous insects, with storage of sperm for periods of days to months or even years. Likewise, as demonstrated by the example of badgers, internally fertilizing animals can also uncouple fertilization from embryonic development if needed. Therefore, if circalunar control of reproduction was indeed a more ancient feature of reproduction, the selective pressure to maintain it would have become more relaxed in species evolving internal fertilization strategies. Other selective advantages might therefore be more relevant for the maintenance/evolution of circalunar reproductive strategies in such lineages, for instance, the ability to limit the chance for predators to prey on the offspring (wildebeest). (ii) The two highlighted species in which copulations are limited (wildebeest and badgers) display a clear seasonality in reproduction. The lunar cycle therefore is not the only relevant cycle governing reproduction but is also integrated with information on the season. It is still unclear if these species use light cues to derive information on both lunar phase and seasonal state, or if other cues (temperature for the season; gravity for the lunar phase) may play a role.

Effects of the Moon on Human Birth Rates?

Classical authors as well as popular mythology also suggest various effects of the moon on human biology. These range from an influence on the menstrual cycle and birth dates to aggressive behavior or an impact on mental health [reviewed in Ref. (67)]. Any of these effects is discussed in a controversial manner. Here, we will mainly review three of these aspects: the question if the moon has an influence on human birth rate, the question if there is a connection between the lunar cycle and sleep, and the question if the lunar cycle affects mental health.

Concerning reproduction, one popular claim is that human births are not randomly distributed over the month, but that birth rates differ over the course of the lunar cycle. As for the cases of animal reproduction mentioned earlier, scientists have begun to systematically analyze such claims in the twentieth century. One of the first studies systematically investigating the frequency of births in a large, longitudinal study (1948–1957, around 250,000 births) concluded that around the full moon, birth rates (calculated as a sliding window of 3-day averages) were between 2 and 3% elevated over the average, whereas the time point around new moon showed a reduced birth rate (2–3% below average). The effect was found to be statistically significant (68) and was also consistent with a subsequent study that was conducted for around 500,000 births over a shorter period (1961–1963) (69). Interestingly, the described differences match well with the differences reported by the aforementioned study by Yonezawa and colleagues in the parturition of cows (65). In the decades to follow, various studies have investigated the correlation between birth rates and lunar phases in independent, and partly larger, datasets. Results, however, varied: some found support for an influence of the lunar phase on birth rates, such as a study by Guillon and colleagues on more than 12 mio births in France

between 1968 and 1982 that confirmed a local maximum around full moons (70) (in addition to non-random distributions around quarter moons). Others, however, do not find evidence for a significant correlation, such as a study by Waldhoer and colleagues on around 2.5 mio births in Austria between 1970 and 1999 (71). One way to interpret these inconsistencies is that there is no real influence of the moon on human births, that earlier studies are to be dismissed as outliers, and/or that their methodology underestimated false positive rates (67, 71). On the other hand, it is worth to consider that there could also be anthropogenic factors that introduce biases, especially in more modern datasets. Menaker and Menaker already commented that they excluded data from private clinics, because they displayed obvious drops in births correlating with weekends (especially Sundays). The authors attributed this to the reduced inclination of private doctors to come in on these days (68). Medical development over the following decades introduced various ways in which deliveries could be artificially induced, for instance, by oxytocin or prostaglandin treatment, or amniotomy. Moreover, the frequency of caesarian sections has increased in many countries, now ranging around 30% in the US, Germany, or Austria, and even higher rates in middle and South America, peaking at more than 50% in Brazil (72). Obviously, any of these techniques offers the possibility to induce birth before the natural date and therefore represent factors that would obscure any small effect on natural birth dates caused by the moon at modern times. Of note, both of the more recent studies report a significant drop of birth rates on weekends in their respective datasets (70, 71).

Possible Lunar Effects on Mental Health and Sleep, and the Role of Artificial Nocturnal Light

Another area that has attracted significant interest is the question if the lunar cycle has any impact on mental state of humans. A connection between the moon and mental health is deeply rooted in etymology: the Latin word for moon (luna) is contained in the German word "Laune" (=mood); likewise, the Oxford Dictionary explains that the old Latin word "lunaticus" gave rise to French "lunatique" or English "lunatic," with the word "monseoc" ("moon-sick") representing an old English equivalent to this term. All of these terms relate to the concept that certain persons exhibit periodic phases of mental illness or mood swings, with the earliest use likely relating to epileptic episodes (73, 74). The question is if the link to the moon represents a mere analogy or mythological connection, or if it reflects a—direct or indirect—influence of the moon on mental states.

Different authors have provided alternative explanations for the origin of this connection, and on the role nocturnal light might play in that context: one line of arguments is that in the pre-industrialized world, moonlit nights—especially the three days surrounding full moon—provided a natural opportunity to perform work, hunt, or travel (73), and that these nights therefore led to a decrease in human night sleep around the full moon until around 200 years ago. As reduced sleep is a common parameter in conditions causing mania in patients with bipolar disorder (75) and can also increase the chance of epileptic seizures (76, 77), such monthly recurring phases of reduced sleep might form the factual core of the popular association between the lunar phase and mental health (67, 73). Following this line of reasoning, the advent of artificial illumination ended the dependence of humans on moon light as an exclusive nocturnal light source, thereby causing more stable sleep–wake patterns, and dissolving any apparent lunar periodicity in mental illnesses. In line with this, modern studies do not find a connection between epileptic seizures and the full moon (78).

While this explanation emphasizes the secondary nature of sleep deprivation (as a consequence of a cultural habit to work on moonlit nights), other authors suggest that the lunar cycle itself has a relevant effect on sleep, thereby reflecting a more direct impact of the moon on human physiology. Whereas this issue still remains controversial, two concepts need to be distinguished. On the one hand, the lunar cycle—for instance, the difference in light intensity—might directly impact on sleep parameters. This is, for instance, consistent with a large study on children in 12 different countries whose activity patterns were monitored by accelerometers. This study reported a significant shortening of sleep around full moon by about 5 min (79). The children in this study were monitored remotely in their home environments, and thereby could have been exposed to moonlight in their bedrooms.

Most studies on that subject, however, result from sleep laboratories, in which external light sources like moonlight were systematically excluded. Surprisingly, even under such conditions, effects of the lunar cycle on human sleep have been reported: Cajochen et al. reported a correlation between human sleep quality and the state of the moon in a dataset comprising sleep recordings from 33 subjects, with deep sleep patterns (−30%) and total sleep time (−20 min) being significantly reduced around full moon (80). As the authors emphasized, the analysis was performed only *post hoc*, such that neither the subjects nor the scientists involved in the original experiments could have been biased. Independent studies also arrived at the conclusion that the lunar phase affected sleep, while differing in the detail: consistent with the study by Cajochen et al., Smith et al. reported a reduction of total sleep time during full moon in a study focusing on 47 volunteers, but reported that this overall effect was driven by the sleep patterns of men (−50 min), pointing at possible differences in sex, at least for young subjects (see below). By contrast, Turányi and colleagues, focusing on patients with sleep disorders, reported a stronger effect on women (81). Likewise, a study by Della Monica et al. on 205 healthy subjects, arrived at the conclusion that women had a significantly reduced total sleep time during full moon, whereas men in this study showed even an increase, such that the net effect (irrespective of sex) was not significant (82). The interpretation of differences in these studies varies: some take them as evidence that effects of the lunar phase on sleep exist, but may vary depending on sex or age. For instance, women in the Della Monica study were primarily postmenopausal, whereas in the study by Smith et al., the individuals were on average 23 years old (82, 83). Such differences could also explain why significant net effects were not observed in a re-analysis of three large sleep datasets (covering together more than 2,000 individuals, not separated by sex) by Cordi and colleagues (84). Conversely, as for the discussion of human birth rates, the argument has been made that smaller

datasets might produce significant correlations merely by chance, and that non-significant results are unlikely to be published, generating a confirmation bias in the published—and publishable record—on that matter (82, 84).

Adding to this discussion, the third possibility also exists: if one accepts the idea that endogenous circalunar clocks could also exist in humans (see below), and that they impacted on sleep structure, it would currently be completely unclear if the subjects in either of the mentioned studies were likely to have synchronized oscillations or if these oscillations were offset between individuals. For instance, if nocturnal light played a role in adjusting an individual's circalunar clock—as evident for several of the marine species discussed earlier—changes in the availability and spectrum of nocturnal light could easily impact on the phase of such a clock. Remarkably, artificial light sources have begun to dramatically change the natural fluctuations of light conditions that organisms have experienced during their evolution (52) and are changing both the spectrum and intensity of nocturnal light, with local increases in intensities of up to 20% per year (85). A comparison of illuminance levels shows that recommended levels for room lights, as well as the illuminance from traffic or mobile phones, far exceed the illuminance even of a bright full moon (**Figure 2**). Moreover, the spectral composition of artificial light sources can strongly deviate from sun or moon light, and blue components in artificial lighting are already known to affect melatonin production and animal circadian clocks, even though the natural entrainment stimulus for these is orders of magnitude more intense (**Figure 2**). Given these considerations, it is clear that the impact of artificial lights on biological clocks or rhythms responsive to moonlight levels could be even more dramatic. In turn, this makes the identification of physiological effects of moonlight on human subjects inherently difficult at modern times.

Whereas the majority of the discussed arguments considers moonlight as the most likely cue that impacts on animal physiology—either directly or *via* circalunar clocks—a recent publication by Wehr (86) argues in favor of another possibility. By analyzing a set of longitudinal studies (up to 7 years) in patients with bipolar disorder, the author found evidence that episodes of rapid switches between mental states (mania to depression or *vice versa*) did not occur randomly. Rather, these episodes—as well as some pronounced switches in their frequency—were coupled to gravitational cycles of the moon. For example, when the author compared the mood cycles of patients with the 14.8-day cycle that characterizes the re-occurrence of the axis of moon, sun, and earth (spring-neap tidal cycle), these cycles had particular phase relationships. In some patients, a complete mood cycle (e.g., depression—mania—depression) occurred every two biweekly lunar cycles (i.e., every lunar month, 29.5 days). In other cases, there seemed to be a resonance between one mood cycle and three biweekly lunar cycles (44.3 days), or other integer relationships (86). Moreover, when the author assessed when switches in these periodic relationships occurred (for instance, major shifts in frequency from shorter mood cycles to longer mood cycles), these repeatedly coincided with the 206-day recurrence of the perigee-syzygy constellation of the Earth–Moon–Sun system ("supermoon") which is marked by the coincidence of a full moon with the closest proximity of the moon on its elliptical orbit around the earth. While these constellations also represent an increase in full moon illuminance by about 30%—a factor that ought to be taken into account by studies on moon light effects as well (87)—the favored interpretation by the author is that the coincidence with mood switches in the patients is caused by some gravitational influence, even though the nature of this effect would currently remain unclear (86).

Circalunar Clocks in Mammals

In summary, the above examples illustrate that there are various indications for an influence of the moon on the physiology of humans and other mammals, and point toward aspects that require more detailed analyses. As to humans, several authors have emphasized the need for more extensive longitudinal studies that could better resolve inter-individual differences [see, e.g., Ref. (82)]. Likewise, light pollution will need to be taken into consideration for both human and animal studies, especially as this phenomenon is increasing on a global scale (85), not only affecting terrestrial but also marine environments (88). As pointed out, light pollution is at least a potential caveat when scientists dismiss early studies on circalunar rhythms or clock phenomena based on more recent studies—which is not to say that older analyses could not have failed.

Controversies about the extent of lunar influence, however, should not distract from another physiological phenomenon that is worth emphasizing at the end of this review: primates including humans clearly possess hormonal cycles with monthly periodicity, indicating that there must be timekeeping mechanisms in humans as well as other mammals that are able to run with a roughly monthly period. The most prominent cycle is the menstrual cycle of women who has been determined to cycle almost precisely with a lunar monthly period (29.5 days) (68). Recent data from human males who were kept isolated in a highly controlled environment indicates that such hormonal cycles are not limited to females, but can occur in males as well (89), arguing that the respective timing mechanisms are general properties of human biology. Also outside primates, estrous cycles with a period length of around a month exist. For instance, the estrous cycle of badgers—mentioned above for the role of the lunar phase on the animals' conception dates—has been reported to take approximately one lunar month (90).

Of course, any of these correlations might be coincidence. But one alternative speculation is that such cycles could also be the remnants of an ancient situation where clocks with a monthly period were indeed synchronized by external factors (such as gravity or nocturnal light). A likely scenario then was that the relevance of that synchronization was secondarily diminished—for instance, by a shift in selective pressure that reduced the advantage of a synchronized reproductive window. As a consequence, the endogenous clocks might subsequently have been uncoupled from their respective entrainment mechanisms, while still persisting as oscillators that structure the timing of mammalian physiology. This scenario does not exclude that there could be a remnant capacity of hormonal cycles to be entrained by nocturnal light, as has been suggested by some experiments for

the human estrous cycle [reviewed in Ref. (20)]. But rather than putting emphasis on such remnant capacities, this hypothesis would make the prediction that the actual timing mechanisms between mammals and non-mammalian animals with circalunar clocks share ancient commonalities.

AUTHOR CONTRIBUTIONS

FR and KT-R conceived and wrote most of the manuscript. HT contributed specific subsections and also provided constructive feedback.

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frontiers in Neurology

MINI REVIEW
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Tanycytes As Regulators of Seasonal Cycles in Neuroendocrine Function

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Annual cycles of physiology and behavior are highly prevalent in organisms inhabiting temperate and polar regions. Examples in mammals include changes in appetite and body fat composition, hibernation and torpor, growth of antlers, pelage and horns, and seasonal reproduction. The timing of these seasonal cycles reflects an interaction of changing environmental signals, such as daylength, and intrinsic rhythmic processes: circannual clocks. As neuroendocrine signals underlie these rhythmic processes, the focus of most mechanistic studies has been on neuronal systems in the hypothalamus. Recent studies also implicate the pituitary stalk (*pars tuberalis*) and hypothalamic tanycytes as key pathways in seasonal timing. The *pars tuberalis* expresses a high density of melatonin receptors, so is highly responsive to changes in the nocturnal secretion of melatonin from the pineal gland as photoperiod changes across the year. The *pars tuberalis* in turn regulates tanycyte function in the adjacent hypothalamus via paracrine signals. Tanycytes are radial glial cells that persist into adulthood and function as a stem cell niche. Their cell soma are embedded in the ependymal lining of the third ventricle, and they also send elaborate projections through the arcuate nucleus, many of which terminate on capillaries in the median eminence. This anatomy underlies their function as sensors of nutrients in the circulation, and as regulators of transport of hormones and metabolites into the hypothalamus. *In situ* hybridization studies reveal robust seasonal changes in gene expression in tanycytes, for example, those controlling transport and metabolism of thyroid hormone and retinoic acid. These hormonal signals play a key role in the initial development of the brain, and experimental manipulation of thyroid hormone availability in the adult hypothalamus can accelerate or block seasonal cyclicity in sheep and Siberian hamsters. We hypothesize that seasonal rhythms depends upon reuse of developmental mechanisms in the adult hypothalamus and that tanycytes are key orchestrators of these processes.

Keywords: tanycyte, season, photoperiod, neuroendocrinology, stem cells

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INTRODUCTION

Investigation of the central mechanisms underlying seasonal cycles in energy balance has provided new insights into the fundamental control systems of appetite and energy expenditure in the brain. Homeostatic mechanisms governing the short-term control of energy balance, for example, the timing of meals and the response to acute fasting, have been extensively studied in laboratory animal models. This body of work has given us great insight into the autonomic and endocrine signals emanating from the gastrointestinal tract and white adipose tissue that communicate to integrative

centers of the hypothalamus and brainstem (1). However, the evidence that changes in homeostatic gene expression underlie long-term season cycles in energy balance is very limited (2, 3). In seasonal mammals, rheostatic mechanisms that govern the long-term control of energy balance reflect a higher order set of processes controlling the neuroendocrine system (4). A key element of this rheostatic system comprises hypothalamic tanycytes (**Figure 1**). These are radial glial cells whose cell soma in embedded in the ependymal lining of the third ventricle (**Figure 1**). They possess elaborate projections that communicate with hypothalamic nuclei implicated in energy balance (5). Subtypes of tanycyte have been identified on the basis of their location and their proximity to hypothalamic nuclei: α1 and α2 tanycytes appose the dorsomedial and ventromedial nuclei, whereas β1 and β2 tanycytes border the arcuate nucleus and median eminence. Interestingly, β2 tanycytes differ from the other subtypes as they have direct access to circulating plasma (6). These tanycytes in the ventral region of the third ventricle are uniquely fenestrated and selectively permeable, allowing passive and active transport of molecules from the circulating blood supply in the median eminence into the cerebroventricular fluid in the third ventricle (7). While there is conflicting evidence for homeostatic-induced gene expression changes in tanycytes, there is consistent evidence between studies and species for seasonal/photoperiodic-induced changes in gene expression (**Figure 2**). In particular, tanycytes have been identified as key determinants of long-term seasonal changes in ingestive behavior and energy metabolism through their role in transport and regulation of thyroid hormone availability in the hypothalamus (8). The aim of this review is to summarize our current understanding of tanycyte biology and outline their key roles in nutrient and hormone sensing, and in directing neuroplasticity, and thereby regulating hypothalamic control of energy metabolism.

HYPOTHALAMIC TANYCYTES AS MEDIATORS OF ENERGY HOMEOSTASIS

The blood–brain barrier (BBB) is a feature of the cerebral vasculature that restricts and regulates access of molecules to the brain, and therefore acts as a gatekeeper to the hypothalamic nuclei and beyond (9–11). However, despite the prominence of tanycytes within the ependymal layer of the third ventricle and their expression of a wide range of hormone receptors and nutrient

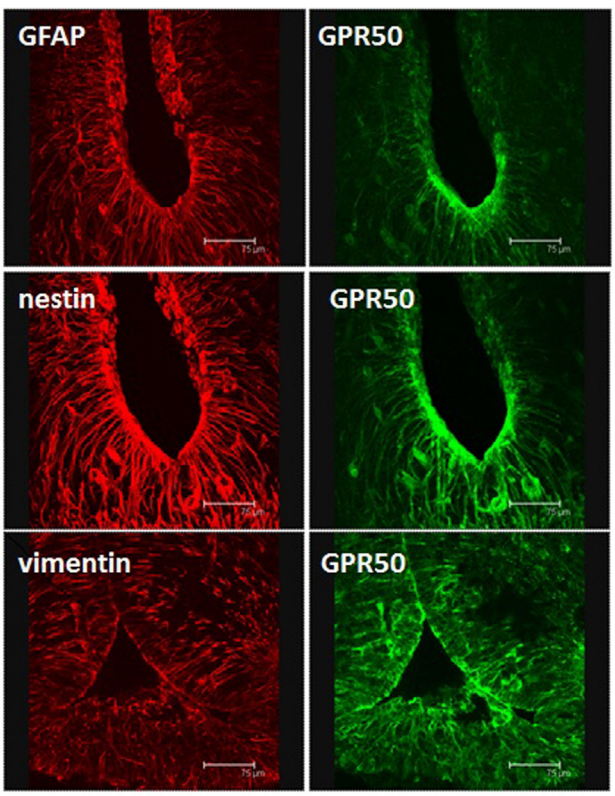

FIGURE 1 | Immunohistochemical identification of tanycytes in coronal sections through the mediobasal hypothalamus of a Siberian hamster. Polyclonal rabbit antisera detect glial fibrillary acidic protein, or the intermediate filaments nestin or vimentin. Sections are also stained with a goat polyclonal directed against the melatonin-related receptor GPR50. Scale bars = 75 μm. Image from Fowler and Ebling, University of Nottingham.

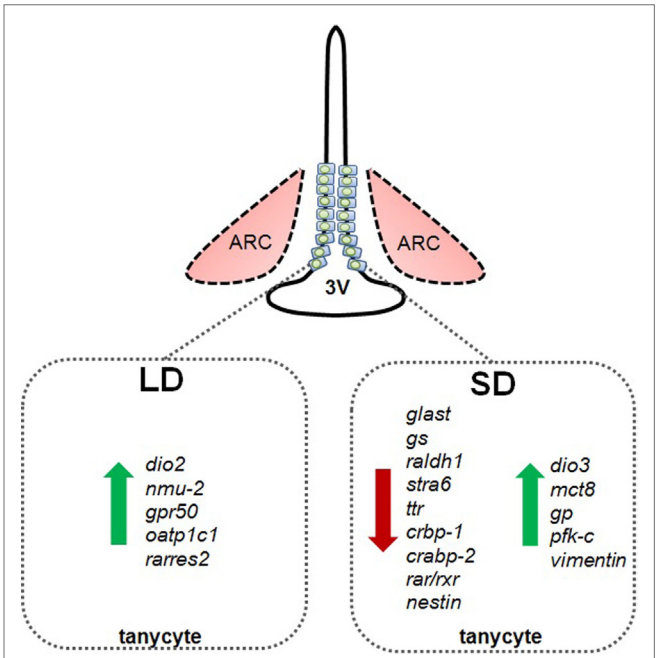

FIGURE 2 | Schematic summary of photoperiod-induced changes in gene expression in tanycytes in Siberian hamsters exposed to long summer photoperiods (LD) or short winter photoperiods (SD). *dio2*, deiodinase 2; *nmu-2*, neuromedin 2; *gpr50*, G-protein-coupled receptor 50 (=melatonin-related receptor); *oatp1c1*, organic anion transporter 1C1; *rarres2*, chemerin; *glast*, glutamate transporter; *gs*, glutamine synthetase; *raldh1*, retinaldehyde dehydrogenase; *stra6*, retinol transport protein stimulated by retinoic acid gene 6 homolog; *ttr*, transthyretin; *crbp-1*, cellular retinol binding protein; *crabp-2*, cellular retinoic acid binding protein-2; *rar/rxr*, retinoic acid and rexinoid receptors; *nestin*, type VI intermediate filament nestin; *dio3*, deiodinase 3; *mct8*, monocarboxylate transporter 8; *vimentin*, type III intermediate filament vimentin; *gp*, glycogen phosphorylase; *pfk-c*, phophofructokinase C.

sensors, their role in energy homeostasis is hotly debated. Early studies on tanycytes focused upon their barrier function (6). In response to food deprivation and a resulting fall in blood glucose, tanycytes undergo morphological changes and increase vascular permeability via enhanced secretion of VEGF-A (10). These reversible morphological alterations at the BBB suggest that nutritional state modulates the access of metabolic signals via tanycytes from the periphery to hypothalamic nuclei critical for energy homeostasis. However, the role of tanycytes as mediators of energy homeostasis extends beyond the morphological to the adaptive homeostatic and neuroendocrine.

In response to a fast, the hypothalamus–pituitary–thyroid (HPT) axis is downregulated through a reduction of thyrotropin-releasing hormone (TRH) synthesis in the paraventricular nucleus (PVN). The neurons of the PVN project to the median eminence and the terminals are in close proximity to the projections of β2 tanycytes. These cells express pyroglutamyl peptidase II (PPII), an ectopeptidase that hydrolyzes TRH, and thus controls the amount of TRH available to cause thyroid-stimulating hormone (TSH) synthesis and secretion in the anterior pituitary. *In situ* hybridization studies demonstrated that PPII and deiodinase 2 (DIO2) were increased in tanycytes following a fast (12). DIO2 removes an outer ring iodine atom, so converts the inactive form of thyroid hormone (thyroxine; T4) into the biologically active form triiodothyronine (T3). This is a common theme in tanycyte biology. Interestingly, increased DIO2 activity in tanycytes suppresses TRH secretion from the PVN via the local increase in T3 availability in the hypothalamus, and subsequent studies demonstrated that DIO2 in tanycytes is essential for regulation of the HPT axis (13–15).

An intriguing feature of tanycyte biology is that these cells are also activated by signals emanating from the adjacent *pars tuberalis* in the pituitary stalk. For example, TSH receptors located in tanycytes are activated by TSHβ produced in the *pars tuberalis*. This signal is transduced via both activation of adenylate cyclase and phosphorylation of extracellular signal-regulated kinases (ERK1/2), resulting in increases in DIO2 mRNA expression (16). In addition to fasting, overnutrition results in changes in tanycyte biology; ghrelin uptake/transport is attenuated following neonatal overfeeding (by reducing litter size) in the mouse (17). The lipopolysaccharide-induced cytokine upregulation of DIO2 expression in tanycytes and the stimulatory actions of pituitary adenylate cyclase-activating polypeptide both occur via increased intracellular cAMP and the NF-κB pathway (18, 19).

Interestingly, tanycytes express the insulin-independent glucose transporters GLUT1 and GLUT2, and also glucokinase. Indeed, in hypothalamic slice cultures, tanycytes respond to exogenously administered glucose, which stimulates Ca^{2+} ion fluxes and ATP release; effects that are then propagated across neighboring cells (20). This is further evidence that they function as nutrient sensors (21, 22). Furthermore, tanycytes express a number of enzymes involved in lipid metabolism, and monocarboxylate transporters, a family of transporters that mediate the facilitated diffusion of lactate, pyruvate, and ketone bodies. This suggests further possible mechanisms, whereby tanycytes mediate neuronal responses in the hypothalamus to changes in peripheral carbohydrate and fat metabolism (23, 24). Recently, a metabolic link between tanycytes and astrocytes, likely to impact hypothalamic lipid sensing, has been suggested (25). In addition, in leptin receptor deficient mice (*db*/*db*) and in mice treated with a leptin antagonist, leptin accumulates in the median eminence but fails to appear in the mediobasal hypothalamus, providing evidence that leptin's signaling cascade begins in tanycytes in the median eminence, and then transitions to hypothalamic nuclei and neurons (26).

Further evidence supporting the neuroendocrine roles of tanycytes is provided by a series of experiments that targeted the fibroblast growth factor receptor 1 c isoform (FGFR1c). It was previously shown that antibody-mediated targeting of the FGFR1c receptor reduced body weight, adiposity, and insulin resistance in animal models of obesity and type II diabetes (27–29). Subsequent *in situ* hybridization studies in the Siberian hamster revealed a high level expression of the FGFR1c in tanycytes, consistent with previous qPCR studies in the mouse (30, 31). Targeting of the FGFR1c in the long day (LD) obese Siberian hamster peripherally and centrally via intracerebroventicular infusion of a monoclonal FGFR1c antibody reduced food intake and body weight, which was associated with a decrease in expression of DIO2 in the ependymal cell layer containing tanycytes (31). This further supports the hypothesis that tanycytes are an important component of the mechanism by which the hypothalamus integrates central and peripheral signals to regulate energy homeostasis. It also highlights a potential role in seasonal metabolic cycles, as the response to tanycyte manipulation was attenuated in short-day (SD) lean animals.

HYPOTHALAMIC TANYCYTES AS MEDIATORS OF SEASONAL CYCLES

In response to seasonal changes in daylength, mammals such as the Siberian hamster and the F344 strain of photoperiodic rat undergo substantive behavioral and physiological adaptations, for example, in body composition, growth, and reproductive activity (32, 33). The retina is crucial to such adaptations; for example, optic nerve transection or bilateral enucleation prevents the synchronicity of seasonal reproduction (34, 35). Photoneuroendocrine pathways, where retinal information is conveyed to the suprachiasmatic nucleus, are well characterized, as is the neurochemical index provided by the secretion of melatonin by the pineal gland in response to changes in daylength (36). More recently, we have begun to appreciate the role and importance of the *pars tuberalis*, part of the pituitary stalk that contains a high density of melatonin receptors in all seasonal mammals and communicates to adjacent tanycytes in the hypothalamus (37). Emerging evidence suggests that tanycytes are an integral part of the mechanism that facilitates seasonal physiology and behavior in seasonal mammals. In addition to melatonin-regulated changes in secretion of paracrine factors including TSHβ and neuromedin U (NMU), this region undergoes structural changes in response to changing photoperiod, particularly in the thyrotrophs, which produce TSH (38–40). One consequence of this is that a significantly lower percentage of cells display exocytotic activity in SD, supporting the hypothesis that the *pars tuberalis* functions as an interface

between photoperiodic stimuli and the endocrine system (41). Furthermore, the regulation of thyrotrophs is a melatonin-dependent process; pinealectomy blocks the SD-induced downregulation of TSHβ production, and treatment with melatonin can mimic the actions of SD (42, 43). As noted above, the TSHβ subunit has been shown to signal to tanycytes, and studies on the Syrian hamster, photoperiodic rat, and sheep have revealed that tanycytes express the TSH receptor, while local infusion of TSHβ into the third ventricle upregulates DIO2 in these glial cells (44, 45). It is of note that in juvenile photoperiodic rats, TSHβ also downregulates deiodinase 3 (DIO3) expression in the ependymal cell layer (44).

DIO3 is an enzyme in the tanycyte cell layer that opposes the action of DIO2, as it removes an inner ring iodine, and therefore deiodinates T4 into reverse T3, which is biologically inactive. Furthermore, it deiodinates T3 into the inactive metabolite diiodothyronine (T2). In the adult Siberian hamster, rather than a LD-induced upregulation of DIO2 (**Figure 2**) that increases the local availability of T3, DIO3 is upregulated in response to SD (**Figure 2**), inactivating T3 or converting the precursor to T2 (32). This phenomenon is not limited to the Siberian hamster, it is also seen in male sheep exposed to SD for 14 weeks (46). It is predicted that the enhanced expression of DIO3 would have the same effect on local thyroid hormone availability in the hypothalamus as the downregulation of DIO2 observed in most other photoperiodic species (32). The biological significance of this predicted change in hypothalamic T3 concentrations was directly tested in the Siberian hamster by surgically inserting micro T3 implants into the hypothalamus, and exposing hamsters to changes in photoperiod. Such implants blocked the SD-induced weight loss and catabolism of fat depots and prevented SD-induced testicular regression (8). Correspondingly, T3-releasing implants stimulated appetite and induced body weight gain and reproductive recrudescence when placed in hamsters previously exposed to SD (47). The T3 microimplants blocked the SD-induced increase in VGF expression in the dorsomedial posterior arcuate nucleus, a potential regulator of seasonal changes in appetite and energy expenditure (8).

In addition to the clear effects of TSHβ derived from the *pars tuberalis* on deiodinase gene expression in tanycytes, other paracrine mechanisms may also be important in the regulation of deiodinases and tanycyte function. For example, ICV infusion of NMU decreases food intake and in obese mouse models increases physical activity, energy expenditure, and thermogenesis. Furthermore, $NMU^{-/-}$ mice exhibit hyperphagia, increased body weight, and reduced energy expenditure. The actions of NMU are conferred by the NMU-2 receptor (48). Interestingly in photoperiodic rats in LD, NMU gene expression is upregulated in the *pars tuberalis*, while its receptor is upregulated in tanycytes (44, 49). It was subsequently shown that local infusion of NMU into the third ventricle of photoperiodic rats held in SD upregulated DIO2, thus mimicking the LD state (44). Similarly, the GPR50 receptor, which is homologous to the melatonin receptor MT1 but does not bind melatonin, is expressed in tanycytes (**Figure 1**) and has been implicated in adaptive thermogenesis and torpor (50). GPR50-null mice are resistant to diet-induced obesity; however, when fasted, they more readily enter a state of torpor. These effects appear to be mediated through TRH, as entry into torpor is reversed by treatment with TRH receptor agonists (51, 52). In the Siberian hamster exposed to SD, GPR50 expression is significantly reduced in tanycytes (**Figure 2**); this may contribute to bouts of adaptative thermogenesis, torpor, and more broadly energy balance (53). In response to SD, the thyroid hormone transporter monocarboxylate 8 (MCT8) is increased in tanycytes in the Siberian hamster, while fasting reversed this effect, further evidence supporting the role of thyroid hormone and tanycytes in the photoperiodic regulation of seasonal biology (54). Additionally, the thyroid hormone transporter organic anion transporter family member 1C1 (Oatp1c1) is photoperiodically regulated in tanycytes so potentially contributes to seasonal alterations in thyroid hormone transport [**Figure 2**; (55)]. Interestingly, the lactate (MCT2) and glutamate (GLAST) transporters, as well as glutamine synthetase, are reduced in tanycytes during SD (**Figure 2**), suggesting glutamate uptake and production of glutamine are diminished. Furthermore, glycogen phosphorylase and phosphofructokinase-C, rate-limiting steps in the metabolism of glycogen to glucose, are increased in tanycytes during SD [**Figure 2**; (56)].

Interestingly, T3 rapidly induces the RA-synthesizing enzyme retinaldehyde dehydrogenase 1 (RALDH1) in tanycytes (57). In photoperiodic rats, RALDH1 and -2 expression is reduced in SD, while the retinol transport protein stimulated by retinoic acid gene 6 homolog (STRA6) is reduced by SD (58, 59). Furthermore, expression of transthyretin (TTR), a common transporter for vitamin A and its metabolite retinoic acid, is downregulated under SD in the tanycytes of photoperiodic rats, while cellular retinoic acid binding protein (CRBP1), a retinoic acid transport protein, is downregulated in SD photoperiods in tanycytes in Siberian hamsters. The latter effects are reversed by pinealectomy, which suggests that the mechanism is dependent upon melatonin (53). Furthermore, cellular retinoic acid binding protein-2 (CRABP-2) and members of the nuclear retinoic acid receptor and retinoid X receptor families are reduced in response to SD in the Siberian hamster (53, 60). Interestingly, retinoic acid regulates the ability of tanycytes to proliferate and generate new cells in the hypothalamus highlighting another possible role for tanycytes (5).

HYPOTHALAMIC TANYCYTES AS A STEM CELL NICHE

A number of studies support the existence of hypothalamic stem cells capable of generating new neurons in a variety of species. However, the location and identity are hotly disputed. Recent *in vitro* and *in vivo* studies have suggested that they are located within the mediobasal hypothalamus parenchyma and could represente NG2-expressing oligodendrocyte progenitor cells (61, 62). Contrasting studies have suggested that subpopulations of tanycytes constitute the source (63–66). This in itself, however, is controversial as both α- and β-tanycytes have been identified as the possible neurogenic niche, as well as a possible role for insulin-like growth factor (63, 65, 67). Interestingly, in one study, exposure of mice to a high fat diet depleted numbers of putative

hypothalamic stem cells, which was associated with impaired glucose tolerance and subsequent obesity (68). However, rather contradictory results were reported in the study that demonstrated increased numbers of cells labeled with the thymidine analog BrdU in the hypothalamic ventricular zone in mice maintained on a high fat diet (64). Furthermore, in the latter study, focused irradiation of the hypothalamus inhibited cell division that was associated with reduced body weight gain on a high fat diet, suggesting that new cells produced in the hypothalamus might have an anabolic function (64). More recently, increased ciliary neurotrophic factor signaling was detected in tanycytes close to the median eminence in obese mice on high fat diet, further supporting the hypothesis that positive energy balance is associated with enhanced hypothalamic neurogenesis (69).

In addition to high fat diet, photoperiodic stimuli regulates cell division in the adult hypothalamus. Exposure to SD increased vimentin labeling in hypothalamic tanycytes of sheep and increased numbers of BrdU-positive cells in the sheep hypothalamus, though a substantive proportion of these expressed a microglia marker so were not destined to become neuronal (70, 71). Following the transition from LD to SD, an increase in cellular proliferation is apparent in the hypothalamus of Syrian hamsters; in the Siberian hamster, the intermediate filament protein, and neural stem cell marker nestin is downregulated during SD (53, 72). Further studies are clearly required to determine whether the reported seasonal changes in BrdU uptake or expression of cell cycle markers such as Ki67 truly reflect altered neurogenesis, or whether new cells integrate into functional circuits in the hypothalamus. However, given the evidence above regarding photoperiod-induced changes in thyroid hormone availability in the hypothalamus, and the extensive evidence that the thyroid hormone system is implicated in neural division and differentiation, it seems very likely that plasticity of cell division and connectivity in the hypothalamus will be identified as a core feature of seasonal cycles (73, 74). Finally, it has been observed that the ability of tanycytes to proliferate postnatally declines with age: incorporation of the S-phase marker BrdU in β-tanycytes deteriorates between P7 and P45, while no incorporation is seen by 12 months of age (63). Furthermore, tanycyte numbers declines by almost 30% with increasing age as well as inducing significant morphological and anatomical changes; processes become thicker and disorganized in the pericapillary zone, with a loss of perpendicular orientation (75). This poses further tantalizing questions regards their metabolic role in relation to aging, and whether seasonal cycles might be considered as arrested or even reversible aging.

CONCLUSION

Identifying the mechanisms by which mammals naturally regulate appetite and body composition across the year should provide insights into how long-term improvements in metabolic health could be promoted in man. Tanycytes are the only cell type in the hypothalamus that shows major changes in gene expression across a seasonal cycle, so are a likely regulator of long-term changes in energy balance. Tanycytes have a privileged position as a nutrient and hormone sensor with projections to the metabolic brain, and potentially function as a neural stem cell niche, highlighting a number of mechanisms that could influence energy intake and expenditure in the long term. Experimental studies in the hamster have already confirmed that changes in thyroid hormone processing by tanycytes are part of this seasonal programming of the hypothalamus.

AUTHOR CONTRIBUTIONS

JL and FE drafted and revised the manuscript.

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Control of Rest:Activity by a Dopaminergic Ultradian Oscillator and the Circadian Clock

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There is long-standing evidence for rhythms in locomotor activity, as well as various other aspects of physiology, with periods substantially shorter than 24 h in organisms ranging from fruit flies to humans. These ultradian oscillations, whose periods frequently fall between 2 and 6 h, are normally well integrated with circadian rhythms; however, they often lack the period stability and expression robustness of the latter. An adaptive advantage of ultradian rhythms has been clearly demonstrated for the common vole, suggesting that they may have evolved to confer social synchrony. The cellular substrate and mechanism of ultradian rhythm generation have remained elusive so far, however recent findings—the subject of this review—now indicate that ultradian locomotor rhythms rely on an oscillator based on dopamine, dubbed the dopaminergic ultradian oscillator (DUO). These findings also reveal that the DUO period can be lengthened from <4 to >48 h by methamphetamine treatment, suggesting that the previously described methamphetamine-sensitive (circadian) oscillator represents a long-period manifestation of the DUO.

Keywords: dopaminergic ultradian oscillator, biological rhythms, circadian clock, dopamine transporter, rest:activity

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INTRODUCTION

Many species on earth have evolved a self-sustaining timing system, likely to facilitate robust 24-h rhythms in physiology and behavior despite non-24-h variations in the environment. This timing system, the circadian clock, has been studied in detail over the past decades, uncovering its cellular and molecular basis (1, 2). In addition to 24-h variations, there are also numerous accounts of cyclic changes in physiology and behavior with periods much shorter than 24 h, i.e., in the ultradian range. Ultradian rhythms with periods of 2–6 h have been reported in the context of locomotion, sleep, feeding, body temperature, and serum hormones levels, in species from the fruit fly to humans (3–13). However, in sharp contrast to circadian rhythms, the biological substrate and mechanistic basis of ultradian rhythm generation has remained elusive.

Ultradian Behavior in Voles and Mice: Hourglass vs. Oscillator

While ultradian range rhythms are often found to be labile when compared to circadian/diurnal rhythms (14, 15), a particular overt and robust expression of ultradian behavior is exhibited by the common vole (*Microtus arvalis*) (**Figure 1A**) (5). This is thought to be due to evolutionary pressures resulting in the emergence of synchronous ultradian day time foraging as a strategy to reduce predation risk: by emerging from the burrows during the daytime every 2–3 h in synchrony, the

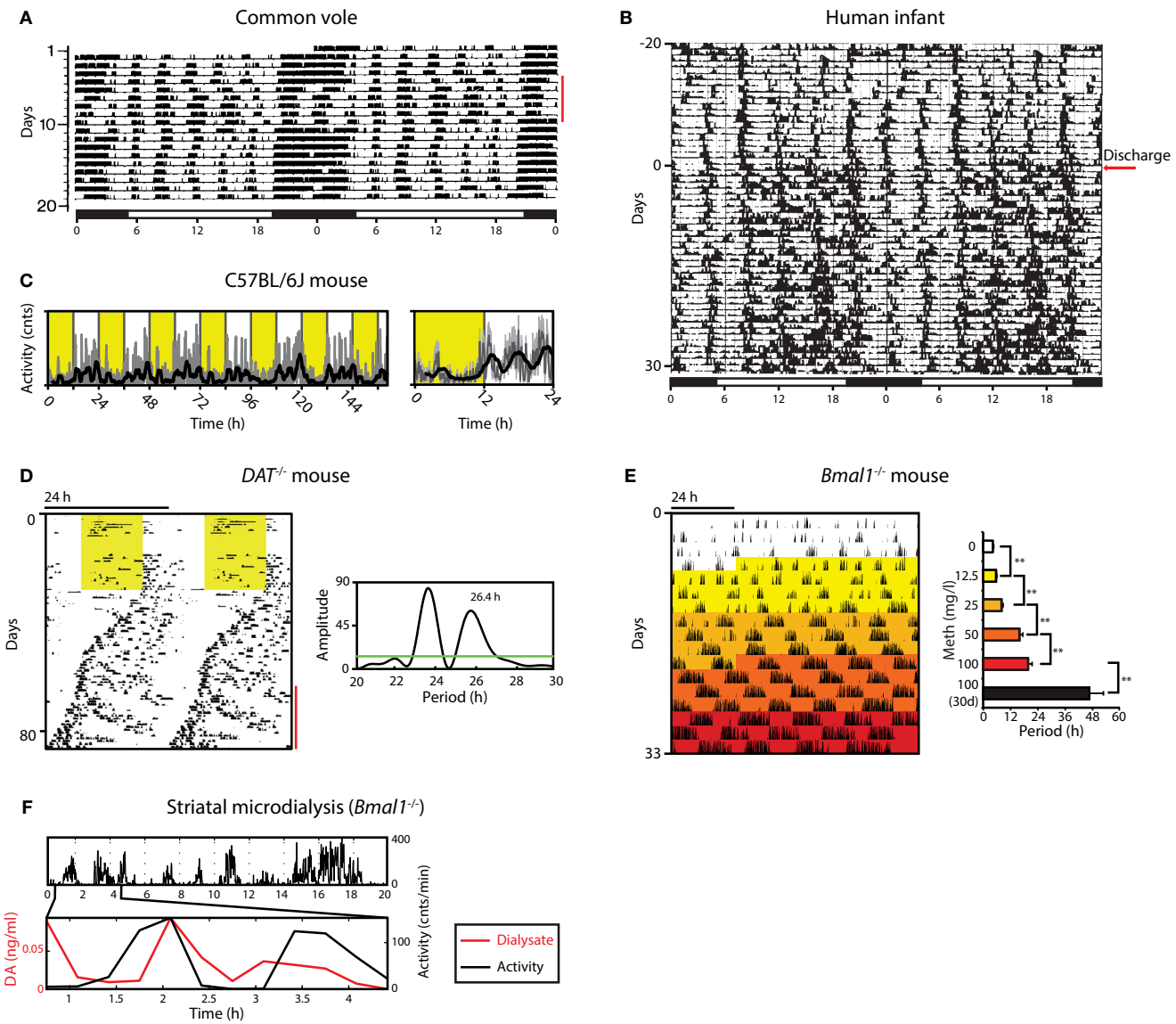

FIGURE 1 | Ultradian rhythms and their manipulation from voles to humans. **(A)** Locomotor activity (LA) rhythms in the common vole in the presence of a running wheel; red bar indicates days when the wheel was blocked; bar on top indicates periods of lights on (white) and off (black); adapted from Ref. (16) with permission. **(B)** Activity record of a preterm infant based on ankle-actigraphy; arrow indicates day of hospital discharge; adapted from Ref. (8) with permission. **(C)** Recording of ambulatory activity in the mouse by telemetry implants; right, average daily activity based on primary data shown on the left; yellow shading indicates lights on. **(D)** Running wheel activity of a DAT$^{-/-}$ mouse; yellow area, lights on; red bar indicates the emergence of a second rhythmic component, supported by periodogram analysis (right). **(E)** Gradual ultradian locomotor period lengthening by increasing methamphetamine concentration in the drinking water of Bmal1$^{-/-}$ mice in constant darkness. **(F)** Extracellular dopamine measured by microdialysis in the striatum fluctuates synchronously with ultradian LA in Bmal1$^{-/-}$ under constant dim red light. Graphs shown in D, E, F are adapted from Ref. (20).

voles are less likely to fall prey to a kestrel (5). Ultradian rhythm expression does not require the circadian timer as rhythms persist in the vole after lesioning of the suprachiasmatic nucleus (SCN), the central circadian pacemaker site (16). While such ultradian behavior could be the output of a discrete rhythm generator, it may as well be driven by physiological demand, such as energy depletion or sleep debt. However, food, water, or sleep deprivation does not affect ultradian locomotor activity (LA) of the vole in substantial ways (5, 17). For instance, if—in the laboratory cage setting—food access is blocked, the voles still engage the food access bar at the same ultradian period as under conditions of *ad libitum* food access (17). Equally, forced lengthening of the active phase by rest deprivation does not lead to a proportional increase in subsequent rest time, which consequentially would result in ultradian period lengthening (17). It appears instead that sleep rebound is facilitated by an increased rest:activity ratio within a given ultradian cycle, instead of changing the cycle length *per se*. Taken together, these data argue against a

role of behavioral output to define or regulate ultradian period but favor an endogenously generated, self-sustained oscillatory process that does not require a "driver," as would be the case if the ultradian rhythmicity is based on an hourglass mechanism (18, 19).

In contrast to voles, ultradian components in LA are less overt but still detectable in circadian intact laboratory mice, exhibiting periods of 3–5 h (7, 20) (**Figure 1C**). Elimination of the master circadian pacemaker by SCN lesion or genetic manipulation renders them readily observable, however, murine ultradian locomotor rhythms are typically less robust compared to the vole, exhibiting a wider frequency range with substantial inter- but also intra-animal variation (20–22).

Ultradian Activity in Humans

Overt ultradian behavior has been also reported for human infants (8, 23–25). Activity recordings based on ankle-actigraphy revealed clear ultradian rhythmicity in preterm infants regardless of whether they were exposed to constant dim light or a 24 h light:dark (LD) cycle (**Figure 1B**) (23). While the periodic activity bouts could potentially result from rhythmic interference by nursing staff, sleep diary recordings of term infants by mothers who breastfed at the infant's will also revealed ultradian patterns in feeding and sleep (25). Of note, these ultradian patterns within the first few months of postnatal life were observed in the majority of the infants tested. These reports also suggest that—in humans—the circadian and/or diurnal control of sleep:wake rhythmicity only establishes over the course of weeks to months postnatally, thereby permitting an "unobstructed" view on ultradian rhythms in the 2–6 h range during this early postnatal period. The actigraphy and sleep diary data suggest that once the circadian and/or diurnal control of sleep:wake is established, both the ultradian and 24-h rhythmic components integrate in a harmonic fashion (see, e.g., **Figure 1B**, bottom half of the record) (8). The resulting compound pattern that is distinctly observable in some cases supports the idea that an ultradian rhythm generator has perhaps evolved or has been evolutionary adopted to promote social synchrony in gregarious species, precipitating for instance a frequency of three major meals per day, which seems to dominate the temporal structure of human food intake.

A CASE FOR A DOPAMINERGIC OSCILLATOR DRIVING ULTRADIAN BEHAVIOR

Monoamines and the Ascending Arousal Pathway

The monoamines histamine, norepinephrine, serotonine, and dopamine have all been associated with the ascending arousal pathway and are considered to be key elements of wakefulness promotion (26, 27). Interestingly however, genetic manipulation of monoamine levels by disrupting their biosynthesis or reuptake systems has only relatively mild effects on LA (28–32) except in the case of dopamine (33, 34). DA reuptake blockade (35) leads to a profound hyperlocomotor (33) phenotype and abolishing dopamine synthesis by tyrosine hydroxylase gene disruption selectively in DA neurons leads to an almost complete loss of spontaneous LA (34, 36). Thus, among the monoamines associated with the ascending arousal pathway, dopamine has the strongest link to LA, which is highly associated with the wake state (37).

DAT Removal Lengthens Ultradian Period

When running wheel activity is monitored long-term, mice deficient of the dopamine transporter *(DAT;* official gene name, *Slc6a3)* exhibit less consolidated, rather erratic activity that nevertheless remained largely confined to the dark period of the LD cycle when compared to wild-type littermates (**Figure 1D**) (20). However, upon switching to constant darkness (DD), periodogram analysis revealed the emergence of a second component of rhythmic activity that persisted over several cycles with a period longer than 24 h, while the primary or circadian component exhibited periods below 24 h as expected for endogenous circadian pacemaking of the C57BL/6J laboratory mouse strain that served as genetic background for the $DAT^{-/-}$ mouse line (**Figure 1D**). Further examination revealed that this second component does not result from a phase dissociation within the SCN clock cell ensemble, which has been shown to account for the split locomotor rhythm observed in hamsters exposed to constant light (38), or for the two component pattern in rats exposed to a 22 h LD cycle (39). If the second, >24 h component observed in $DAT^{-/-}$ animals indeed results from the very oscillator that normally accounts for ultradian activity, then upon elimination of the circadian pacemaker, these mice would be expected to show lengthened ultradian activity cycles. Indeed, when running wheel activity of $DAT^{-/-}$ mice is monitored in constant darkness following SCN-lesion or genetic disruption of the circadian clock, a profound lengthening of the ultradian locomotor period is observed, from the typical 2- to 4-h period to ~12 h (20).

Striatal Dopamine Fluctuates in Step with Ultradian Activity

It was further found that extracellular dopamine levels in the striatum of $Bmal1^{-/-}$ mice kept in DD fluctuate in synchrony with ultradian LA (**Figure 1F**), and that extracellular levels of striatal DA strongly correlate with ultradian period (20). Together, these findings are in support of dopamine acting as an ultradian oscillator output and at the same time as a period determinant, arguing for a central role of dopamine in the ultradian rhythm generation process. Hence, the name dopaminergic ultradian oscillator (DUO) was coined (20).

DA NEURONS, SITE OF ULTRADIAN RHYTHM GENERATION?

As DAT is only found in DA neurons and given that selective chemogenetic stimulation of DAT-expressing midbrain neurons leads to ultradian locomotor period lengthening (20), and because of the observation of striatal, extracellular dopamine fluctuating at ultradian periods, midbrain DA neurons could plausibly act as

the site of ultradian locomotor rhythm generation. However, the current data are also consistent with an ultradian rhythm generator located elsewhere, which regulates extracellular dopamine levels by, for instance, rhythmic metabolic conversion, and whose period depends on dopamine tone. However, the DA degrading enzyme catechol-*O*-methyltransferase (COMT), which converts DA into 3-methoxytyramine and which is found in various brain regions including the striatum, seems not to have a significant role in clearing striatal extracellular DA upon evoked dopamine overflow based on the study of COMT deficient mice (40). As the striatum has been the site of detection of ultradian DA fluctuations (20), this finding argues against extracellular DA enzymatic conversion as a means to convey ultradian oscillator output. Interestingly, lesions to the retrochiasmatic, paraventricular, and/or arcuate nucleus regions greatly perturbs or even abolishes ultradian rhythm generation in the common voles, indicating that these brain areas either participate in rhythm generation or affect oscillator output (16, 41). Because DAT-expressing dopamine neurons are also found in the arcuate nucleus region (42, 43) and along the walls of the hypothalamic third ventricle (44), it is possible that these hypothalamic DA neurons contribute to rhythm generation as part of a network of DA neuronal populations that together make up the DUO oscillator (**Figure 2A**). However, selective and chronic *in vivo* activation of midbrain DA neurons using a chemogenetic strategy (20) led to a sustained lengthening of the ultradian period, suggesting that extra-midbrain DA neurons are not critical for ultradian rhythm generation/period determination.

Of note, gonadotropin-releasing hormone (GnRH) is released in a pulsatile fashion by GnRH neuron terminals at the portal vessels of the median eminence, a structure located at the base of the arcuate nucleus (45). Interestingly, serum levels of luteinizing hormone, whose release is controlled by GnRH, have been shown to fluctuate with an ultradian period of 2–3 h in male rhesus monkeys (12, 46) and luteinizing hormone levels were shown to rise about every 6 h in the mid luteal phase of the menstrual cycle in women (47). Given that the GnRH projections originating from the preoptic area traverse the retrochiasmatic area and arcuate nucleus to reach the median eminence, it is conceivable that the hypothalamic lesions affect ultradian rhythmicity in the vole by severing GnRH neuronal processes, and thus their ability to contribute to the ultradian locomotor rhythm generation by means of their role in pulse generation. However, the LH pulse frequency has been shown to differ substantially between female [1 pulse per 1 h (48)] and male [1 pulse per 2–3 h (49, 50)] mice. Because no such sexual dimorphism is reported for the ultradian locomotor periodicity, these findings argue against a key role of the GnRH pulse generator in ultradian locomotor rhythm generation. Pulsatory secretion is also a key characteristic of the hypothalamic–pituitary–adrenal axis (HPA) (51). Corticotrophin-releasing hormone (52, 53), adrenocorticotropic hormone (54, 55), as well as the glucocorticoids (CORT) (56, 57) are all rhythmically secreted into the circulation with pulse frequencies typically in the hourly range in rat (56–58) and man (59–61). Thus, as in case of GnRH/LH, also HPA axis pulse generation may not be involved in the production of ultradian locomotor rhythm which are characterized by multi-hour periodicities.

THE METHAMPHETAMINE-SENSITIVE (CIRCADIAN) OSCILLATOR (MASCO) RHYTHM REFLECTS A SPECIFIC STATE OF THE DUO

Several decades ago, it was found that treatment with the psychostimulant methamphetamine *via* the drinking water leads to the expression of a second rhythmic component in addition to the daily circadian component. Because this component exhibited periods in the circadian range (62) it was dubbed the MASCO (63). As SCN lesion (62) or genetic disruption of clock function (64) does not prevent the expression of methamphetamine-dependent rhythmicity, it was concluded that the MASCO rhythm expression does not require the known circadian clock machinery (64, 65). When methamphetamine-treated SCN-lesioned rats were given timed intraperitoneal injections with the antipsychotic haloperidol, which binds to the dopamine receptor 2 found on midbrain dopamine neurons, it shifted the rhythm phase, with the directionality of the shift depending upon the relative time point (with regard to activity onset) of haloperidol injection (66). Notably, this early finding already pointed to a critical role of dopamine in the oscillator process driving these methamphetamine-induced rhythms.

The observation that methamphetamine is not only capable of gradually lengthening the ultradian locomotor period of *Bmal1*$^{-/-}$ mice from ~4 to ≥48 h (**Figure 1E**) (20), but to similarly affect the ultradian oscillator in circadian intact mice, causing the 3 night-time activity peaks to transition into 2 and then 1 single peak (20) now argues that the methamphetamine-induced rhythmicity described earlier in fact represents a long period manifestation of a highly tunable ultradian oscillator, the DUO.

INTERACTION OF THE DUO AND SCN CIRCADIAN TIMER

Studies on the SCN-intact common vole specifically in constant darkness showed that the ultradian rhythms in LA and feeding are phase-locked with the circadian clock, indicating coupling of the two oscillator systems (67). It was suggested that the ultradian rhythm is reset daily by the circadian clock and that it is not directly sensitive to light cues, and that phase resetting by light is instead mediated through the circadian timer. Further support for interaction between the SCN and ultradian timer comes from the observation of a phase-dependent change in oscillator speed, which is also known as relative coordination if the speed change does not lead to stable entrainment between two oscillatory processes (68, 69). For instance, under conditions of methamphetamine treatment: the second (>24 h) locomotor component frequently seems to "slow down" when overlapping with the "primary," SCN-driven bout in methamphetamine-treated animals (63, 70) (see **Figure 2C**,c for illustration). In addition to an influence of the circadian clock on the ultradian

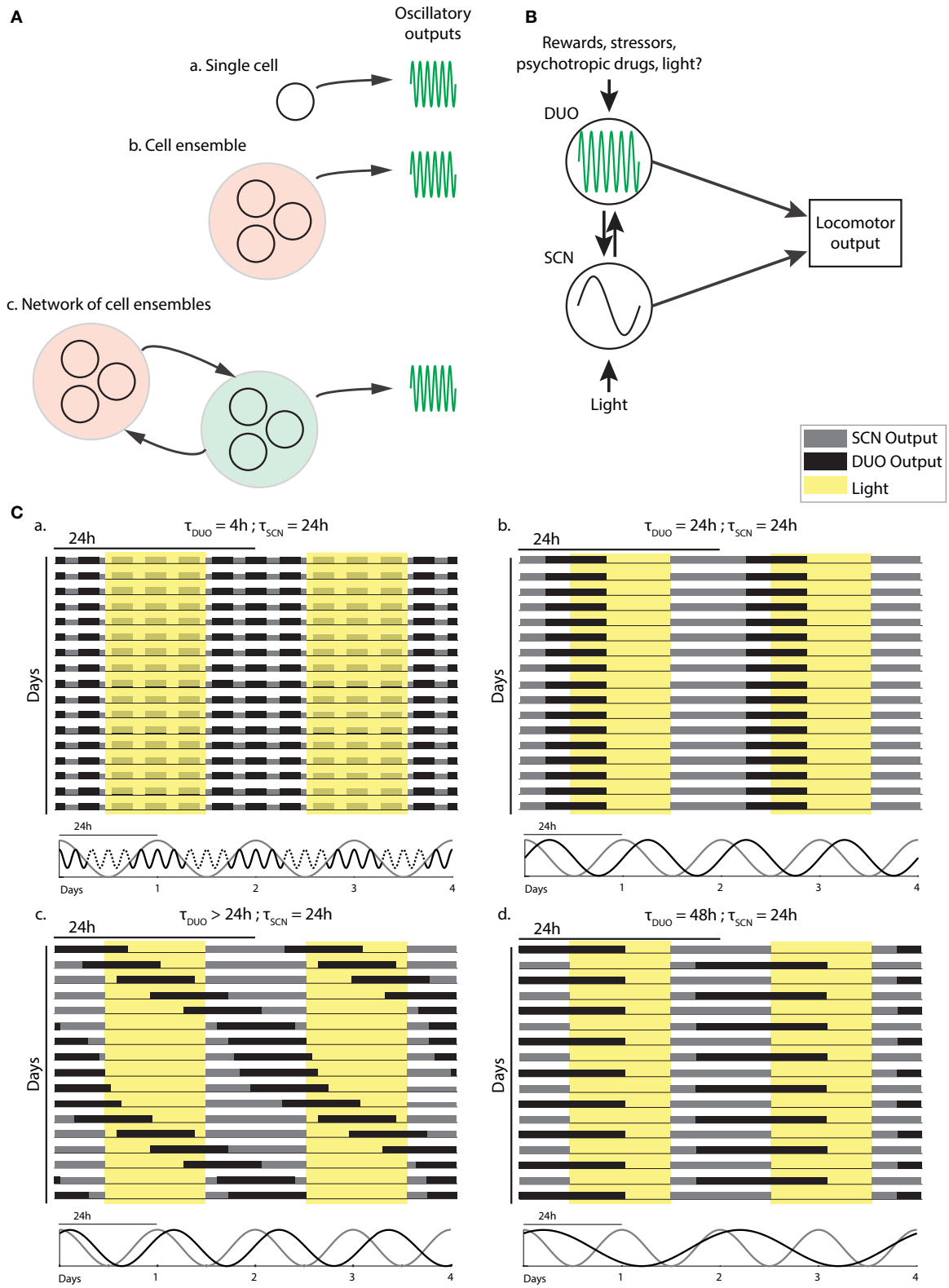

FIGURE 2 | Dopaminergic ultradian oscillator (DUO) make up and output integration. **(A)** Structural basis of the DUO: ultradian rhythm generation may be cell autonomous (a), require a cell ensemble (b), or rely on a network of cell ensembles (c). **(B)** Possible DUO/circadian clock [suprachiasmatic nucleus (SCN)] interaction and output integration. LA, locomotor activity. **(C)** Schematic representation of typical LA patterns found in mice with and without dopamine system interference. The periodicities of the SCN and DUO oscillators suggested to underlie the activity patterns are illustrated below each actogram.

oscillator, there is also evidence for the inverse: the emergence of the second long period (>24 h), likely DUO-driven component, in *DAT*$^{-/-}$ mice is associated with a simultaneous period lengthening of the SCN-driven (~24 h) component (**Figure 1D**, DD portion of the graph). Similar observations have been made in methamphetamine-treated animals where the SCN-component delays its phase in the presence of the second (methamphetamine dependent) component (63). Thus, it seems as if both the DUO and SCN clock produce signals for their reciprocal entrainment which may or may not lead to full entrainment between both oscillators. Of note, mice with reduced expression of DAT have been reported to exhibit a lengthened circadian LA period (71). While ultradian rhythmicity has not been explicitly probed, the authors did not rule out the possibility that the observed period lengthening could be due to the action of a dysregulated DUO as proposed by Blum et al. (20).

Genetic ablation of the orexins have been reported to attenuate the ultradian amplitude in daily locomotor behavior, heart rate, and body temperature (72), suggesting a modulating role of these peptides on DUO function. As with the monoaminergic systems, orexins and the orexin-expressing neurons are part of the ascending arousal pathway (26), receiving input from the SCN *via* the dorsomedial hypothalamic nucleus, and projecting to the midbrain area where the DA neurons reside (73). Orexins could thus serve as mediators of circadian clock control onto the DUO.

ULTRADIAN AND CIRCADIAN OSCILLATOR LOCOMOTOR OUTPUT INTEGRATION

The data presented in Blum et al. (20) suggest that a second oscillator is operative in the mammalian brain (**Figure 2B**) which fundamentally differs from the circadian timer due to its high, frequency tunability. **Figure 2C** illustrates how this feature can explain the profoundly deviating patterns in daily LA that are observable upon manipulation of the dopamine system.

When unchallenged, the DUO cycles at an ultradian period of, e.g., 2–4 h alongside the circadian timer, producing activity bouts throughout the 24-h cycle in voles or infants, but accounts only for the three night-time activity peaks in mice, likely due to strong daytime inhibition of DUO locomotor output by the SCN timer (**Figure 2C**,a). Methamphetamine treatment or DAT disruption lengthens the DUO period. This lengthening may reach 24 h, a period at which the DUO can cycle harmoniously with the SCN timer/LD cycle (**Figure 2C**,b). The relative phasing between the SCN timer/LD cycle and the DUO will depend on the entrainment capacity of the SCN timer/LD cycle and the free-running period of the DUO, i.e., the period the DUO would adopt in the absence of the SCN timer, e.g., the longer the DUO free-running period, the more delayed the phase of entrainment with the SCN timer/LD cycle will be (**Figure 2C**,b). If the SCN/LD cycle is incapable to fully entrain a long-period (>24 h) DUO, the DUO will free-run in the presence of the SCN/LD cycle; however, as a consequence of partial entrainment, its speed will be altered in a phase-dependent manner, resulting in relative coordination (**Figure 2C**,c). Further DUO period lengthening may lead to entrainment at 48 h likely because this frequency is again harmonious with the SCN timer/LD cycle and thus 24-h entrainment cues cause a sufficient phase shift to stably entrain the DUO at the 48-h frequency (**Figure 2C**,d).

OUTLOOK

The finding that DAT removal has a profound period lengthening effect on ultradian LA rhythms together with the discovery of synchronous fluctuations in extracellular dopamine provides a first framework for the molecular underpinnings of the oscillatory process that underlies ultradian rhythmicity. The current data indicate a central role for DA neurons in the rhythm generating process; however, it remains to be seen if rhythm generation is cell autonomous, as in case of the circadian oscillator or instead requires one or more interconnected cell ensembles (**Figure 2A**). Intriguingly, at least some of the LA patterns observed in rodents upon dysregulation of the dopamine system show striking similarities to the aberrant sleep:wake behavior associated with psychopathologies such as bipolar disorder (74, 75) or schizophrenia (76, 77). Given the strong concordance of LA and wakefulness for both rodents and humans (37, 78) the pattern similarities between rodent models and these human subjects indicate that the study of the DUO may have important implications in understanding the etiology of these sleep abnormalities and perhaps the psychopathologies themselves.

AUTHOR CONTRIBUTIONS

CB and K-FS contributed equally to the writing of this review.

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Disrupted Ultradian Activity Rhythms and Differential Expression of Several Clock Genes in Interleukin-6-Deficient Mice

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The characteristics of the cycles of activity and rest stand out among the most intensively investigated aspects of circadian rhythmicity in humans and experimental animals. Alterations in the circadian patterns of activity and rest are strongly linked to cognitive and emotional dysfunctions in severe mental illnesses such as Alzheimer's disease (AD) and major depression (MDD). The proinflammatory cytokine interleukin 6 (IL-6) has been prominently associated with the pathogenesis of AD and MDD. However, the potential involvement of IL-6 in the modulation of the diurnal rhythms of activity and rest has not been investigated. Here, we set out to study the role of IL-6 in circadian rhythmicity through the characterization of patterns of behavioral locomotor activity in IL-6 knockout (IL-6 KO) mice and wild-type littermate controls. Deletion of IL-6 did not alter the length of the circadian period or the amount of locomotor activity under either light-entrained or free-running conditions. IL-6 KO mice also presented a normal phase shift in response to light exposure at night. However, the temporal architecture of the behavioral rhythmicity throughout the day, as characterized by the quantity of ultradian activity bouts, was significantly impaired under light-entrained and free-running conditions in IL-6 KO. Moreover, the assessment of clock gene expression in the hippocampus, a brain region involved in AD and depression, revealed altered levels of *cry1*, *dec2*, and *rev-erb-beta* in IL-6 KO mice. These data propose that IL-6 participates in the regulation of ultradian activity/rest rhythmicity and clock gene expression in the mammalian brain. Furthermore, we propose IL-6-dependent circadian misalignment as a common pathogenetic principle in some neurodegenerative and neuropsychiatric disorders.

Keywords: interleukin 6, circadian activity, mouse, behavior, clock gene, hippocampus

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INTRODUCTION

Changes in the diurnal oscillations of the periods of activity and rest are in the spotlight of basic and applied biomedical research on circadian rhythms in humans and other animals (1). The interest in analyzing these changes in active wakefulness and quiescent rest rhythmicity relates to the fact that alterations of these rhythmic fluctuations are associated with a wide spectrum of pathologies,

ranging from metabolic and cardiovascular dysfunctions to tumorigenesis and cancer. In the neurosciences, the consequences of circadian disruptions and chronic misalignments have been most prominently studied with regards to their effects on cognitive and emotional functions within the framework of some of the most severe neurological and psychiatric illnesses. Specifically, strong clinical and experimental evidence supports a link between disturbances of the sleep–wake cycle and other physiological functions regulated by the circadian system in the pathophysiology of Alzheimer's disease (AD) and major depression (MDD). These dysfunctions include interruptions of the wakefulness during the day and bursts of activity during the night in individuals suffering from AD (2–6).

In addition, it has been described that part of the clinical symptomatology in AD patients is exacerbated at particular periods of the day, most commonly in the early evening (2, 7–10). In addition, a derangement in the circadian rhythmicity of several physiological functions (including the regulation of body temperature and hormone release) is frequently observed (11–15).

Similarly, MDD patients often report disrupted sleep–wake cycles and impairments in the diurnal patterns of other physiological processes [as reviewed in Ref. (16)]. In parallel to the reported "sun downing" in AD, MDD patients often also show significant diurnal mood swings with depressive symptoms usually being strongest in the morning (1).

At the molecular level, polymorphisms and expressional changes in several clock genes, the genetic elements constituting the molecular machinery organizing endogenous circadian rhythmicity, have been identified in postmortem samples of AD and MDD patients and animal models thereof (15, 17–29). Together with the shared involvement of circadian disruptions, both MDD and AD have been associated with altered inflammatory states (30, 31). The pro-inflammatory cytokine interleukin 6 (IL-6) (32), which is linked to circadian clock-related inflammation (33), is considered to play a central role in the pathophysiology of MDD and AD (30, 31, 34–39). Indeed, IL-6 has been proposed as a molecular bridge between circadian and inflammatory processes in a chronobiological animal model of depression (40) and is implicated in circadian rhythmicity (41) and in the circadian regulation of sleep drive (42, 43). Moreover, its secretion is determined by a marked diurnal pattern (44–46), and several clock genes are known as regulator of its production (47, 48).

However, the specific relationship between IL-6 and the diurnal rhythms of activity and rest remain poorly understood as varying observations regarding IL-6 levels under physiological and pathology conditions emerge from literature. These apparent discrepancies may be a consequence of species-specific effects and/or depend on the sample type or methodological approaches employed (31, 44–46, 49). Hence, further investigations using specific, genetically engineered animals are warranted. We here, therefore, set out to examine the involvement of IL-6 in the regulation of behavioral circadian rhythms by studying the changes in the diurnal patterns of locomotor activity in constitutive IL-6 knockout mice (IL-6 KO) in comparison with their wild-type (WT) littermate controls. To determine the impact of IL-6 deletion on the orchestration of circadian rhythmicity at the molecular level, the expression of 19 clock and clock-controlled genes was analyzed in the hippocampus, a brain region importantly implicated in the pathophysiology of MDD and AD.

MATERIALS AND METHODS

Animals

Experiments were carried out in male adult IL-6 KO (B6.129S2-Il6tm1Kopf/J) and WT littermate control mice (Jackson Laboratories, Bar Harbor, ME, USA) (n = 9–11 per group). All mice were 8- to 10-week old at the time of experiments. Mice were housed individually in Nalgene cages equipped with running wheels (15 cm in diameter; Actrimetrics, Evanston, IL, USA) in a sound-attenuated room with constant temperature of 22 ± 2°C. Before experimental assessment of the circadian activity all animals were kept on a light/dark (LD) cycle of 12:12 h with lights on at 6 a.m. and off at 6 p.m. During the light phase, mice were exposed to a light intensity of ~200 lux. During conditions of constant darkness [dark/dark (DD)] defined as LD cycle of 0:24 h, the cage cleaning and animal care taking was carried out under dim red light (15 W). Mice were supplied with food and tap water *ad libitum* throughout the experimental period. All experiments were designed to minimize animal suffering and the number of animals used. Animal procedures were approved by the Austrian ethical committee (BMWF-66.009/0069-II/36/2011) on animal care and use conducted in accordance with international laws and policies.

Assessment of Circadian Wheel-Running Activity

Acquisition

Wheel revolutions were recorded using the ClockLab computer software, with sampling epochs of 1 min (Actimetrics, Evanston, IL, USA). After 1 week of habituation to the vivarium, the light-entrained daily activity was assessed for 14 days during LD followed by the evaluation of the free-running circadian activity during DD. On day 29, DD was briefly interrupted by a light pulse (30 min, 300 lux) at circadian time (CT) 16 (4 h after activity onset) for the induction of a phase-shift response to evaluate the response of the endogenous circadian pacemakers to external *zeitgebers*. After 7 additional days of DD, all mice were exposed to LD for 7 days before sacrifice on day 46 (**Figure 1**).

Analysis

Wheel-running activity was analyzed using the ClockLab software package (Actimetrics, Evanston, IL, USA) as previously described (27, 50). The default software settings were used to determine the activity onsets, which were manually edited when appropriate. Measures of the entrainment period (T) in LD and circadian period (tau) in DD and the total activity were derived from regression lines fit to the activity onsets. Activity bouts were defined as periods during which activity never reached less than 1 count per minute (bout threshold) for longer than 18 min (maximum gap length) at a time. All parameters were determined for

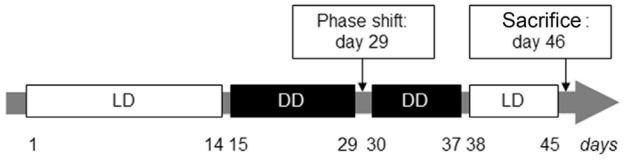

FIGURE 1 | Experimental paradigm for the evaluation of light-entrained and free-running circadian rhythms in interleukin (IL)-6 knockout (IL-6 KO) and wild-type (WT) mice. Illustration of the temporal course (in days) for the analysis of circadian behavioral locomotor activity in IL-6 KO and WT mice under light-entrainment [light/dark (LD): 12 h light and 12 h dark phase; white boxes] and during settings of free-running rhythms [dark/dark (DD): 24 h constant darkness, black boxes].

each animal under LD and DD conditions. Phase-shift responses were evaluated by comparing the predicted activity onset for the day after light pulse treatment from extrapolated lines of the activity onsets of the days preceding the light pulse and 7 days after the pulse.

Gene Expression Analysis

Brain Dissection

All brain dissections were carried out during the light phase of the circadian cycle (between 9 a.m. and 11 a.m.). Mice were sacrificed by neck dislocation, and brains were rapidly dissected over ice and total hippocampi were bilaterally collected and stored in RNA later® (Ambion, Austria, Austin, TX, USA) at −20°C until used for RNA isolation or kept at −80°C for protein expression studies.

RNA Isolation, cDNA Synthesis, and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

RNA was isolated from hippocampal tissues using the miRNeasy kit (Qiagen®, USA, Hilden, Germany) following the instructions of the manufacturer. Briefly, 900 ng of total RNA was used for cDNA synthesis using the MMLV reverse transcriptase first-strand cDNA synthesis kit G1 (Biozym®, Hessisch Oldendorf, Germany) following the manufacturer's instructions. The resulting cDNA reaction mix (1:10 dilution) was used for PCR amplification using the Fast SYBR Green Mastermix (Applied Biosystems, Foster City, CA, USA) on a StepOnePlus real-time PCR system (serial no. 271000455; Applied Biosystems, Foster City, CA, USA). All reactions were carried out in duplicates. Primer sequences for all clock were analyzed: *brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1* (*bmal1*), *circadian locomotor output cycles kaput* (*clock*), *cryptochrome 1/2* (*cry1/2*), *deleted in esophageal cancer 1/2* (*dec1/2*), *neuroD1*, *neuronal PAS domain-containing protein 2* (*npas2*), *period 1–3* (*per1–3*), *reverse erythroblastosis virus* α/β (*rev-erb*α/β) and *RAR* (*retinoic acid receptor*)*-related orphan receptor* α-γ (*ror*α-γ) and clock-controlled genes *D site of albumin promoter* (*albumin D-box*) *binding protein* (*dbp*)*, E4 promoter-binding protein 4* (*e4bp4*)*, inhibitor of DNA binding 2* (*id2*), and *neuronal differentiation 1* are listed in the Supplementary Table 1 of Ref. (27).

The C(t) values of β-actin were used for calculation of ΔC(t), representing the relative quantification of mRNA amounts in each sample. This further allowed the calculation of ΔΔC(t), subtracting mean ΔC(t) value of the WT from the mean ΔC(t) value for the KO. ΔΔC(t) was then used to express the fold change of mRNA levels observed between WT and KO mice, using the formula $2^{-\Delta\Delta C(t)}$.

Statistical Analysis

BioStat software (AnalystSoft Inc., Alexandria, VA, USA) was used for statistical analysis. Comparisons between two groups were determined using unpaired two-tailed Student's t-test. In addition, two-way analysis of variance (ANOVA) (light condition × genotype) was employed for statistical evaluation of locomotor activity (alpha, rho, and total) and for bout analysis (number of bouts/day, bout length and counts/bout). The level of significance was set at $p < 0.05$ in all instances.

RESULTS

IL-6 KO Mice Present with Fragmented Daily Activity Patterns under LD and DD Conditions

To characterize the effects of genetic IL-6 deficiency on behavioral rhythms of rest and activity, wheel-running activity was monitored in IL-6 KO and WT littermate control mice. The investigation of light-entrained rhythms under LD conditions indicated unaltered length of the entrainment period (T) (**Figure 2A**) in IL-6 KO mice. Similarly, the amount of wheel-running activity was comparable between IL-6 KO and WT mice during periods of inactivity (rho) and activity (alpha) within the circadian cycle (**Figures 2B–D**). IL-6 deletion, however, was associated with an increased quantity of activity bouts ($p < 0.05$) with unchanged duration and amount of activity/bout (**Figures 2E–G**). Calculations of activity onsets and offsets revealed no differences between genotypes, and the duration of the active period was not statistically different between groups under LD conditions (Figure S1 in Supplementary Material).

To determine circadian locomotor patterns during free-running rhythms, daily behavioral activity was further analyzed under DD conditions. In the same way as for the light-entrained rhythms, the circadian period, as well as the amount of wheel-running activity, was undistinguishable between IL-6 KO and WT mice (**Figures 3A–D**). Consistent with the results from the LD paradigm, the number of activity bouts was enhanced in IL-6 KO mice under DD conditions ($p < 0.05$), whereas no differences were seen in the duration and quantity of activity/bout or in the phase shift response in comparison with WT controls (**Figures 3E–H**). In addition, the duration of the active period was shorter in IL-6 KO mice under DD conditions ($p < 0.05$) (Figure S1 in Supplementary Material).

Hence, the temporal architecture of the ultradian rhythms is disrupted in IL-6 KO mice under both LD and DD conditions as illustrated in the respective actograms of the two genotypes (**Figures 4A,B**). Further examples of representative actograms are provided in Figure S2 in Supplementary Material.

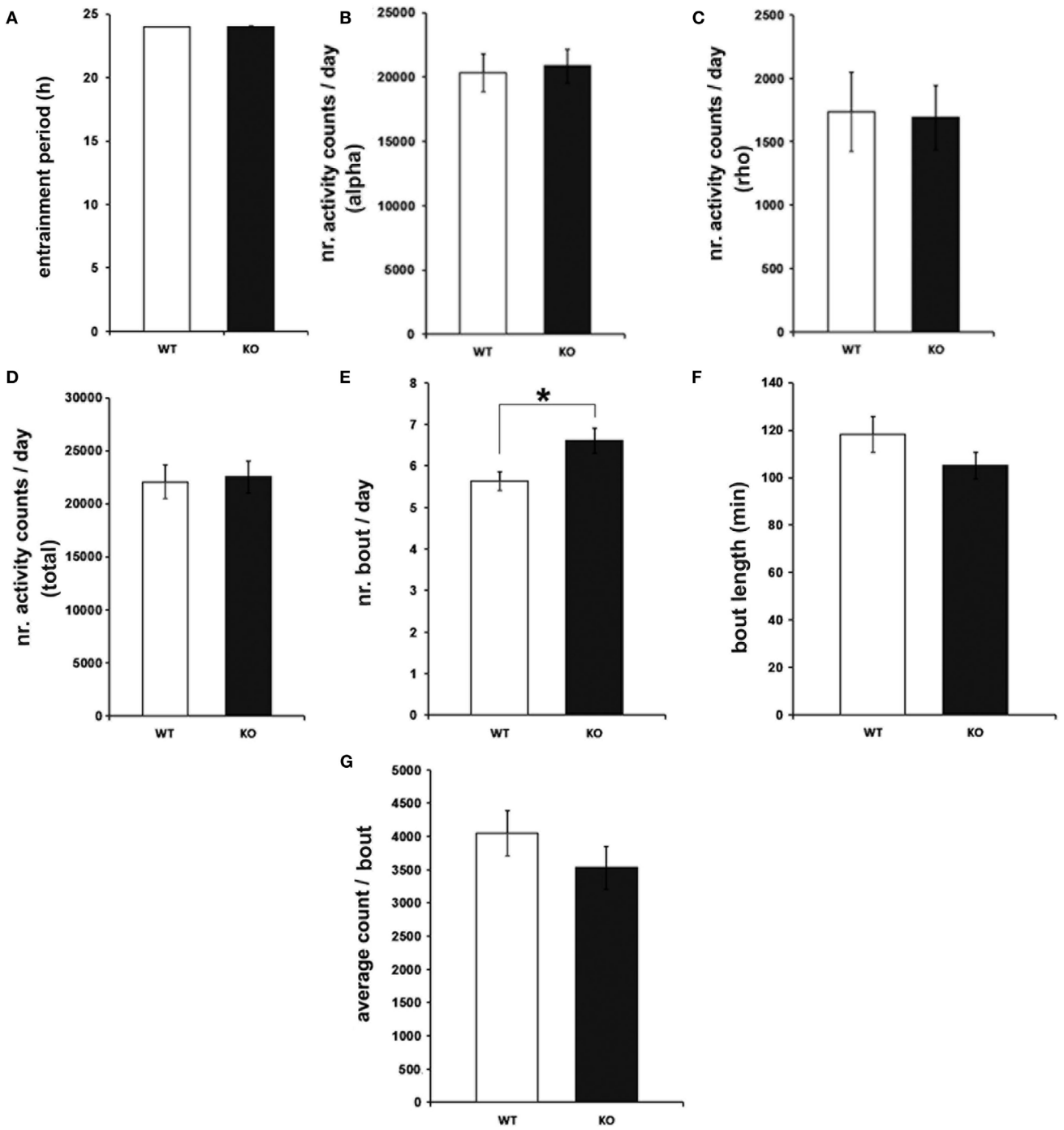

FIGURE 2 | **Entrainment period (*T*), wheel-running activity, and bout analysis in under light-entrained [light/dark] conditions in interleukin (IL)-6 knockout (IL-6 KO) and wild-type (WT) mice**. Analysis of the light-entrained circadian behavioral locomotor activity in IL-6 KO and WT mice (n = 9–11 per group) demonstrating comparable **(A)** *T* and wheel-running activity during the **(B)** alpha and **(C)** rho phase and in **(D)** total amounts. **(E)** Significantly increased quantity of activity bouts in IL-6 KO compared with WT mice with unaltered **(F)** bout length and **(G)** activity counts/bout. All data are displayed as mean ± SEM; $^*p < 0.05$.

In addition, two-way ANOVA analysis (light condition × genotype) has been carried out to examine the possible effect of the light condition and its interaction with the genotype. The following main effects have been observed: for overall activity significant main effects of light condition for alpha: $F_{(3,43)} = 88.54$, $p < 0.001$ and rho: $F_{(3,43)} = 178.17$, $p < 0.001$. The characterization of the

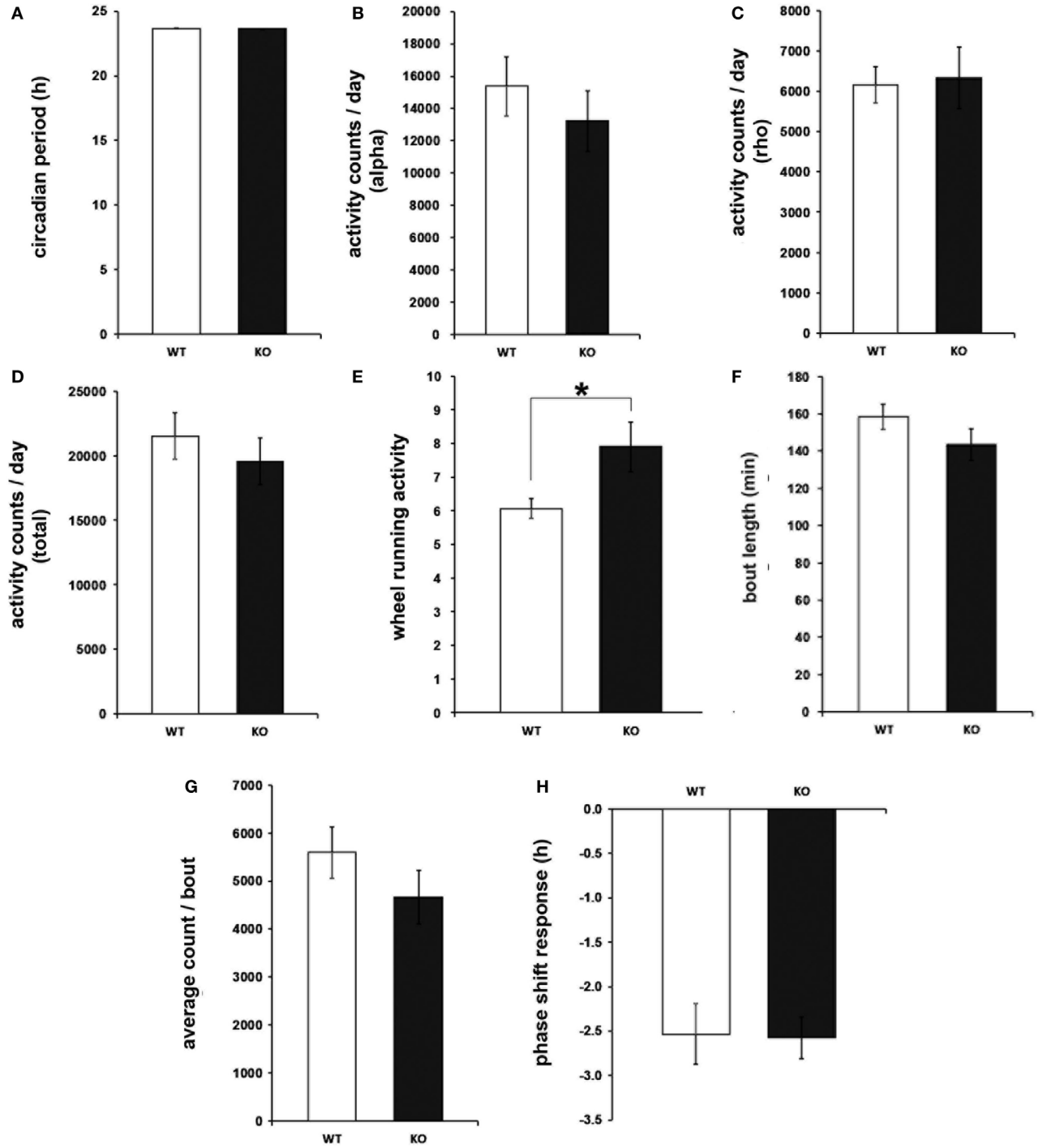

FIGURE 3 | Circadian period (tau), wheel-running activity, bout analysis, and phase shift response under free-running (dark/dark) conditions in interleukin (IL)-6 knockout (IL-6 KO) and wild-type (WT) mice. Analysis of the free-running circadian behavioral locomotor activity in IL-6 KO and WT mice (n = 9–11 per group) demonstrating comparable **(A)** tau and wheel-running activity during the **(B)** alpha and **(C)** rho phase and in **(D)** total amounts. **(E)** Significantly increased quantity of activity bouts in IL-6 KO compared with WT mice with unaltered **(F)** bout length and **(G)** activity counts/bout. **(H)** Unaltered phase shift response to a brief light pulse at CT14 is in IL-6 KO mice. All data are displayed as mean ± SEM; *$p < 0.05$.

bouts revealed a significant main effect of genotype [$F_{(3,43)} = 10.47$, $p < 0.01$] for bouts per day and significant main effects of light condition for bout length: $F_{(3,43)} = 29.98$, $p < 0.001$ and counts/bout: $F_{(3,43)} = 8.57$, $p < 0.01$. The duration of the active periods revealed a significant main effect of genotype [$F_{(3,43)} = 7.17$, $p < 0.05$]. No other significant main effects or interactions were found.

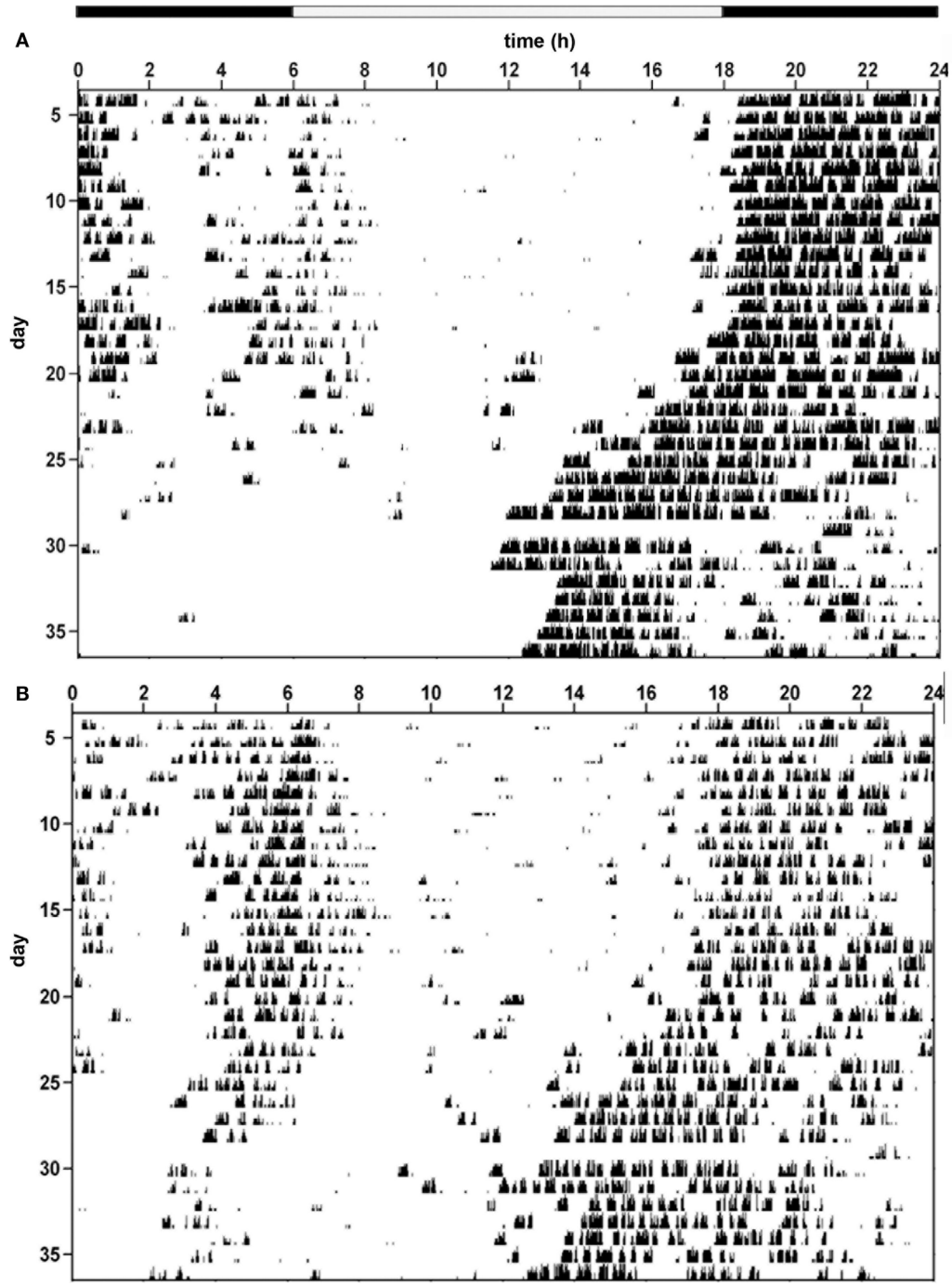

FIGURE 4 | Behavioral actograms exemplifying circadian locomotor activity patterns in interleukin (IL)-6 knockout (IL-6 KO) and wild-type (WT) mice. Sample actograms illustrating wheel-running activity in **(A)** WT and **(B)** IL-6 KO mice.

Aberrant mRNA Expression of Cry1, Dec2, and Rev-erb-Beta in the IL-6 KO Mouse Hippocampus

With regard to the molecular mediators of the observed alterations in the rhythmic oscillation of rest and activity patterns, mRNA levels of 19 clock (*clock*, *cry1/2*, *npas2*, *per1–3*, *rev-erb*α/β, and *ror*α-γ*)* and clock-controlled genes (dbp, e4bp4, id2, and neuroD1) were assessed in the hippocampus of IL-6 KO and WT mice. qRT-PCR analysis revealed a significant increase in levels of *cry1* ($p < 0.05$) and *dec2* ($p < 0.01$), whereas expression of *rev-erb-beta* ($p < 0.01$) was reduced in IL-6 KO compared with WT

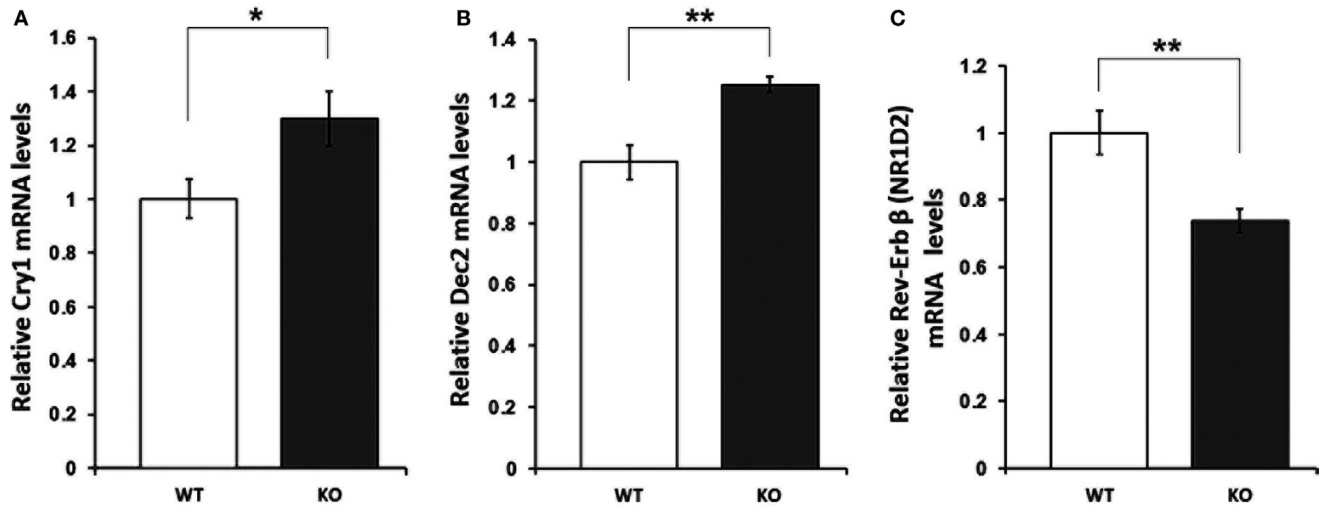

FIGURE 5 | mRNA levels of clock genes with significantly different expression in hippocampal tissue of interleukin (IL)-6 knockout (IL-6 KO) compared with wild-type (WT) mice. Relative expression of **(A)** *cry1*, **(B)** *dec2*, and **(C)** *rev-erb beta* in hippocampal tissue of IL-6 KO compared with WT mice (n = 6–9 per group). All data are data displayed as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

controls (**Figure 5**). No differences in the mRNA of any of the other clock genes investigated were found (**Table 1**).

TABLE 1 | Clock and clock-controlled genes with comparable mRNA levels in hippocampal tissue of knockout (KO) and wild-type (WT) mice.

Gene name	WT (rel. expression)	KO (rel. expression)	*p* Value
clock	1.000 ± 0.1293	1.0197 ± 0.0038	0.6
cry2	1.000 ± 0.1332	0.9949 ± 0.0168	0.9
dbp	1.000 ± 0.0916	1.0272 ± 0.0140	0.4
dec1	1.000 ± 0.1414	0.9893 ± 0.0406	0.8
e4bp4	1.000 ± 0.1375	0.9952 ± 0.0084	0.8
id2	1.000 ± 0.0902	1.0385 ± 0.0371	0.4
neuroD1	1.000 ± 0.0673	1.0045 ± 0.0038	0.7
npas2	1.000 ± 0.0759	0.9993 ± 0.0192	0.9
per1	1.000 ± 0.0841	1.0516 ± 0.0468	0.3
per2	1.000 ± 0.1055	1.0291 ± 0.0105	0.2
per3	1.000 ± 0.2047	1.0170 ± 0.0206	0.7
rev-erbα/β	1.000 ± 0.0740	1.0367 ± 0.0181	0.2
ror-α	1.000 ± 0.1685	0.9714 ± 0.0344	0.5
ror-β	1.000 ± 0.0731	0.9714 ± 0.0191	0.2
ror-γ	1.000 ± 0.1034	0.9829 ± 0.0092	0.2
bmal1	1.000 ± 0.1180	1.0225 ± 0.0131	0.3

Fold change values in KO mice (normalized to WT means for each transcript) of clock and clock-controlled (gray) genes are displayed as mean ± SEM (n = 6–9 per group). p Values represent results of statistical analyses using two-tailed Student's t-tests.

DISCUSSION

Most species living on the surface of earth have evolved under conditions of rhythmically changing daily variations in fundamental environmental constituents, such as light. To anticipate and respond to these oscillating physical properties, organisms have developed systems to accordingly fit their physiology. Hence, the most essential functions of the body, including those of the nervous and the immune systems, are determined by these intrinsic timing regulations. Thus, the association between disruption in "biological clocks" and pathologies of the brain (31, 51–53) and the immune response is unsurprising [see for review Ref. (54)]. Indeed, the circadian regulation of the behavioral states of activity/rest (as fundamental output of brain function) is well described. Similarly, evidence for the impact of the endogenous clockwork on the most pivotal elements of the body's defense mechanisms, such as the release of immune modulatory substances, is augmenting (55–58) [see for review Ref. (59)].

The current report is, to the best of our knowledge, the first comprehensive, long-term assessment of the impact of a genetic deficiency in a central element of the immune response (the pro-inflammatory cytokine IL-6) on circadian wheel-running activity rhythms in the mouse. This interrelationship is particularly noteworthy within the framework of diseases and disorders in which all these functions are of pathophysiological relevance, as is the case for the neurodegenerative AD and the neuropsychiatric MDD, where the involvement of the circadian and the immune systems have been extensively demonstrated (31). In the case of both these mental illnesses, frequent presentations of aberrant diurnal oscillations of behavioral activity have been reported in patients and in subjects of the respective experimental animal models (15–29, 31, 60–62).

In the herein studied IL-6 KO mice, traditional parameters of diurnal behavioral rhythmicity were unaltered under light-entrained and free-running conditions, as tau and the amount of activity during active and inactive phase were comparable with those of WT controls but were determined by the light conditions (LD versus DD) for both genotypes. Interestingly, the duration of

the active period was shortened in IL-6 KO mice. In a previous short-term evaluation of home cage behavior, higher activity of IL-6 KO compared with WT mice has been reported (63). However, the analysis of home cage activity does characterize a behavioral output distinct from circadian wheel-running activity (64). Although home cage activity reflects the baseline activity, wheel running is an elective action, which is driven by additional endogenous factors, such as motivation (64). However, it is the only system to reliably address some distinct features of the internal timekeeping system, such as the modulation of the endogenous circadian machinery by environmental stimuli. Indeed, an unaltered phase-shift response in IL-6 KO mice indicated an intact responsivity of the endogenous CT keeping system to an external *zeitgeber*. Hence, the 24-h structure of the behavioral locomotor rhythm seemed largely preserved IL-6 KO mice. However, a close examination of the activity bouts as indicators of units of ultradian activity revealed a significant difference in the number of bouts between genotypes, independent of the external lighting conditions: IL-6 KO mice presented with an augmentation in the number of bouts/circadian day, while the bout length and activity/bout remained unchanged. This result is also reflected in the two-way ANOVA analysis, which revealed a significant main effect of genotype for the number of bouts, whereas interestingly the bout length and activity/bout were significantly dependent on the light conditions for both WT and KO mice.

The nature and regulation of ultradian rhythms and activity bouts is less well described than is the case for the classical indicators of diurnal rhythms, e.g., length of the circadian period tau and activity onsets and offsets, which are largely dependent on the suprachiasmatic nucleus (SCN) of the hypothalamus as a central circadian pacemaker (65–70). The SCN also orchestrates rhythmic activities in other regions of the brain and peripheral parts of the body with synchronization of clock gene expression as a pivotal molecular event.

To examine potential neurobiological mechanisms contributing to the observed phenotype of IL-6 KO mice, we decided to focus on the hippocampus, a brain region involved in the pathophysiology of AD (71–73) and MDD (74, 75). Examination of the expression of major clock genes as molecular mediators of circadian rhythmicity revealed a selective effect of genetic IL-6 deficiency on the hippocampal mRNA levels of *cry1*, *dec2*, and *rev-erb-beta*.

Although the statistically significant expressional differences between IL-6 KO and WT mice were modest in magnitude, they may be well of biological relevance considering the role of these genes in the tightly controlled feedback loops of transcription–translation from which circadian rhythms are generated at the molecular level (20, 24, 28, 76). The increased levels of *cry1* in IL-6 KO are paralleling observations in plasma levels of sepsis patients were an increase in IL-6 was associated with a decrease in *cry1* mRNA (77). A modulatory influence of several immune mediators on the expression of *dec2*, which is here to be reported significantly reduced in the hippocampal tissue of IL-6 KO mice, has been described. Interestingly, IL-6 is a direct activator of AMP-activated protein kinase (78), which has been found to mediate the regulatory effects of *dec2* in several tissues (79).

Previous work reports that *rev-erb* expression in peripheral blood leukocytes of human subjects, together with several other clock genes (including *cry1*), is dampened by endotoxin treatment, which leads to a concomitant increase in circulating levels of IL-6. This description is in line with our observation on augmented *rev-erb-beta* and *cry1* levels in IL-6 KO.

Alternatively or additionally to a mechanistic involvement of clock gene expression, the alteration in the ultradian architecture of behavioral activity in IL-6 KO mice may relate to the direct regulatory effect of IL-6 on the serotonin transporter (SERT) (80). Indeed, multifaceted interactions between the circadian and the serotonergic systems have been demonstrated with a proposed role of these interrelationships for several mental illnesses, including MDD [see for review Ref. (81, 82)]. However, although a defined role for dopamine and the dopamine transporter in the regulation of ultradian rhythms of locomotor behavior have been proposed (83), a potential involvement of SERT in the control of ultradian activity architecture remains to be examined in future studies.

Some conceptual restrictions, which were imposed by the study design, such as the determination of clock gene expression at a single time of the day in *a priori* selected brain region of interest have to be considered for the interpretation of the results obtained. Hence, the observed differences in clock gene expression between IL-6 KO and WT mice do not allow for conclusions regarding the diurnal oscillation in the expression of these genes in the two genotypes, an important mechanistic insight that will be addressed in follow-up investigations. Within this framework, however, this study allows for the deduction of three major conclusions: first, IL-6 is not required for diurnal time keeping of the circadian period under either light-entrained or free-running conditions; second, genetic IL-6 deficiency is associated with aberrant ultradian activity patterns as reflected in an increased number of activity bouts with unaltered length and activity counts per bout, independent of the external light conditions; and third, a selective modulation of hippocampal clock gene expression proposes an involvement of disrupted mRNA levels of *cry1*, *dec2*, and *rev-erb-beta* in the circadian phenotype of IL-6 KO mice.

Collectively these data suggest a potential pathophysiological involvement of the pro-inflammatory cytokine IL-6 in the circadian alterations associated with severe neurological and psychiatric disorders and invite further investigations on the underlying molecular mechanisms.

AUTHOR CONTRIBUTIONS

FM co-designed experiments, analyzed data, and co-wrote the manuscript; AC and JA analyzed data; IE and OH conducted behavioral experiments; WD carried out gene expression analysis; and DP and MG conceived the study, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fneur.2017.00099/full#supplementary-material.

FIGURE S1 | Duration of the active period (*alpha*) and activity onsets and offsets in interleukin-6 (IL-6) and wild-type (WT) mice. Analysis of the length of the active period in IL-6 and WT mice (n = 9–11 per group) under **(A)** light/dark and **(B)** dark/dark conditions. **(C)** Activity onsets and **(D)** offsets in circadian hours in IL-6 compared with WT mice. All data are displayed as mean ± SEM; $^{*}p < 0.05$.

FIGURE S2 | Behavioral actograms exemplifying circadian locomotor activity patterns in interleukin-6 (IL-6) and wild-type (WT) mice. Sample actograms illustrating wheel-running activity in **(A)** WT and **(B)** IL-6 mice.

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Molecular Mechanisms Regulating Temperature Compensation of the Circadian Clock

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An approximately 24-h biological timekeeping mechanism called the circadian clock is present in virtually all light-sensitive organisms from cyanobacteria to humans. The clock system regulates our sleep–wake cycle, feeding–fasting, hormonal secretion, body temperature, and many other physiological functions. Signals from the master circadian oscillator entrain peripheral clocks using a variety of neural and hormonal signals. Even centrally controlled internal temperature fluctuations can entrain the peripheral circadian clocks. But, unlike other chemical reactions, the output of the clock system remains nearly constant with fluctuations in ambient temperature, a phenomenon known as temperature compensation. In this brief review, we focus on recent advances in our understanding of the posttranslational modifications, especially a phosphoswitch mechanism controlling the stability of PER2 and its implications for the regulation of temperature compensation.

Keywords: circadian clock, temperature compensation, phosphorylation, phosphoswitch, period2

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The main advantage of having an intact circadian clock system is to anticipate and alert our physiological mechanisms to prepare for daily changes in the environment imposed by light–dark cycle of the earth. At the organism level, the circadian clock is a hierarchical multioscillator network, where in mammals, the suprachiasmatic nuclei (SCN) is the master oscillator. The SCN in the hypothalamus of brain is entrained by the light–dark cycle through the eye and neuronal retinal ganglion cells. Synchronized highly interconnected neurons in the SCN oscillate and transmit their rhythm to peripheral oscillators such as liver, lung, and kidney *via* systemic cues including neuronal, neuroendocrine, and behavioral pathways. This clock network entrains physiological processes including the sleep–wake cycle, liver metabolism, and body temperature (1–3). At the molecular level, the circadian clock is composed of transcriptional and translational feedback loops that oscillate in cycles of approximately 24-h to create the circadian rhythms we see at the organism level. In the core loop, the positive transcriptional activators Clock and Bmal1 bind to E-box motifs and activate the expression of many targets, including their own negative regulators, Period (Per1, 2, and 3) and Cryptochromes (Cry1 and Cry2). As the negative feedback proteins Per and Cry increase in abundance, they multimerize, enter into the nucleus, and bind to the heterodimeric Clock and Bmal1 complex to inhibit their transcriptional activity. This generates a 24-h cycle that is cell autonomous. This clock machinery is broadly functional in all mammalian tissues (1–3).

The three major hallmarks of circadian clocks are their ~24-h oscillation in the absence of any external stimuli, entrainment by external stimuli, and temperature compensation. Entrainment allows the master clock to synchronize with seasonally and geographically changing light–dark cycles. In mammals, light entrains the central clock *via* retinal ganglion cells that communicate with the SCN *via* the retinal–hypothalamic tract. Homeothermic animals such as mammals

maintain a nearly constant body temperature with a narrow range of fluctuations in most part of the body, whereas poikilotherms such as frogs have body temperature, which can vary in wide range (4, 5). However, even in mammals, peripheral clocks can be entrained by small daily oscillations in internal body temperature (1, 3, 6).

Although the circadian clock system can be entrained by fluctuations in temperature, it remains fairly resistant to ambient temperature-induced changes in circadian period (5, 7). According to the Arrhenius equation of temperature dependence on reaction rate, in any (bio)chemical reaction, a rise in temperature increases the rate of the reaction (8), which eventually reduces the reaction time. But in the case of the circadian biochemical system, in spite of changes in ambient temperature, the period length remains essentially constant at approximately 24-h. Thus, Pittendrigh demonstrated that the *Drosophila* rhythm of eclosion (emergence of the adult fly from the pupa) retained a 24-h rhythmicity in total darkness over a temperature range of 16–26°C (5). This phenomenon is referred to as temperature compensation (5, 9). The temperature compensation of circadian period is evolutionarily conserved from light-sensitive cyanobacteria to homeothermic mammals, and surprisingly, even an *in vitro* circadian clock reconstituted with KaiABC proteins of cyanobacteria shows temperature compensation between 25 and 35°C, suggesting that it is a core design feature of the molecular clock (5, 10–13). More recently, using tissue explants and cell culture, it has been demonstrated that temperature compensation is a tissue and cell autonomous property. For example, the circadian oscillators controlling melatonin synthesis in the retina of golden hamsters are temperature compensated between 27 and 33°C (14), and *Per1*^{Luc} fibroblasts maintain ~24-h period length despite changes in temperature over the range of 28.5–36.5°C (12). These findings also confirm peripheral cells as *bona fide* model systems to study the temperature compensation mechanism of the circadian clock (12).

MODELS OF TEMPERATURE COMPENSATION

How the active process of temperature compensation is achieved by organisms is an area of intense research interest to both chronobiologists and mathematical modelers. Hastings and Sweeney almost 60 years ago proposed that temperature compensation could be achieved if two temperature-dependent reactions oppose each other, although at the time there was no inkling of what those reactions might be (9). This conceptual model was extended by Ruoff with the notion that positive and negative feedback loops of the oscillators might act as the opposing reactions and lead to temperature compensation in any kinetic oscillator model (15). As specific molecular members of the clock were identified, Hong et al. first proposed that PER protein dimerization might regulate temperature compensation (16). Ten years later, as the complexity of the clock mechanism became clearer, many of the newly described regulatory steps have been tested in mathematical models of the clock to assess their potential contribution to temperature compensation. For example, Hong et al. suggested that switch-like mechanisms acting on sensitive parameters such as phosphorylation, ubiquitination, or complex formation controlling PER protein might regulate temperature compensation (17). Others have suggested using modeling that the concentration of a rate-limiting enzyme involved in processes like phosphorylation can determine temperature compensation (18).

In *Neurospora*, the core clock gene *frequency* (*frq*) undergoes alternative splicing that is temperature sensitive. The resulting two isoforms have opposing effects on clock speed and was once proposed to underlie temperature compensation (19–21). More recently, casein kinase 2 in *Neurospora* was implicated in temperature compensation. Decreased CK2 activity, or mutation of a specific CK2 phosphorylation site, leads to altered temperature compensation, probably due to an altered balance of phosphorylation at distinct sites. Interestingly, CK2 itself had a normal Q10, i.e., its activity changed twofold with a 10°C increase in temperature (22). In this system, casein kinase 1 (CK1) was important for clock speed but not temperature compensation. Although these studies have provided some insights for understanding the mechanisms of temperature compensation, either they lack good experimental evidence to support their mathematical model or these models are not tested in mammalian system.

PHOSPHORYLATION OF PER2 CONTROLS CLOCK SPEED

Many of the mathematical models suggested that temperature compensation could be due to two opposing reactions acting on a rate-limiting step of the circadian clock machinery (9, 18). The reversible multisite phosphorylation of PER2 is a potential target in this regard due to its rate-limiting role in regulating clock speed (**Figure 1**) (23, 24). The importance of phosphorylation in the control of circadian rhythms was demonstrated first by the finding of short- and long-period mutations in *Drosophila* that both mapped to the Dbt kinase gene, the ortholog of mammalian CK1δ and CK1ε (25, 26). CK1 is a family of serine/threonine kinases with seven different isoforms in mammals that are encoded by distinct genes (α, β, γ1, γ2, γ3, δ, and ε), which are involved in diverse biological functions including circadian rhythms, Wnt signaling, membrane trafficking, cytoskeleton maintenance, DNA replication, DNA damage response, RNA metabolism, and parasitic infections (23, 27–30). The first circadian clock phenotype in mammals was found in *tau* hamsters with 20-h short period (31). Later, it was identified that a missense mutation in hamster CK1ε^{tau}(Arg178Cys) is to underlie the short-period phenotype of the *tau* hamster (32). Subsequently, point mutation of a CK1δ/ε-regulated motif in human PER2 [S662G, familial advanced sleep phase (FASP) site] (33) and a point mutation of CK1δ were found in families with FASP syndrome (34). A body of evidence suggests that CK1δ is the major driver of clock timing, but that CK1ε plays an important role as well.

The mechanism by which CK1 regulates phosphorylation of PER2 is complex and is slowly being teased apart. Phosphorylation of PER2 by CK1ε leads to recruitment of the ubiquitin ligase, β-TrCP, and proteasomal-mediated degradation of PER2 (35). But

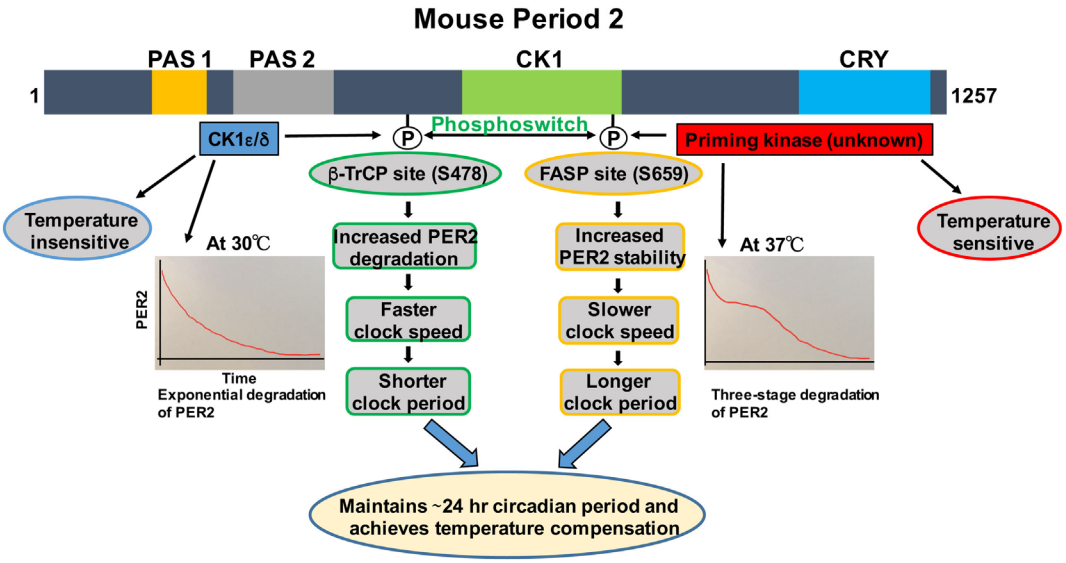

FIGURE 1 | Regulation of PER2 phosphorylation, degradation, and its role in temperature compensation by the phosphoswitch mechanism. Lower temperature increases relative phosphorylation at the β-TrCP site of PER2, leading to faster degradation and shorter period. Higher temperature increases relative familial advanced sleep phase (FASP) site phosphorylation, enhancing PER2 stability and lengthening the period. The degradation pattern of PER2 at 30°C is largely exponential, while at 37°C, three-phase degradation is seen. This has important implications for temperature compensation (see text for details). Domain architectures are shown in colors. PAS1, PAS domain 1 (orange); PAS 2, PAS domain 2 (grey); CK1, Casein kinase 1-binding domain (green); CRY, Cry binding site (blue).

the impact of CK1ε activity on the clock speed has been puzzling, due to opposing observations that reduced CK1 activity shorten (32, 34) and lengthen the circadian period (35). To solve this puzzle, mathematical modeling was applied and then experimentally confirmed the non-intuitive prediction that the short-period *tau* mutation of CK1ε is in fact functionally a gain of function, not a loss of function mutation. It was further reported that the CK1ε^{tau} is a highly specific gain of function for its substrate PER2, which gets phosphorylated and degraded much faster, resulting in a faster clock and shorter circadian period (36). These studies emphasized the value of combining experimental studies with predictive mathematical models to advance our understanding of the clock and how changes in kinase activity can alter the clock.

A PHOSPHOSWITCH REGULATES PER2 DEGRADATION

We and others have shown that there are two phosphorylation sites, the FASP and the β-TrCP site, regulating stability of mammalian PER2 (**Figure 1**) (35, 37). The FASP site is a missense mutation at S662G (S659 in mouse) associated with FASPS, which prevents priming phosphorylation by an unknown priming kinase. Priming phosphorylation of S659 (FASP site) is required for the phosphorylation of four immediate downstream serines of PER2 (659-**S**VVSLTSQCSYSS-671) by CK1ε/δ (33, 37). The second functional phosphorylation site is β-TrCP site that is also a CK1ε-dependent phosphorylation site (S478 in mPER2), but that seems to be independent of priming phosphorylation (35). It has been identified that surprisingly PER2 undergoes three distinct stages of degradation upon addition of the protein synthesis inhibitor cycloheximide during the PER2 accumulation phase (CT 14–26) of the circadian cycle. Mathematical modeling predicts that a phosphoswitch generates the three-stage degradation of PER2 (38). Accordingly, the first rapid decay phase is β-TrCP site phosphorylation dependent, the second slow plateau phase is dependent on FASP site phosphorylation, and in the third and falling phase, PER2 protein is degraded in a CK1δ/ε-independent manner that is not well understood. Importantly, the model was experimentally confirmed (38). Further experiments showed that CK1ε^{tau} has decreased activity on the FASP site, leading to an increased activity on the β-TrCP (S478) site. This explains how CK1ε^{tau} is a gain of function on phosphorylation at S478 and further supports the phosphoswitch between the two sites (the FASP and the β-TrCP site) (38).

PERIOD2 PHOSPHOSWITCH UNRAVELS THE MECHANISM OF TEMPERATURE COMPENSATION

Before CK1ε was even identified as a clock component, its role in temperature compensation was suggested by the observation that retinas from *tau* mutant hamsters have significantly impaired temperature compensation (14). Isojima et al. subsequently reported that unlike virtually all other kinases, CK1ε/δ are temperature insensitive (39). Therefore, they proposed that CK1ε/δ-dependent phosphorylation process might play a central role in temperature compensation of the circadian clock (39). Indeed, in further study, the CK1ε/δ phosphorylation of a β-TrCP peptide

was temperature insensitive (39). The mathematical model of Kim and Forger, building on the pioneering work of Forger and Peskin in understanding the mammalian clock system using mathematical tools (40–42), predicted a potential role for the phosphoswitch mechanism in temperature compensation. A key feature of the model requires that there are two sites involved in the phosphoswitch, the FASP and the β-TrCP sites (**Figure 1**) (38). Since CK1 is relatively temperature insensitive (39), the model assume that priming of the FASP site has normal temperature sensitivity, i.e., its activity increases with increasing temperature, while CK1ε/δ phosphorylation of the β-TrCP site is temperature insensitive, i.e., the rate of phosphorylation is constant regardless of temperature. Incorporating this differential kinase temperature sensitivity into the mathematical model indeed predicted that this could underlie temperature compensation. This model was then experimentally tested in immortalized *Per2*^{Luc} mouse embryonic fibroblasts (MEFs). It was found that at higher temperatures, increased FASP site phosphorylation by the priming kinase leads to slow second-phase degradation and more accumulation of PER2, eventually lengthening and compensating period length. Similarly, *Per2*^{Luc} MEFs at 30°C showed a marked decrease in second-phase degradation, whereas first-stage degradation remained intact. These findings underscore the importance of the relative rates of phosphorylation of the two phosphoswitch sites in temperature compensation (38). Additional experiments indirectly tested if an intact phosphoswitch mechanism is necessary for temperature overcompensation. An abnormal temperature compensation was observed in CK1ε^{tau}; *Per2*^{Luc} MEFs, and also in *Per2*^{Luc} MEFs treated with a CK1ε/δ inhibitor, further supporting a role for CK1ε/δ and an intact phosphoswitch mechanism as a prerequisite for temperature compensation. The studies also support the value of a robust mathematical model that makes testable predictions about complex systems when biological intuition has reached its limits (38).

Recently, it has been reported that cells with knockouts of specific circadian clock components retain temperature compensation (43). The authors concluded that temperature compensation is likely determined by a rate-limiting process(es) that are temperature sensitive, consistent with the phosphoswitch mechanism (43). Another mathematical model for temperature compensation has recently proposed a temperature insulation mechanism where oscillation period is determined by very few temperature-independent or only slightly temperature-dependent parameters, but where other parameters remain strongly temperature dependent (44). This model is analogous to the proposed phosphoswitch mechanism in which the CK1ε/δ is temperature independent or slightly dependent, whereas the priming kinase is temperature dependent (38).

There are a number of unresolved issues. The priming kinase has not been identified yet. It also remains unclear what happens to PER2 phosphorylation over the full 24-h day, in part because the methods to study this in mammalian systems are not suitably sensitive. This is relevant to another unsolved question: how PER2 is degraded in the third phase of three phase decay, when neither CK1 nor proteasome inhibitors impact PER2 loss? Moreover, further study is necessary to understand whether fluctuations in body temperature, which can entrain the clock, do so in part *via* the phosphoswitch mechanism in addition to the proposed heat shock factor 1 (HSF1) mechanism (7, 45). Finally, it is also important to address whether the mechanisms regulating temperature compensation in peripheral cells and the central pacemaker (SCN) cells are the same and whether temperature-induced changes in peripheral clocks can feed back to the central clock.

THE OUTLOOK

It is remarkable that the complex yet robust phenomenon of temperature compensation is regulated by subtle differences in phosphorylation of the same protein at different sites. Notably, this finding is in general agreement with predictions of earlier mathematical models that suggested that opposing outputs with switch-like mechanisms might control temperature compensation (9, 17). In the future, it will be important to identify the priming kinase that plays a central role in the phosphoswitch model. This phosphoswitch mechanism of temperature compensation may be a core feature of clocks in many species, as a similar interaction of phosphorylation sites is operative in *Drosophila* and *Neurospora* as well (46–48).

AUTHOR CONTRIBUTIONS

RN and DV were involved in conceptualizing, researching and writing this review.

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REVIEW
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Systems Biology-Derived Discoveries of Intrinsic Clocks

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A systems approach to studying biology uses a variety of mathematical, computational, and engineering tools to holistically understand and model properties of cells, tissues, and organisms. Building from early biochemical, genetic, and physiological studies, systems biology became established through the development of genome-wide methods, high-throughput procedures, modern computational processing power, and bioinformatics. Here, we highlight a variety of systems approaches to the study of biological rhythms that occur with a 24-h period—circadian rhythms. We review how systems methods have helped to elucidate complex behaviors of the circadian clock including temperature compensation, rhythmicity, and robustness. Finally, we explain the contribution of systems biology to the transcription–translation feedback loop and posttranslational oscillator models of circadian rhythms and describe new technologies and "–omics" approaches to understand circadian timekeeping and neurophysiology.

Keywords: systems biology, models, theory, RNA sequencing, neurophysiology, circadian rhythm, ribosome profiling

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SYSTEMS BIOLOGY—A BRIEF HISTORY

In contrast to a reductionist approach, systems biology emphasizes the interaction of components rather than the components themselves: to see the forest for the trees. This holistic approach is not a modern idea, but can be traced as far back as the Greek Aristotle "…the totality is not, as it were, a mere heap, but the whole is something besides the parts…" In the modern era, Karl Ludwig von Bertalanffy is generally credited as one of the founders of general systems theory with his model of individual cell growth in the early 20th century (1). Later, the Dutch physicist Balthasar van der Pol working with electric circuits developed his eponymous equation to describe relaxation oscillations (2), which was used for theoretical models of neuronal systems (3, 4). In the 1950s, Alan Hodgkin and Andrew Huxley described the first mathematical model of an action potential propagating along a neuron, which famously predicted the existence of ion channels before their experimental discovery (5), and Alan Turing proposed a reaction–diffusion system in "The Chemical Basis of Morphogenesis" to explain how an initially homogenous system—the embryo—forms patterns through the action of morphogens (6).

These early systems models of cellular behavior were overshadowed by the excitement of the molecular biology revolution. Geneticists and biochemists learned to devise assays to measure the impact of single genes and single enzymes. In the 1970s, Ronald Konopka in Seymour Benzer's lab used chemical mutagenesis to screen fruit flies for defects in their rhythmic emergence from the pupae state. He discovered three alleles of the Period gene, which is one of the earliest examples of a gene determining behavior in an organism (7). For the next 30 years, circadian biologists mostly pursued reductionist approaches similar to Konopka's strategy to examine circadian

behaviors in different organisms by knocking out single genes or isolating individual tissues.

The era of functional genomics and next-generation sequencing has begun to shift the balance back toward systems biology. In the following sections, we review the contributions of mathematical models, microarray technology, RNA sequencing, proteomics, and neurophysiological approaches to systematically dissect circadian behavior and uncover new modes of regulation (for an overview, see **Figure 1**).

MODELING THE SYSTEMS PROPERTIES OF CIRCADIAN RHYTHMS

The circadian clock is an interconnected network—a network of small molecules and metabolites, a network of genes and proteins, and a network of cells, neurons, and tissues. At each level, the interacting network of components can create complex behaviors. These systems-level properties include three defining characteristics of circadian rhythms: (1) periodicity—rhythms are autonomous with a period that matches the daily 24-h rotation of the Earth, (2) entrainment—rhythms can be reset by environmental cues such as light, temperature, or food intake, and (3) temperature compensation—periodicity of rhythms persistent despite fluctuations over physiologically relevant temperature ranges.

Before genetics led to the identification of molecular components governing a transcription and translation feedback loop that underlies the mechanism of circadian oscillation in many organisms, theoretical studies sought to model how oscillation, periodicity, entrainment, and temperature compensation could arise. The first was Goodwin's model of a molecular oscillator using negative feedback (8–10). Understanding the different types of behavior in networks have enabled mathematical biologists to make predictions about which biological processes affect circadian rhythm behavior such as period length and temperature compensation. For example, in a hypothetical biochemical network with negative feedback, there are necessary constraints on reaction rates for the generation of instability at steady state (11). Using this constraint and other ideas from signal processing in the Goodwin model for circadian oscillation, it could be shown that transcription and translation rate are not important for setting period length, but instead a critical feature is the degradation rate of the repressor (12). These studies highlight the fundamental contributions of systems modelers even without knowledge of the molecular network underpinning circadian rhythms.

Identification of the molecular components of circadian rhythms led to an explosion of models incorporating these proteins and functions. Goldbeter's model used non-linearity of Hill-type equations in the Goodwin model when he reported the first model of circadian rhythms based on observations of PERIOD phosphorylation and degradation in *Drosophila* (13). Non-linearity in feedback repression could occur through cooperative binding of multiple repressors to a promoter or via repressive multisite phosphorylation of a transcriptional activator. Derivations of this type of model have been used to examine *Drosophila* (13–19), *Neurospora* (14, 16, 20, 21), and mammalian circadian rhythms (22–30). In the next subsections, we discuss how these and other models contributed to our understanding of the systems properties of circadian rhythms.

Periodicity and Design of the Transcription–Translation Feedback Loop

The period of a biological rhythm is tied to the 24-h rotational movement of the Earth. Organisms across different domains of life evolved timing mechanisms called biological clocks to coordinate function and behavior to specific times of the day (31). Each day environmental cues such as light and temperature reset your biological clock in a process called entrainment (32). Food can also entrain biological rhythms by affecting clock machinery in the liver (33, 34). Entrainment allows us to recover from the jet lag inducing effects of airplane travel by either advancing or delaying the phase of the circadian clock. Response to external cues is not instantaneous—timekeeping of the circadian clock persists, which is why we feel jet lagged in the first place.

Flexibility in period length was apparent from the earliest studies of mutant organisms (7, 35, 36). Systematic screening of chemical libraries also revealed chemical compounds that could alter period length by targeting specific clock proteins (24, 37–44). Pharmacological and/or genetic perturbation could extend the range of periods in the fibroblast from 27 to 54 h (41) and suprachiasmatic nucleus (SCN) from 17 to 42 h (45). Investigating why some mutant organisms have short or long periods revealed the molecular mechanisms of circadian rhythms and researchers could begin to test models by designing and manipulating components in the circuit. They were perhaps inspired by synthetic bacteria genetic circuits that recapitulate transcriptional oscillations (46) and bistable switches (47). For circadian rhythms, mathematical modeling guided construction of a synthetic 26-h oscillator based on siRNA-based silencing of a tetracycline-dependent transactivator (48). Construction of a mammalian promoter/enhancer database allowed researchers to identify high-scoring or low-scoring cis-elements and validate high- or low-amplitude expression, respectively, in cells (49), which enabled synthetic reconstruction of different circadian phases in cells by mixing combinations of promoter elements (50, 51). Researchers have also implemented artificial photic input pathways to clock cells to investigate singularity behavior, in which the circadian clock is reset after perturbations of different strengths and timing (52). More recently, researchers have succeeded in replacing the endogenous repressor in mice with a tunable one (53) and artificially manipulating the molecular circuitry of pacemaker cells in the brain (54, 55) to alter period length. These synthetic biology reconstruction experiments probe the sufficiency of circadian networks to generate oscillations and oscillations of different periods as well as test ideas about how network components interact and function within cells.

Periodicity and the Rise of the Posttranslation Circadian Oscillator

Scientists originally thought that a transcription–translation feedback network was required for 24-h rhythms. But then, a remarkable study was published. Working in cyanobacteria,

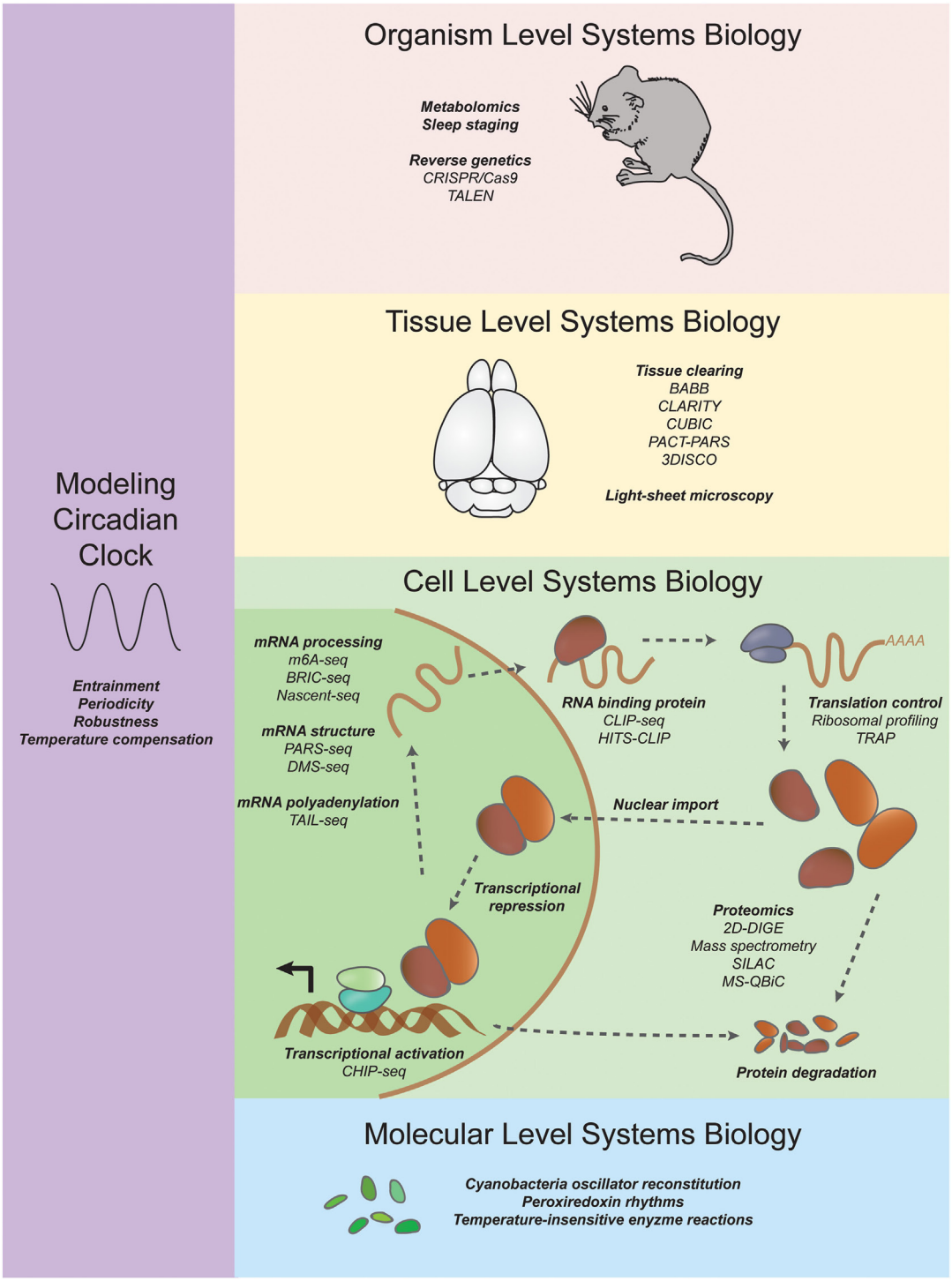

FIGURE 1 | **Systems approaches to studying circadian rhythms**. On an organism level, researchers are using CRISPR/Cas9 and TALEN coupled with new sleep staging techniques to uncover mutations in genes that increase or decrease sleep. On a tissue level, new tissue clearing techniques such as CLARITY and CUBIC are enabling researchers to investigate the neuroanatomical basis of behavior (see Systems Neurophysiology). On a cell level, systems transcriptomics experiments have revealed not only rhythmic mRNA levels through microarrays and RNA sequencing but also other molecular details such as chromatin state, mRNA structure and modification, ribosome binding, and rhythmic protein abundance (see Systems Transcriptomics, Systems Proteomics and Metabolomics, and Systems Approaches to Study Translation Regulation in Circadian Rhythms). On a molecular level, reconstitution of a cyanobacteria posttranslational oscillator and the discovery of transcription/translation independent peroxiredoxin rhythms have expanded our understanding of circadian oscillations (see Periodicity and the Rise of the Posttranslation Circadian Oscillator). Systems modelers have discovered insights into constraints and parameters necessary for unique features of the circadian clock such as entrainment, periodicity, robustness, and temperature compensation (see Modeling the Systems Properties of Circadian Rhythms).

Kondo and colleagues mixed a small number of cyanobacterial proteins KaiA, KaiB, and KaiC, and ATP in a test tube to produce rhythmic 24-h oscillations in KaiC protein phosphorylation (56). In a manner similar to simple chemical reaction–diffusion systems creating Turing patterns, 24-h periodicity could be established in the absence of a transcription–translation negative feedback loop architecture.

A few years later, it was discovered that an antioxidant enzyme called peroxiredoxin in cultured human red blood cells undergoes temperature-independent circadian cycles of hyperoxidation. Because red blood cells lack a nucleus and peroxiredoxin rhythms persist in the presence of transcription and translation inhibitors, these rhythms prove the existence of a non-transcriptional-based circadian oscillator in mammals (57) and was later found to be conserved in a wide range of species (58). In mice, rhythmic peroxiredoxin oxidation is thought to occur through hemoglobin-dependent H_2O_2 generation and proteasome degradation (59), but it remains unclear how rhythmic oxygen delivery occurs in isolated cells and how the rhythms of peroxiredoxin oxidation are temperature compensated. In the future, a more detailed understanding of the relationship between rhythmic peroxiredoxin oxidation and canonical circadian clocks is needed.

The reconstitution of a phosphorylation oscillator in cyanobacteria (56) prompted modelers and synthetic biologists to question what the minimal components are for a circadian oscillator. In cyanobacteria, biochemical studies have driven our understanding of the mechanism of the oscillator. KaiC was discovered to be both a kinase and a phosphatase (60–62). KaiC autophosphorylation is triggered by allosteric activation by KaiA (63, 64) and regulated through feedback inhibition by KaiB (60, 65). Importantly, a sequential ordering of phosphorylation at two sites on KaiC is necessary for oscillation (66, 67) and remarkably, when Kai protein complexes from different starting phases are mixed, the phosphorylation state of the population remains in synchrony (68).

Several models have been proposed to explain the mechanism of oscillation (69–71) and synchrony of the cyanobacteria oscillator on a population level (67, 72, 73). A central idea is that there is monomer shuffling between KaiC hexamers, which was proposed in mathematical models (72, 74) and by experiments from the Kondo laboratory (65, 68), and confirmed elsewhere by FRET experiments (75). Other models do not explicitly rely on monomer exchange for synchrony (67, 73), but rather synchrony arises as an emergent property of the system based on KaiA's affinity for different phosphorylated forms of KaiC. Of course, concepts such as differential affinity and monomer exchange have been incorporated together into more sophisticated models of cyanobacteria rhythms (76, 77).

Studies in cyanobacteria provide a foundation to understand the requirements (ordered phosphorylation, synchrony, etc.) for a generic phosphorylation oscillator. Most models of non-circadian phosphorylation oscillators require additional mechanisms for rhythmicity such as protein synthesis and degradation (78) or allosteric feedback from substrate (79, 80). However, a theoretical study demonstrated that autonomous circadian oscillations are possible with a single substrate reversibly phosphorylated at only two sites (81) and suggested that a well-defined ordering of phosphorylation states and sequestering checkpoints for enzyme activity could be design principles for single-molecule oscillators for the circadian clock and potentially other cellular oscillators. The Jolley model (81) results in a substrate with four possible modification states similar to MAPK (82) and cyanobacteria models (67). While a general phosphorylation oscillator has not yet been built based on these models, the reconstruction of temporal (56) and spatial (83) oscillators from purified components provide inspiration for future work. Furthermore, the recently reported success in transplanting the circadian clock from cyanobacteria into the non-circadian bacterium *Escherichia coli* (84) implies some amount generality for the network and design principles upon which circadian rhythms lie.

Temperature Compensation

Insensitivity to temperature was originally identified as an essential characteristic of biological time-measuring systems in bees, flies, and marine organisms (85–88) and references therein. In particular, it was postulated that temperature independence was the result of a temperature compensation mechanism involving the opposing effects of enzyme activities in response to changes in temperature (87). Researchers began to identify genetic mutants with defects in temperature compensation in *Neurospora* (89, 90) and *Drosophila* (91, 92). In flies, repressor dimerization was thought to be involved in temperature compensation because loss of the repressor's dimerization domain caused the period to strongly depend on temperature (91). Researchers incorporated these ideas into models of circadian rhythms by suggesting that nuclear import of the repressor decreases with temperature and repressor dimerization increases with temperature (93, 94). Other models emphasized the importance of degradation of the repressor (95, 96) and other parameters needed for temperature compensation (97). The conceptual point of these models is that for circadian rhythms to be temperature compensated, some biochemical reactions accelerate circadian oscillations, while other biochemical reactions decelerate circadian oscillations. The balance model supposes that the former acceleration reactions are less sensitive to temperature, whereas the latter deceleration reactions are more sensitive to temperature. A molecular basis for this type of temperature compensation was proposed in plants (98) and also formulated mathematically as a balance equation (99) to explain how *Neurospora* repressor stability decreases with an increase in temperature (95, 100), which is ultimately caused by phosphorylation-dependent degradation from a kinase (101).

In 1968, Pittendrigh and colleagues argued against a balancing model in which temperature shortens a reaction in the first half of a circadian cycle while simultaneously lengthening a reaction in the second half of the cycle in their experiments with *Drosophila* (102). They used short light pulses to shift the phase of *Drosophila* pupae at different temperatures and showed that the period and wave form of the phase response curve changes only a little bit with temperature. They proposed a model where circadian output from a temperature-dependent oscillation is subjected to feedback inhibition from another temperature-dependent reaction

(102, 103). These early studies suggested a model in which the enzymatic reactions that comprise the clock are temperature compensated. However, the idea of a temperature-compensated enzyme is counterintuitive because most chemical processes are temperature dependent. In cyanobacteria, the kinetic profile of the phosphorylation to dephosphorylation ratio is temperature compensated *in vitro* (56, 104). This was the first indication that temperature compensation could occur through the enzymes themselves as opposed to compensation that occurs through competing biochemical reactions.

The canonical transcription–translation feedback loop underlying circadian rhythms in eukaryotes may also be affected by temperature-insensitive enzymatic reactions. In eukaryotes, it was first discovered in mammals that the phosphorylation-dependent degradation rate of the repressor is temperature insensitive in cells, and temperature-insensitive phosphorylation is preserved *in vitro* (41). This suggests that temperature-insensitive enzymatic reactions can influence the circadian transcription–translation network. In addition to component-level temperature compensation (41), detailed examination of the degradation of the repressor revealed three distinct stages of degradation that depend on when during the circadian cycle protein translation is arrested (105). The authors in this study suggested that temperature-insensitive and -sensitive phosphorylation at different sites of the repressor are responsible for temperature compensation. In the future, it will be particularly interesting to uncover the mechanisms and structural basis of temperature compensation in these individual reactions and to synthetically engineer temperature compensation in circadian clocks similar to synthetically temperature-compensated genetic networks in bacteria (106).

Robustness to Gene Dosage

Circadian rhythms are surprisingly robust to changes in gene dosage—there has been much discussion about why knockout of core clock genes only results in subtle period lengthening or shortening (107). There have been efforts to understand networks effects by systematically altering individual gene levels (108) or by globally altering transcription levels with drugs (109). Resistance to internal noise from the stochastic nature of biochemical networks in the cell is an essential property for a robust circadian clock network (110). Theoretical models suggested that intercellular coupling between individual oscillator cells is necessary for synchrony and noise resistance (111). Indeed, dissociated SCN neurons and isolated cells from tissues such as lung and liver are arrhythmic compared to intact tissues with altered rhythmicity (112, 113). Robustness is also ensured by interlocking-feedback loops at the genetic circuit level, for review, see Ref. (114), and has been featured in models of circadian rhythms from different organisms (17, 115–118). In mammals, genetic (119–122) and pharmacological (38, 44) perturbation of the secondary feedback loop showed that it primarily served as a stabilizing mechanism.

Modeling approaches have revealed that activator and repressor complex formation are necessary for noise resistance (123) and that a 1:1 stoichiometric balance of repressors binding activators rather than binding DNA is important for robust circadian timekeeping (124). Experiments in mammals seem to support these models because rhythm generation in mouse embryonic fibroblasts can be abolished by constitutive expression of the mammalian repressor (125) or by artificially altering the stoichiometry between activators and repressors (126). Indeed, the natural stoichiometry between activators and repressors in a mouse liver is close to 1:1 as measured by western blotting (127) and mass spectrometry (128).

The difference in repression mechanisms—Hill-type nonlinearity from models based on the Goodwin oscillator or protein-based sequestration leads to subtle differences in the activity of the activator in circadian models as the concentration of the repressor increases. For Hill-type models, there is an all-or-none switch that occurs when multisite phosphorylation or cooperative binding reaches some critical level. The activator is like a light bulb that is on until it suddenly gets switched off. For protein-sequestration models, the activity of the activator linearly decreases as a function of the molar ratio between activator and repressor, which is like a light bulb slowly turned down by a dimmer. These differences can affect the synchronized period between coupled heterogeneous oscillators compared to the mean period of uncoupled oscillators (129). Importantly, understanding the differences in repression mechanisms for coupled oscillators can lead to testable predictions on how clock components interact with other proteins, such as regulation of the tumor antigen p53 (130).

SYSTEMS TRANSCRIPTOMICS

Identification of the Components of the Circadian Clock

On a tissue level, the central clock in mammals is located in a structure of the brain called the SCN. Ganglion cells in the retina detect light signals through a photopigment called melanopsin and relay this information to the SCN. SCN neurons project to different regions of the brain and synchronize biological clocks in peripheral tissues by secretion of hormones as previously reviewed (131, 132). However, most tissues in an organism have the core transcriptional architecture for circadian rhythmicity including liver, lung, and muscles (133) as well as cultured cells (134–136).

The genetic network for circadian rhythms is based on delayed feedback repression of transcription. Briefly, a CLOCK:BMAL1 heterodimer activates transcription at promoter elements called E-boxes. A protein called PERIOD (PER) heterodimerizes with another protein CRYPTOCHROME (CRY) and translocates to the nucleus where it represses transcription of the Period gene and other genes that activate Period transcription, reviewed elsewhere extensively (137–140). Several components of the core transcriptional network were identified in forward-genetics screens (i.e., random mutation of an organism's genome and searching for mutants with abnormal rhythms) including Period (7) and Timeless (141) in *Drosophila*, Frequency (35) in *Neurospora*, and Clock (142, 143) in mice.

Systems approaches have been successful in identifying other core clock components such as Bmal, which was identified using an iterative search for other bHLH proteins (144, 145). Genomics-based strategies helped to identify activators of Bmal such as Rora (122) and Nr1d1 (121), and functional genomics strategies in *Drosophila* revealed Clockwork Orange (146–148) as a homolog of the mammalian Dec1 and Dec2 (149).

Systems Experiments to Study the Transcriptome

Some of the earliest systems approaches to study circadian rhythms were to simply analyze all the mRNA in a tissue or organism to determine which mRNAs had cyclic expression. These studies used microarrays to identify cycling mRNAs in *Drosophila* (150, 151), in the mouse liver, heart, and SCN (152–155), rat pineal gland (156), isolated fibroblasts (157, 158), and in plants (159). There was considerable tissue specificity in rhythmic genes because only approximately 10% of cycling genes were common to at least one other tissue (160). Additionally, there are approximately 100-fold fewer cycling transcripts in NIH3T3 and U2OS cell culture models compared to mice tissue (161). This study also revealed 12-h oscillatory transcripts in liver, heart, lungs, and other tissues, but not in cultured cells (161). These "harmonic" rhythms are perturbed by a disrupted circadian clock in the SCN (162). Rhythmicity of the core clock component PER2 in these tissues could be confirmed with luminescent reporter mice (163).

Recent studies have begun to use RNA sequencing to measure steady-state mRNA expression in tissues such as the mouse liver (164–166) or to identify transcription factor-binding sites using chromatin-immunoprecipitation coupled with RNA-sequencing (CHIP-seq) (164–170). Comparative genomic approaches revealed the importance of E-boxes, D-boxes (171), and RREs (155, 171) in timing circadian mRNA expression, which have allowed ensemble-based predictions of phase response from combinations of these elements (25).

Systems Experiments Analyzing Chromatin State

Next-generation sequencing experiments revealed both circadian initiation and recruitment of RNA polymerase II (RNAPII) to circadian promoters (164, 168) and concomitant circadian changes in chromatin state (164, 166, 168). In particular, H3K4me3 histone methylation have circadian oscillations that slightly lag RNAPII occupancy (168). Circadian regulation of chromatin state was first observed in an increase in phosphorylation of histone H3S10 in the SCN in response to light (172). Additionally, rhythmic acetylation of histone 3 was observed in the promoters of Per1, Per2, and Cry1 in mouse liver (173, 174). CLOCK itself has intrinsic histone acetylase activity (175) and is rhythmically recruited to circadian promoters (174, 176). CLOCK can acetylate other non-histone proteins including BMAL1, which promotes recruitment of CRY1 and thus BMAL1–CLOCK inactivation (177). SIRT1, a sirtuin histone deacetylase whose activity depends on the coenzyme nicotinamide adenine dinucleotide (NAD^+), interacts with CLOCK and can deacetylate BMAL1 (174) and PER2 (178). SIRT1 also controls H3K4me3 methylation through circadian deadenylation of the histone methyltransferase mixed-lineage leukemia 1 (179). Circadian regulation results in cycles of NAD + biosynthesis (180), NAD + recycling (181), alters Clock and Bmal1 binding (182), and NAD redox rhythms have been observed directly in cells (183). Together, these studies suggest a direct link between metabolism and epigenetic regulation of circadian rhythms.

MicroRNAs (miRNAs) in Circadian Rhythms

In addition to discovering cycling transcripts, systems transcriptomics experiments have uncovered other cycling RNAs such as long non-coding RNAs (lncRNAs) and miRNAs. For example, CHIP-seq experiments revealed clock proteins such as Clock, Bmal1, and Nr1d1 binding at sites outside of canonical gene promoters (166, 167, 169, 170, 184, 185), which suggested circadian regulation of non-protein-coding transcripts. MiRNAs bind target mRNAs typically in 3′ untranslated regions (3′ UTRs) to inhibit translation and destabilize the mRNA, for review see Ref. (186–189). Microarray studies uncovered miRNA expression inversely correlated with circadian activators Clock and Bmal1 and positively correlated with circadian suppressors Per, Cry1, and Nr1d1 (190), and other miRNAs that have diurnal expression patterns (191). MiRNAs are regulated by circadian proteins such as CLOCK (170, 192, 193) and NR1D1 (194) and modulate the expression of circadian genes such as Bmal1 (195–198), Clock (193, 199, 200), the circadian polyA deadenylase Nocturnin (201), Per1 and Per2 (202–204), Clockwork Orange (205), Timeless in *Drosophila* (206), and Cry1 (207). Knockout of the core miRNA-processing machinery in mouse liver revealed that ~30% of the rhythmic transcriptome is posttranscriptionally modulated by miRNAs (208).

lncRNAs in Circadian Rhythms

In addition to miRNAs, next-generation sequencing experiments have revealed extensive transcription of lncRNAs (209, 210) and circadian expression of lncRNAs (166, 211, 212). An in depth study revealed differential expression of 112 lncRNAs in the rat pineal gland, and light expression at night could modulate the level of some of these lncRNAs (213). A study of mouse liver revealed 19 out of 123 lncRNAs detected with robust oscillations and detected antisense transcripts associated with Per2 (166). Antisense transcription of Per2 in mice liver has been reported by others (164, 165) and originally in the silk moth (214), but it remains unclear what the function of antisense Per2 is for circadian rhythms. In *Neurospora*, the antisense transcript of frequency (called Qrf—Frq, spelled backward) is important for entrainment to light, oscillates in a reciprocal pattern to Frq, and promotes Frq gene silencing via heterochromatin formation (215–217). Deletion of a lncRNA associated with Prader–Willi syndrome in mice results in increased energy expenditure and altered expression of circadian genes such as Clock, Cry, and Per (218). Additionally, a lncRNA highly upregulated in liver perturbs the expression levels of Clock, Cry, and Per in hepatoma cells (219). Together, these studies suggest a role for non-protein-coding transcripts in the regulation of circadian rhythms.

Posttranscriptional Regulation of Circadian Rhythms

Next-generation sequencing studies have also examined to what extent rhythmic steady-state mRNA transcripts result from *de novo* rhythmic transcription versus rhythms via post-transcriptional regulation. By analyzing expression of introns as an indicator of pre-mRNA levels, a study by Koike et al. determined that the majority circadian mRNAs do not undergo rhythmic transcription (164). Another method to directly assess *de novo* transcription called Nascent-seq confirmed this result and further showed that many mRNAs with *de novo* rhythmic transcription do not have rhythms in steady levels of mRNA (165). A similar nascent-seq study in *Drosophila* also revealed a considerable posttranscriptional contribution to cycling mRNA amplitudes (220).

There are a variety of mechanisms for posttranscriptional regulation of circadian rhythms including splicing, mRNA export, polyadenylation, mRNA stability, methylation, and regulated translation—for review, see Ref. (221). The first indication of posttranscriptional regulation of circadian rhythms was that stability of *Drosophila* Per mRNA oscillates (222), which was also later observed in mammals (223). Posttranscriptional regulators such as LARK bind to the 3′ UTR of Per1 mRNA to enhance PER1 translation (224, 225). LARK also promotes alternative translation of the casein kinase homolog Doubletime in *Drosophila* (226). Researchers have uncovered other proteins that regulate translation of clock components. For example, the heterogenous nuclear ribonucleoprotein Q (hnRNP Q) modulates translation of Nr1d1, Per1, Per3, Cry1, and the rate-limiting enzyme in melatonin synthesis AANAT (227–232). Cry1 mRNA stability is also regulated by AU-rich element RNA-binding protein (AUF1) also known as hnRNP D (233, 234), and Per2 mRNA stability was found to be modulated by polypyrimidine tract-binding protein also known as hnRNP I (235).

mRNA PolyA Tail Length and Circadian Rhythms

Other mRNA processing mechanims may also posttranscriptionally regulate circadian rhythms. The 3′ end of newly transcribed pre-mRNA in the nucleus is cleaved and a polyA tail is added at one of the several possible sites (236). Deadenylation of this polyA tail in the cytoplasm by enzymes such as the poly(A)-specific ribonuclease and the Ccr4-Not complex can shorten tail length and accelerate mRNA degradation (237, 238). Daily variation in polyA tail length was first observed for vasopressin mRNA in the SCN (239). In *Xenopus*, another deadenylase called Nocturnin was discovered in a screen to detect rhythmically expressed mRNAs in retinal photoreceptors (240, 241) and was later shown to be expressed in multiple mouse tissues (242). Nocturnin is one of the few mRNAs that remain rhythmic after the liver clock is conditionally inactivated by drug-mediated Bmal1 expression (243) and can be posttranscriptionally regulated by miR-122 (201). Mice lacking Nocturnin do not have any obvious circadian behavior deficiencies, but are resistant to diet-induced obesity (244). However, in *Drosophila*, loss of Nocturnin results in abnormal behavior rhythms in constant light (245). A microarray method to measure polyA tail length suggested that rhythmic nuclear adenylation is coupled to rhythmic transcription and that rhythmicity in polyA tail length is related to rhythmic protein expression (246). These studies suggest that posttranscriptional regulation by deadenylation may be important for proper circadian rhythms and that next-generation sequencing techniques such as polyA tail profiling (247, 248) will be critical for fully understanding the contribution of polyA tail length to circadian rhythms.

Systems Experiments to Measure mRNA Modification, Structure, and RNA-Binding Proteins

Besides polyadenylation, mRNA processing by other mechanisms may contribute to circadian rhythms. A recent study showed that reduction of Mettl3, an m^6A mRNA methylase involved in mRNA processing and nuclear export, reduces m^6A methylation of circadian transcripts and extends period (249). Next-generation sequencing studies of m^6A methylation may reveal other contexts in which methylation of mRNA is important for circadian rhythms (250). In addition, other RNA-sequencing techniques to probe RNA secondary structure such as dimethyl sulfate sequencing—DMS-seq and parallel analysis of RNA structure—PARS-seq (251, 252), BRIC-seq for mRNA stability (253, 254), and various methods to analyze RNA-binding sites of specific RNA-binding proteins such as CLIP, CLIP-seq, HITS-CLIP, iCLIP, and PAR-CLIP (255–260) will be critical for understanding how mRNA processing is involved in circadian rhythms. For example, CLIP-seq of mRNAs bound to cold-inducible binding protein, which is required for high-amplitude circadian gene expression, revealed binding to Clock and other circadian transcripts (261).

SYSTEMS PROTEOMICS AND METABOLOMICS

Circadian Proteomics

Researchers are beginning to use systems approaches to study the circadian proteome and metabolome. Using two-dimensional difference gel electrophoresis (2D-DIGE), Reddy and colleagues revealed that approximately 20% of the soluble proteins in the mouse liver oscillate. Surprisingly, for many rhythmic proteins, the corresponding mRNA was not rhythmic, which suggests translational and posttranslational control of protein rhythms (262). 2D-DIGE has also been used to investigate circadian differences in the mouse retina (263) and day and night differences in the mouse heart (264). In addition to mice, 2D gel-based mass spectrometry has been used to investigate chronological changes in eukaryotic algae (265, 266) and in plants (267, 268).

Other groups have employed stable isotope labeling by amino acids in cell culture (SILAC) to compare two groups of samples—one mixed with "heavy" amino acids and one mixed with "light" amino acids based on the composition of different element isotopes (269). SILAC-based quantitative mass spectrometry has been used to uncover cycling proteins in the mouse liver (270, 271) and SCN (272). Traditional SILAC approaches use chemical synthesis of peptides with isotopically labeled amino acids (269,

273) or gene expression systems in *E. coli* (274, 275). However, cell-free protein synthesis systems are potentially a more cost-effect tool to express isotope-labeled peptides because the volume of the reaction is much lower and purification is easier because there is no need for culturing, harvesting, and disrupting cells (275–278). Recently, a cell-free protein synthesis system called the PURE system (279) coupled with high-resolution mass spectrometry in a workflow called MS-QBiC was used to quantify 20 selected circadian clock proteins over a 24-h time series (128). This study estimated the absolute number of protein molecules for core clock components per cell and the delay between steady-state levels of mRNA (measured by qPCR) and protein copy number (128).

In addition to SILAC, label-free approaches such as MaxLFQ (280) have been used to quantify proteins in mouse skeletal muscle (281). Mass spectrometry has been used to examine the global proteome in cyanobacteria (282). Mass spectrometry has also been used to analyze the global phosphoproteome and revealed ~5,000 phosphosites that significantly oscillate in the mouse liver (283) and ~3,000 phosphosites in *Arabidopsis* (284). Given the widespread discrepancies between transcript and protein rhythmicity in a number of organisms, in the future, it will be useful to understand the role of translation and posttranslational regulation as well as cycling protein modification states (e.g., phosphorylation) to circadian networks.

Circadian Metabolomics

Researchers have looked at rhythmic metabolites in humans (285–288) and in mice (289–292) and have shown that circadian proteins directly regulate metabolism (44, 184). Researchers have also used comprehensive metabolite profiling to analyze diet effects in mice (293–295) and the effects of sleep loss in humans (296–299). Computational databases have been developed to compare published transcriptomes, proteomes, and metabolomes (292). Metabolic profiling is still quite noisy compared to transcriptome data at least for identifying tissue-specific signatures (300), and many challenges remain including identification of unknown metabolites, standardization of data repositories and reporting methods, and integration with other types of data. Researchers are beginning to use metabolic profiling over larger time courses and with higher resolution in cell culture lines (301). In the future, coupling these methods with gene knockout or knockdown of core clock components will enable researchers to identify connections between circadian rhythms and metabolism. For example, are there harmonics in metabolite rhythms (i.e., multiples of a 24-h rhythm like 8- and 12-h rhythms) similar to the harmonics of mRNA rhythms (161, 162), and would these rhythms be influenced by circadian genes?

One benefit of systems studies is the development of a molecular timetable to detect an individual's body time based on a single time point assay. Molecular timetables have been developed with mice transcriptome data (212, 302) and applied to mice (291) and human (288) metabolite data, proteomic data (128), and even human breath (303). In theory, metabolite timetables could enable researchers to hone chronotherapeutic strategies for clinical conditions. However, despite the strong evidence that circadian timing effects xenobiotic metabolism, bioavailability, and drug efficacy and that many of the most successful drugs in the United States target proteins with circadian rhythm components (212), ongoing clinical trials rarely exploit time-of-day-dependent drug delivery (304).

SYSTEMS APPROACHES TO STUDY TRANSLATION REGULATION IN CIRCADIAN RHYTHMS

Although 10% of genes are rhythmic in the liver (152), *de novo* transcription is only responsible for a small fraction of this rhythmicity (164). Thus, gene expression studies using microarrays and RNA-sequencing may not correlate with translation of the corresponding mRNA nor with protein abundance (305). In the mouse liver, systems studies of the proteome are unable to detect low-abundant components of the core circadian circuit (270, 271), unless special care is taken to examine a particular protein on a case-by-case basis (128). Thus, researchers have begun to use next-generation sequencing techniques of mRNA attached to mRNA in monosomes and polysomes (306, 307) and with affinity purification (308–310) as a proxy for protein abundance and to understand how translation regulation affects protein abundance.

It has been known for more than 50 years that perturbation of translation disrupts circadian rhythms (311). Until recently, there has been a shortage of good tools to measure translation directly. In 2009, Nicholas Ingolia in Jonathan Weissman's lab developed a technique called ribosomal profiling, which uses RNA sequencing of ribosome-bound mRNA protected from RNAse degradation, to determine the location and abundance of ribosomes in the yeast transcriptome (312). Researchers have begun to use this method to study circadian rhythms in ribosomal occupancy (313, 314). These studies discovered a class of rhthmically translated mRNAs without corresponding steady-state mRNA rhythms (313, 314), which in the case of mouse liver may be a result of rhythmic ribosomal biogenesis (315). Researchers have previously observed that global translation is rhythmic in the mouse liver (316, 317), which is probably a result of activation of the TORC1 pathway (315, 318–320). Interestingly, diurnally regulated translation in the mouse liver is only moderately affected by knockout of the core clock component Bmal1 and many genes that contained 5′-terminal oligo pyrimidine tract or translation initiator of short 5′ untranslated region (5′-UTR) sequence have rhythms in ribosomal occupancy independent of trancriptional rhythms (321). These studies in addition to previous research (322–326) suggest that feeding rhythms can synchronize the liver in the absence of cues from neuronal pacemaker cells in the SCN.

The Janich and Jang studies (313, 314) also revealed widespread circadian translation of upstream open reading frames (uORFs) in 5′ UTRs. Translation of uORFs globally represses translation efficiency—a measure of the ratio of ribosomal occupancy, determined by ribosomal profiling, to steady-state mRNA, measured by RNA-sequencing (314). Interesting, many circadian mRNAs also have uORFs in their 5′ UTRs (**Table 1**), which may disrupt translation of the downstream coding sequence by ribosomal pausing on the mRNA, alternative translation, or other mechanisms (327). Ribosome pausing on uORFs may be

alleviated by the action of the non-canonical initiation factors density regulated protein (DENR) and multiple copies in T-cell lymphoma (MCT-1), which act to promote translation reinitiation downstream of uORFs (328, 329). Depletion of DENR by shRNAs in NIH3T3 cells shortens the period by 1.5 h, which suggests that uORFs may be relevant for circadian function (314). In other biological contexts, repression of translation by uORFs can be regulated by trans-acting factors. For example, in *Drosphila*, the master switch gene Sex-lethal (Sxl) is important for sex, for review see Ref. (330–332). SXL-binding downstream of a short uORF on *male-specific lethal (msl)-2* enhances translation repression by the uORF on downstream reading frame translation (333). During mitosis, one of the most translationally repressed mRNAs is early mitotic inhibitor 1 (Emi1) that inhibits the activity of the anaphase-promoting complex (334). Emi1 has multiple transcript isoforms and the isoform with several uORFs in the 5′ UTR is severely crippled for translation initiation in single-molecule reporter experiments (335). These studies suggest that uORF-mediated translational repression is important in a variety of biological functions and may have an unexplored role in circadian rhythms.

What is the consequence of disrupted translation for circadian rhythms? One clue came when researchers showed that codon usage affects circadian function in cyanobacteria (336), *Neurospora* (337), and *Drosophila* (338). While cyanobacteria with codon-optimized Kai genes have enhanced circadian rhythmicty at cooler temperatures, this modification impairs cell growth, which suggests that non-optimal translation could provide an adaptive response to changes in the environment (336). In *Neurospora*, codon optimization of Frq alters FRQ protein structure, which impairs circadian rhythms (337). Similarly, in *Drosophila*, codon optimization results in conformational changes of the *Drosophila* PER protein altering PER phosphorylation, stability, and impairs behavioral rhythms (338). Additionally, it is becoming clear that translation control is interlinked with both circadian rhythms and sleep disorders. For example, Ataxin2 functions as a critical translation activator of Per2 in flies (339, 340), and individuals with disease mutations in human Ataxin2 have disturbed rapid eye movement sleep (341, 342).

TABLE 1 | Number of upstream open reading frames (uORFs) in common circadian clock genes.

Gene name	Ref Seq ID	Number of uORFs	uORF length (nt)
Bhlhe40	NM_011498	1	18
Bmal1	NM_007489	4	72; 42; 21; 33
	NM_001243048	2	201; 171
Clock	NM_007715	3	66; 48; 30
	NM_001289826	4	339; 66; 48; 30
Cry1	NM_007771	2	36; 24
Cry2	NM_009963	0	—
CK1d	NM_139059	2	27; 21
CK1e	NM_013767	0	—
	NM_001289898	0	—
	NM_001289899	2	126; 66
Dbp	NM_016974	2	12; 42
Nfil3	NM_017373	3	15; 51; 12
Nr1d1	NM_145434	3	117; 192; 21
Nr1d2	NM_011584	3	120; 120; 117
Per1	NM_011065	1	15
	NM_001159367	1	15
Per2	NM_011066	1	6
Per3	NM_011067	4	63; 30; 84; 48
	NM_001289877	4	63; 30; 84; 48
	NM_001289878	4	63; 30; 84; 48
Rorc	NM_011281	0	—
Tef	NM_017376	1	291
	NM_153484	0	—

SYSTEMS NEUROPHYSIOLOGY

Systems neurophysiologists are beginning to connect the circadian circuit to more complex outputs from the clock such as activity rhythms. Forward genetics in mice have already uncovered core components in the circadian network (142), and researchers have begun to use forward genetics for complex behavior such as sleep (343). On the other hand, the development of TALEN (344), Zinc-Finger Nucleases (345), and CRISPR/Cas9 (346, 347) gene knockout systems have accelerated the pace at which researchers can pursue reverse genetics in mice. In particular, CRISPR/Cas9 systems have been extensively modified to improve targeting efficiency and specificity (346–353). However, the need for invasive techniques such as electroencephalography and electromyography to characterize sleep hampers high-throughput phenotyping. To facilitate rapid phenotyping, researchers have developed a respiration-based, sleep staging system in combination with redundant CRISPR targeting to reveal new genes important for sleep regulation (354, 355). In particular, researchers generated and analyzed more than 21 different KO mice and discovered different ion channels that could increase or decrease sleep duration (355). These studies have revealed the genetic bases for behaviors such as sleep, but do not show how neural networks and structures in the brain are wired to carry out such behavior. In the past, researchers have used conventional histology and immunohistochemistry of sliced brain sections to reveal the when and where of gene function, but recent advances in tissue clearing have begun to enable direct imaging of intact organs (356).

Optical sectioning using light-sheet microscopy in combination with recently developed tissue-clearing techniques is a potent strategy to begin to explore the neuroanatomical basis of behavior (357–362). Image analysis algorithms, automated comparative analysis, and feature extraction will enable researchers to quickly test and analyze neural activity in different parts of the brain with different mutant mice and under a variety of experimental conditions. These approaches will be useful to determine what areas of the brain are affected by sleep/wake pharmaceutical reagents such as methamphetamine and to develop a whole-brain anatomical atlas to catalog and characterize every individual cell in the brain.

CONCLUSION

Systems experiments from modeling to metabolomics have significantly increased our understanding of circadian rhythms, but many challenges remain. For modeling, we still do not have a comprehensive understanding of temperature compensation nor the role individual enzymes have in temperature-independent and -dependent reactions. We do not understand the contribution of temperature-compensating reactions at the molecular, cell, tissue or organism level and how these temperature-compensating systems interface with one another. At an enzymatic level, we could learn much by designing and building *de novo* temperature-compensated reactions or by converting temperature-sensitive enzymes into temperature-compensating ones. There is also a need for understanding how recently discovered posttranslational oscillators such as the peroxiredoxin system interface with the genetic circadian circuit, and for an evolutionary investigation into how and why these distinct circadian timekeeping systems arose. Modeling is needed to make connections between different timekeeping systems, different organization levels of timekeeping from molecule to tissue, and between circadian rhythms and other rhythms such as the cell cycle.

For systems "–omics" researchers, there is a large variation in the rhythmicity of transcripts, metabolites, and proteomes detected even with similarly defined experimental systems. This may be in part due to how different algorithms detect rhythmicity (153, 363, 364), differences in sampling intervals (every 2, 3, 4, or 6 h), sampling duration, environmental conditions, and biological variability (365). As surveys of the circadian proteome increase, there has been an increasing realization of the widespread gap between transcript rhythms and protein levels. Posttranscriptional and posttranslational studies that examine mRNA structure and processing, translation, and protein modification will enhance our understanding of how transcriptional rhythms become protein rhythms, and how rhythms could evolve without genetic underpinnings.

For systems neurophysiologists, there is a pressing need to develop fast and reproducible assays that connect behavioral phenotypes to particular features and neurons in the brain and other tissues. Developments in computational processing power, data storage, and deep learning approaches will aid researchers in handling and analyzing the overwhelming amount of data generated by systems studies. Nevertheless, it will be important to validate findings with molecular techniques, case studies, and synthetic biology approaches to reconstitute behavior. Finally, can we translate this knowledge base to relevance in the clinic? It will be important to develop new assays and algorithms for body time estimation from samples at one or two time points. A combination of transcriptome, metabolome, and proteome timetables may further reduce the need for additional samples and increase accuracy of body time estimation. Integration of chronotherapeutics to clinical trial design and dosing protocols may enhance the success of drug candidates and perhaps lead to a reevaluation of the timing of drug delivery to achieve the greatest benefit to patients.

AUTHOR CONTRIBUTIONS

Discussed and conceived of the direction of the review: AM and HU. Illustrated the table and figure and wrote the paper: AM.

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Phosphorylation Regulating the Ratio of Intracellular CRY1 Protein Determines the Circadian Period

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The core circadian oscillator in mammals is composed of transcription/translation feedback loop, in which cryptochrome (CRY) proteins play critical roles as repressors of their own gene expression. Although post-translational modifications, such as phosphorylation of CRY1, are crucial for circadian rhythm, little is known about how phosphorylated CRY1 contributes to the molecular clockwork. To address this, we created a series of CRY1 mutants with single amino acid substitutions at potential phosphorylation sites and performed a cell-based, phenotype-rescuing screen to identify mutants with aberrant rhythmicity in CRY-deficient cells. We report 10 mutants with an abnormal circadian period length, including long period (S280D and S588D), short period (S158D, S247D, T249D, Y266D, Y273D, and Y432D), and arrhythmicity (S71D and S404D). When expressing mutated CRY1 in HEK293 cells, we show that most of the mutants (S71D, S247D, T249D, Y266D, Y273D, and Y432D) exhibited reduction in repression activity compared with wild-type (WT) CRY1, whereas other mutants had no obvious change. Correspondingly, these mutants also showed differences in protein stability and cellular localization. We show that most of mutants are more stable than WT, except S158D, T249D, and S280D. Although the characteristics of the 10 mutants are various, they all impair the ratio balance of intracellular CRY1 protein. Thus, we conclude that the mutations caused distinct phenotypes most likely through the ratio of functional CRY1 protein in cells.

Keywords: period length, phosphorylation, cryptochrome, subcellular localization, protein stability

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INTRODUCTION

To adapt to dramatic changes in environmental conditions, living organisms from fungi to humans have evolved an internal biological clock (1). In mammals, the circadian clock is an endogenously driven 24-h cycle affecting behavior, physiology, and metabolism (2). The core circadian oscillator is a transcription/translation feedback loop (TTFL) in which CLOCK and BMAL1 are activators that dimerize and promote the expression of *cryptochrome* (*CRY*) and *period* (*PER*) genes. After translation, CRY and PER form heterodimers and translocate to the nucleus where they act as repressors and inhibit their own expression. When nuclear localized CRY and PER proteins are degraded, the inhibition is relieved; the next circadian cycle is subsequently initiated (3).

Although the mechanism by which CRY and PER repress the transcriptional activation of CLOCK:BMAL1 is not well understood, post-translational modifications, such as the phosphorylation of CRY and PER proteins, are closely linked to the inhibition of transcription (4). The

phosphorylation of PER proteins regulates their stability and their subcellular localization. In addition, different phosphorylation events can lead to phenotypes of opposite periodicity, such as the mutation in patients suffering from familial advanced sleep phase syndrome (FASPS) (5). In mammals, the CRY proteins, CRY1 and CRY2, are essential for the maintenance of circadian rhythms, and their absence results in arrhythmicity in constant darkness. In addition, CRY1 and CRY2 play different roles in regulating the circadian clock because mice lacking CRY1 or CRY2 exhibit short or long periods, respectively (6, 7). CRY1 and CRY2 are highly conserved proteins consisting of an N-terminal photolyase homology (PHR) domain, which binds to the flavin adenine dinucleotide (FAD) cofactor and divergent C-terminal tails (8). In CRY2, the phosphorylation of ser265 and ser553/ser557 may affect FAD positioning and electron transport, and proteasome degradation resulting in a shortened circadian period (9, 10). CRY1 phosphorylation in the PHR domain and C-terminal tail indicates that protein stability is linked to abnormal circadian rhythms (11, 12). Recent studies have shown that the stability of CRY proteins is regulated by two competing SCF E3 ligase complexes. The FBXL3 complex mediates degradation of CRY protein in the nucleus, while the FBXL21 complex protects CRY from FBXL3 degradation in the nucleus and promotes CRY degradation in the cytoplasm (13, 14). Although post-translational modifications of CRY1 are crucial for circadian rhythms, little is known about which CRY1 phosphorylation sites have the most impact. Therefore, we conducted a cell-based screen to identify phosphorylation residues in mCRY1 that rescue rhythmicity in *CRY1/CRY2* double-deficient cells (DKO cells) to better understand the role of phosphorylated CRY1 in clock function. We identified phosphorylation sites that cause long periods, short periods, or even arrhythmicity.

MATERIALS AND METHODS

DNA Plasmids and Cells

P(*Cry1*)-CRY1 was constructed by replacing the P(*CMV*) promoter of pcDNA3.1-Cry1-Flag with the mCRY1 native promoter (1.5 kb) and the first intron (15). All of the mutations were generated using the KOD-plus-mutagenesis kit and confirmed by sequencing. HEK293 cells were purchased from the American Type Culture Collection (ATCC).

Kinetic Bioluminescence Recording

Real-time circadian reporter assays were performed as previously described (16, 17). One the day prior transfection, approximately 3–5 × 10^4 DKO cells were plated onto 35-mm culture dishes. Cells were cotransfected using the X-treme GENE HP DNA transfection reagent (Roche) with 1 μg of pGL3-P(*Per2*)-dLuc reporter plasmid and 50 ng of a CRY expression plasmid. Three days after transfection, the cells were treated with 0.1-mM dexamethasone (Sigma) for 2 h and then placed in XM medium as previously described (18). The kinetic bioluminescence was recorded using a Lumicycle luminometer (Actimetrics, Inc.) at 36°C.

Luciferase Repression Assay

HEK293 cells were grown and transfected in 96-well plates. For transfection, 10 ng of the reporter plasmid pGL3-P(*Per2*)-dLuc was combined with 5 ng of a CRY expression plasmid, 10 ng of BMAL1, and 15 ng of the CLOCK plasmid. Empty vector pcDNA3.1 was added as necessary to obtain total DNA concentration of 200 ng per well. Twenty-four hours after transfection, cells were prepared for the Dual-Luciferase Reporter Assay System (Promega).

Luciferase Complementation Assay

Luciferase complementation assay is used to determine the interaction of proteins (19). The N-terminal luciferase fragment was fused to the N-terminus of mCRY1 [wild type (WT) or mutant] and the C-terminal luciferase fragment to the C-terminus of mFBXL3 (or mPER2). mCRY1 (WT or mutant) and mFBXL3 (or mPER2) were co-expressed as fusion proteins with luciferase fragments in HEK293 cells. Twenty-four hours after transfection, cells were prepared for the Dual-Luciferase Reporter Assay System (Promega).

Global Protein Stability Assay

Assays using the global protein stability (GPS) system were performed as described, with minor modifications (20). The GPS system was used to detect the stabilization of WT CRY1 and mutants. The lentiviral reporter construct contains a single promoter and an internal ribosome entry site (IRES) that permits the translation of two fluorescent proteins (DsRed and EGFP) from one mRNA transcript. DsRed served as an internal control, whereas EGFP was expressed as a fusion with our protein of interest. When integrated into the genome, the ratio of EGFP/DsRed can be quantified by fluorescence-activated cell sorting (FACS), producing a ratio that represents the stability of target proteins. The d1EGFP and d4EGFP represent half-life at 1 and 4 h, respectively.

Subcellular Localization Assay

HEK293 cells were transfected with a plasmid encoding GFP-mCRY1 (WT or mutant). Twenty-four hours after transfection, the cells were stained with Hoechst 33258 (Sigma) and fixed with 4% paraformaldehyde in PBS. Samples were observed using Zeiss confocal LSM800 with a 63× water-immersion objective, and the data were analyzed using Image J software.

Statistical Analysis

In all experiments, unless noted, error bars represent SEM ($n \geq 3$ for each experiment). Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons test when comparing each mean to a control mean. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, USA). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

The Phosphorylation of CRY1 Regulates Circadian Rhythms

The importance of core clock protein phosphorylation in the mammalian circadian system is widely accepted (4). However,

little is known about the amount or location of phosphorylated CRY1 protein residues or how phosphorylation affects the molecular clockwork. In this study, we performed a cell-based screen to identify the phosphorylated residues in mCRY1 critical for rescuing rhythmicity in DKO cells. CRY1-mediated rescue of clock oscillation in DKO cells has been observed after transfection with CRY1 DNA concentrations ranging from 3 to 800 ng (**Figure 1A**). Our data show that 3 ng of CRY1 DNA was insufficient to rescue circadian rhythmicity, while 800 ng of CRY1 DNA restored circadian rhythms with low amplitude that were quickly dampened. Using 50 ng of CRY1 DNA, we rescued a circadian rhythmicity of approximately 24.6 h (**Figure 1G**).

In our screen, phosphomimetics were created to identify potential phosphorylation sites in human, mouse, and rat (**Table 1**). We found 10 mutants with abnormal periodical phenotypes (**Figures 1B–F**). Our data demonstrate the following: (1) substitution of serine (S) 71 and 404 with aspartic (D) prevented the rescue of circadian rhythmicity (**Figure 1B**); (2) the phosphomimetic mutants for S158D, S247D, and T249D restored circadian rhythms with short periods (S158D, −1.3 h; S247D, −3.5 h; S249D, −3.3 h) (**Figure 1G**); (3) the mutants with Y266D, Y273D, and Y432D rescued circadian rhythms with short periods (Y266D, −1.9 h; Y273D, −2.7 h; Y432D, −1.8 h), low amplitudes, and quick damping (**Figure 1G**); (4) the S280D and S588D mutants restored circadian rhythms with long periods, especially S588D (S588D, +3.2 h; S280D, +1.6 h) (**Figure 1G**), which is consistent with previous reports (12); and (5) the phosphorylation of serines 281, 575, 595, and 604 had no obvious effect on circadian rhythms (**Figure 1G**). Thus, we established that most of the phosphorylation sites on CRY1 play distinct roles in the mechanism of the molecular clockwork.

To determine whether non-phosphorylation of the phosphorylated sites alters CRY1's function, we generated non-phosphomimetic mutants of CRY1 with Ser/Thr/Tyr changed to Ala or Phe. Our results showed that all of the non-phosphomimetic mutants (S71A, S247A, T249A, S280A, Y266F, Y273F, Y432F, S404A, and S588A) exhibited lack of effect on the circadian period (**Figure 1**).

Effects of Mutant mCRY1 on BMAL1: CLOCK-Induced Transcriptional Activation

To determine how CRY1 phosphorylation affects the molecular clockwork, we used a transcriptional assay to analyze the functional significance of the phosphomimetic mutants. Co-expression of CLOCK and BMAL1 stimulated E-box element-dependent transcription of a luciferase reporter gene in HEK293T cells, which was markedly suppressed by the expression of WT mCRY1 under the control of cytomegalovirus (CMV) promoter [P(*CMV*)] or the native mCRY1 promoter [P(*Cry1*)] (**Figures 2A,B**). We then constructed mutants by replacing phospho-acceptor Ser/Tyr/Trp residues with Asp, which mimics phosphorylation. The phosphomimetic mCRY1 mutants were used to determine the role of each residue in inhibiting transcription. Compared with WT mCRY1, the mCRY1 mutants fell into two phenotypic groups: strong repression (>60% of WT repression activity) that had similar repression activity to WT and weak repression (<20% of WT repression activity) with a significant reduction in repression activity (**Figures 2A,B**). The results showed that WT mCRY1 had strong repression activity that repressed the transcriptional activation to 3% driven by CMV promoter and to 20% driven by mCRY1 promoter. In addition, six Asp mutants (S71D, S247D, T249D, Y266D, Y273D, and Y432D) repressed the transcriptional activation to 16–50% and to 53–85%, respectively, under the control of CMV and mCRY1 promoter, exhibiting significant reductions in repression activity ($p < 0.01$, ANOVA). However, the other four Asp mutants (S158D, S280D, S404D, and S588D) exhibited no obvious change in repression activity ($p > 0.05$, ANOVA), although the repression activity of S404D was slightly stronger than WT (**Figures 2A,B**).

Effects of Phosphomimetic Mutation on mCRY1 Protein Stability and Interactions with FBXL3 and PER2

Previous reports have shown that phosphorylation of mCRY1 at S247 does not affect protein stability (9). Therefore, we investigated whether phosphorylation of other residues that regulate mCRY1's function (**Figures 2A,B**) alters protein stability. The GPS system utilizes an internally normalized fluorescent-based reporter system combined with FACS to detect real-time protein stability at the level of individual living cells (20, 22). GPS vectors expressed a single transcript encoding DsRed and EGFP target separated by an IRES (**Figure 3A**). The coding sequence for DsRed–IRES–EGFP–mCRY1 (WT or mutant) was cloned into a lentiviral vector. After infection by the lentivirus, HEK293 cells stably expressing DsRed and EGFP–mCRY1 (WT or mutant) were analyzed by flow cytometry. The EGFP/DsRed ratio acts as a reporter for stability of the expressed WT or mutant mCRY1. The d1EGFP ($t_{1/2} = 1$ h) and d4EGFP ($t_{1/2} = 4$ h) represent the half-life markers. Our results show that the half-life of WT mCRY1 is similar to that of d1EGFP (**Figure 3B**, top). The S158D, T249D, and S280D mutations exhibited no obvious change compared to WT mCRY1 (**Figure 3B**, middle). Surprisingly, the S71D, S404D, Y266D, Y273D, Y432D, and S588D mutations displayed half-lives longer than that of WT mCRY1 (**Figure 3B**, bottom), despite variations in the rescued period length (**Figure 1G**).

The crystal structure of mCRY1 reveals that binding sites for mPER2 and FBXL3, which partially overlap, are involved in transcriptional repression and protein stability (19, 23). To determine whether mCRY1 phosphorylation affects interactions with mFBXL3 and PER2, we used a luciferase complementation assay to determine how mFBXL3 or PER2 interact with phosphomimetic mCRY1 mutants (**Figure 3C**). WT or mCRY1 mutants and mFBXL3 (or PER2) were co-expressed as fusion proteins with N- and C-terminal luciferase fragments in HEK293 cells (19). Formation of mCRY1-FBXL3 (or PER2) complexes produces functional luciferase and that can be recorded in luciferin-containing medium. Data showed that, to varying degrees, all of the mutations reduced mFBXL3 binding. In particular, the S71D, Y266D, Y273D, S404D, and Y432D mutations drastically reduced mFBXL3 binding to 4, 11, 23, 32, and 11%, respectively (**Figure 3C**, top). By contrast, mPER2 binding was unaffected by the S158D, T249D, S280D, S404D, and S588D mutations, while the S71D, Y266D, Y273D, and Y432D mutations weakened the

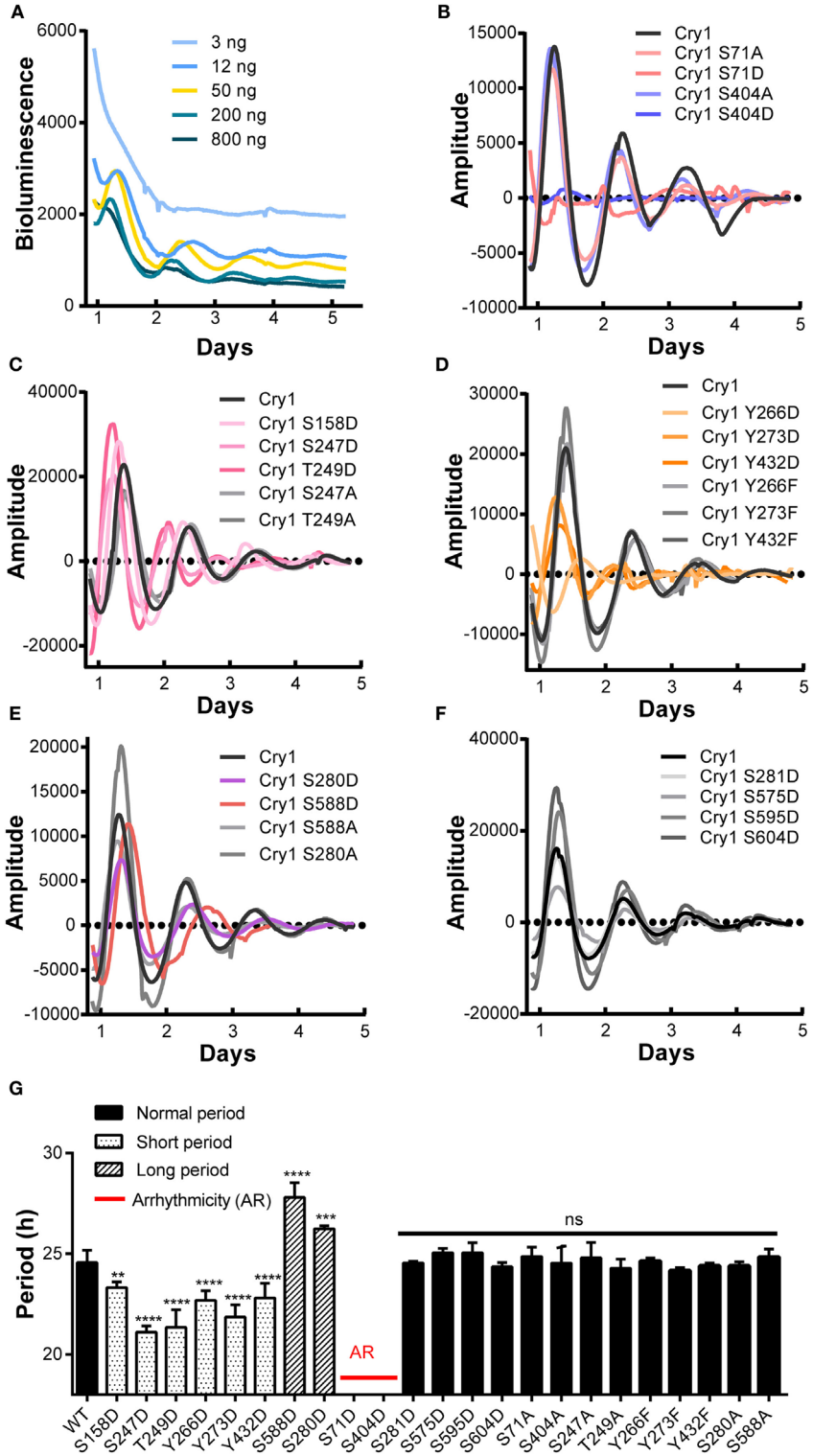

FIGURE 1 | A cell-based screen to identify critical phosphorylation residues on mCRY1 through rescuing rhythmicity in *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ fibroblasts (DKO cells). **(A)** Dosage-dependent rescue of circadian rhythms in DKO cells by mCRY1. The mCRY1 expression vector was cotransfected into cells with the P(*Per2*)-dLuc reporter vector. Three days after transfection, the cells were synchronized by dexamethasone treatment and then moved to luciferin-containing medium for 5–6 days of bioluminescence recording. **(B–F)** The P(*Per2*)-dLuc reporter rhythms (baseline subtracted) from DKO cells transfected with WT or mutant mCRY1, as noted in the legend. Experiments were performed as in **(A)**. **(G)** Quantitation of the period length from WT and mutant mCRY1 transfected cells that showed a distinct period phenotype as noted in the legend. Error bars represent SEM ($n \geq 5$, $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, ANOVA).

TABLE 1 | Phosphorylated residues of CRY1 (mouse, human, and rat) are written in red.

	Mouse		Human		Rat
S71-p	ANLRKLNsRLFVIRG	S71	ANLRKLNSRLFVIRG	S71	ANLRKLNSRLFVIRG
S158-p	KRFQTLVsKMEPLEM	S158	KRFQTLISKMEPLEI	S158	KRFQTLVSKMEPLEM
S247-p	NANSLLAsPtGLSPY	S247	NANSLLASPTGLSPY	S247	NANSLLASPTGLSPY
T249-p	NSLLAsPtGLSPYLR	T249	NSLLASPTGLSPYLR	T249	NSLLASPTGLSPYLR
Y266	CLSCRLFYFKLTDLY	Y266	CLSCRLFYFKLTDLY	Y266-p	CLSCRLFyFKLTDLy
Y273	YFKLTDLYKKVKKNs	Y273	YFKLTDLYKKVKKNS	Y273-p	yFKLTDLyKKVKKNS
S280-p	YKKVKKNssPPLSLY	S280	YKKVKKNSSPPLsLY	S280	yKKVKKNSSPPLSLY
S281-p	KKVKKNssPPLSLYG	S281	KKVKKNSSPPLsLYG	S281	KKVKKNSSPPLSLYG
S285	KNssPPLSLYGQLLW	S285-p	KNSSPPLsLYGQLLW	S285	KNSSPPLSLYGQLLW
Y432	NGDYIRRYLPVLRGF	Y432-p	NGDYIRRyLPVLRGF	Y432	NGDYIRRYLPVLRGF
S575-p	HSLKQGRsSAGTGLS	S555	HLLKQGRSSMGTGLS	S557	NPLKQGRSSMGTGLS
S588-p	LSSGKRPsQEEDAQs	S568-p	LSGGKRPsQEEDTQS	S570	LSSGKRPSQEEDAQS
S595-p	sQEEDAQsVGPKVQR	S575	sQEEDTQSIGPKVQR	S577	SQEEDAQSVGPKVQR
S604-p	GPKVQRQsSN_____	S584	GPKVQRQSTN_____	S586	GPKVQRQSSN_____

The resource is from PhosphoSitePlus database (21).

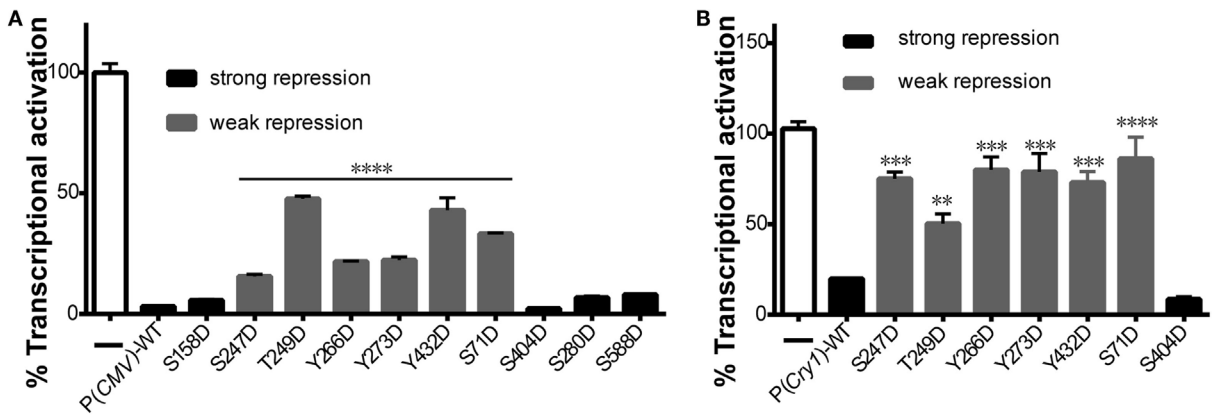

FIGURE 2 | Effects of mutant mCRY1 on BMAL1: CLOCK-induced transcriptional activation. **(A)** HEK293 cells were cotransfected with BMAL1 (10 ng plasmid), CLOCK (15 ng) expression plasmid, and Per2-dluc (10 ng) reporter with WT or mutant mCRY1 (5 ng) as noted. Twenty-four hours after transfection, the cells were changed to luciferin-containing medium for end-point bioluminescence recording. The raw data were normalized such that the reporter control without mCRY1 (WT or mutant) transfection was equal to 100%. Compared with WT mCRY1, the mutants exhibited two profiles: strong repression with similar repression activity to WT and weak repression with a significant reduction in repression activity. Mean and error bars (SEM) of three independent transfections are shown (****$p < 0.0001$, ANOVA). Two additional experiments gave similar results. **(B)** DKO cells were cotransfected with Per2-dluc (10 ng) reporter and WT or mutant mCRY1 (50 ng) as noted. Experiments and data analysis were done as in **(A)**. Compared with WT, the mutants exhibited two profiles: strong repression with similar repression activity as WT and weak repression with a significant difference. Mean and error bars (SEM) of three independent transfections are shown (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ANOVA). Two additional experiments gave similar results.

interactions with mPER2 to 4, 10, 43, and 18%, respectively (**Figure 3C**, bottom). We conclude that phosphomimetic mutations affect the stability and transcriptional repression activity of mCRY1 by antagonizing with mFBXL3 and PER2.

Effects of Phosphomimetic Mutation on mCRY1 Protein Subcellular Localization

The stability of CRY1 protein is regulated by two competing SCF E3 ligase complexes: FBXL3 mediates degradation in the nucleus, while FBXL21 protects CRY1 in the nucleus and facilitates degradation in the cytoplasm (13, 14). Therefore, we sought to determine whether phosphomimetic mutations alter the subcellular localization of mCRY1. To determine the subcellular distribution pattern of the mutants, we generated a GFP-tagged mCRY1 (WT or mutant) expression construct. Representative images of GFP-mCRY1 (WT or mutant), as detected by GFP fluorescence, are shown in **Figure 4A**. The ratio of cells with subcellular distribution and the colocalization of GFP-mCRY1 (WT or mutant) proteins with nuclei are shown in **Figures 4B,C**. In transient transfection assays using HEK293 cells, the mutants were predominantly localized in the nucleus and cytoplasm. However, 5–65% of S158D, S249D, S280D, or S404D-GFP were localized exclusively in the nucleus, similarly to WT, with a colocalization efficiency of more than 75%. In contrast, 6–42% of S71D, Y266D, Y273D, Y432D, or S588D-GFP were only observed in the cytoplasm, with a nuclear colocalization efficiency of less than 63%, especially S71D (~29%) (**Figures 4B,C**). Based on these data, we conclude that the phosphorylation of mCRY1 at amino acid sites S71, Y266, Y273, Y432, and S588 alter the subcellular localization that is critical for the rhythmicity of circadian clock.

Although the mutants S158D, S247D, and T249D showed similar periodical phenotypes with Y266D, Y273D, and Y432D,

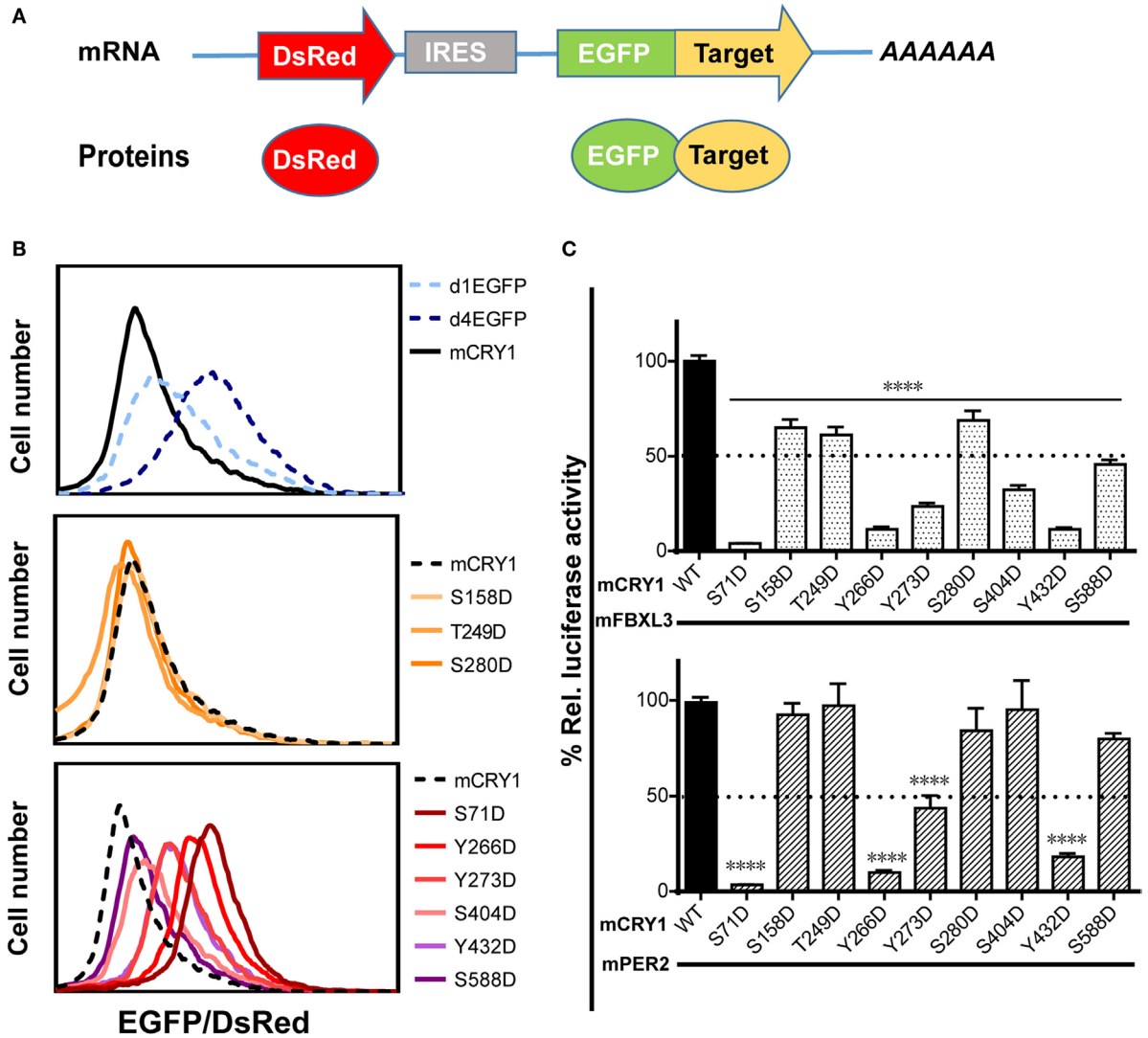

FIGURE 3 | Effects of phosphomimetic mutation on mCRY1 protein stability and interactions with FBXL3 and PER2. **(A)** Global protein stability (GPS) reporter system. The DsRed–IRES–EGFP target element was cloned into a lentiviral vector, and the fluorescent reporter proteins were co-expressed from a single mRNA *via* an internal ribosomal entry site (IRES). **(B)** HEK293 cells were infected with lentivirus of pLv–DsRed–IRES–EGFP–mCRY1 (WT or mutant) for 24 h, and then the fluorescent protein signals were analyzed by flow cytometry. The EGFP/DsRed ratio acts as a reporter for stability of the expressed WT or mutant mCRY1. The d1EGFP and d4EGFP are markers for 1- and 4-h half-lives, respectively. **(C)** Luciferase complementation assay. mCRY1 (WT or mutant) and FBXL3 (or PER2) were co-expressed as fusion proteins with luciferase fragments in HEK293 cells. Experiments were done as in **Figure 2A** and the data presented relative to mCRY1 (WT)-mFBXL3 (or mPER2). Mean and error bars (SEM) of three independent transfections are shown (****$p < 0.0001$, ANOVA). Two additional experiments gave similar results.

the subcellular location of the proteins had no obvious change, suggesting that the mechanisms were different. Indeed, the identified phosphorylated sites were located in different functional regions of mCRY1 (**Figure 4D**), supporting our conclusion that the phosphorylation of these sites with different repression activity, protein stability, and subcellular location results in various periodical phenotypes by distinct mechanisms.

DISCUSSION

In the basic TTFL model, both positive and negative regulatory elements are important for generating the autoregulatory feedback loop. Post-translational regulation of the activity, degradation, and localization of these regulators, most notably phosphorylation influences the circadian rhythms (4, 25). Although many CRY1phosphorylation sites have been identified, their contribution to clock function was unclear. We conducted a cell-based screen in CRY-deficient (DKO) cells and identified 10 phosphomimetic mutants of mCRY1 that induce abnormal circadian periods, including long period (S280D and S588D), short period (S158D, S247D, T249D, Y266D, Y273D, and Y432D), and even arrhythmicity (S71D and S404D). The period length of the circadian clock in cells is complicated because many genes participate in regulating the circadian period (11, 26–28).

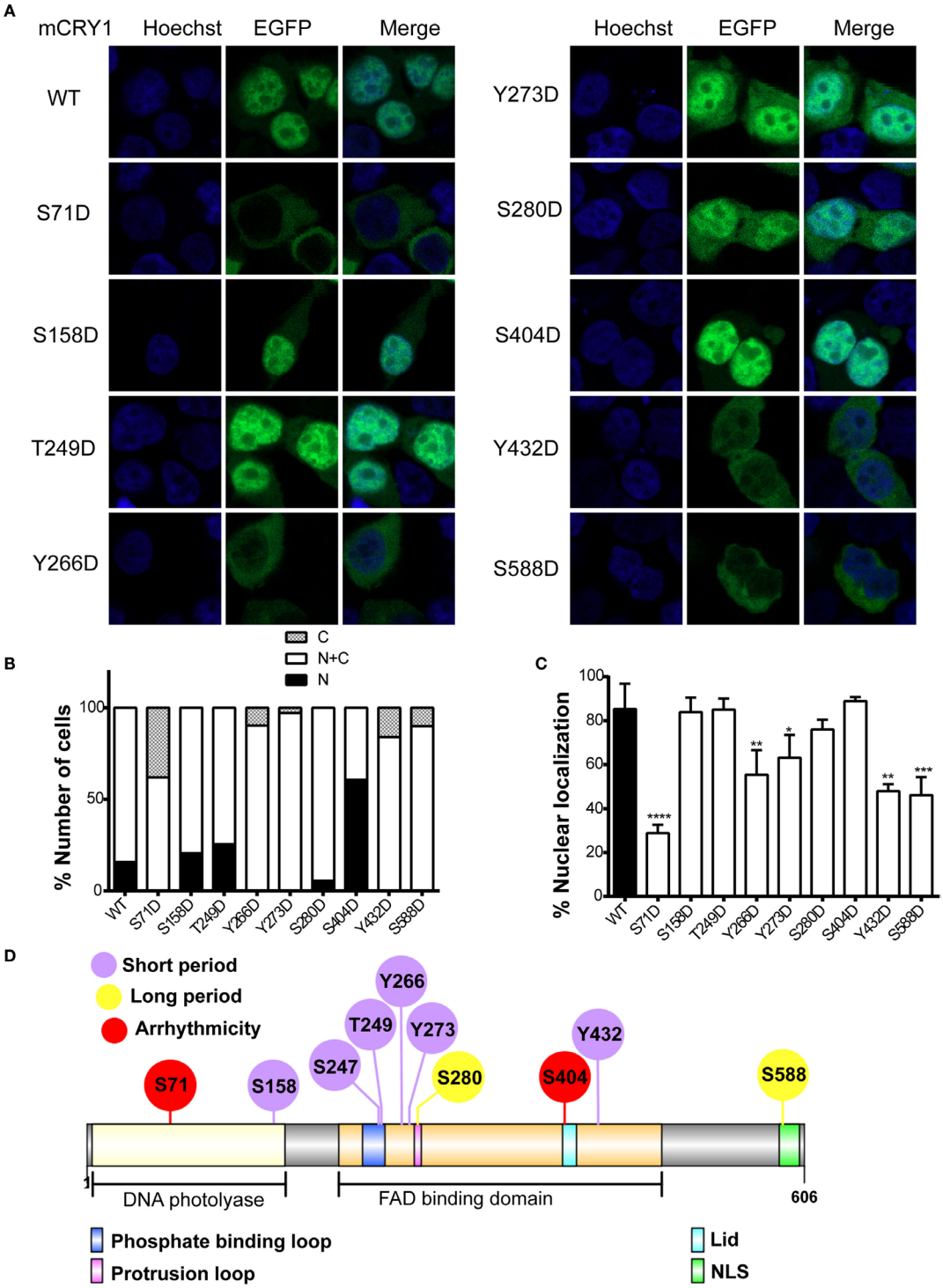

FIGURE 4 | **Effects of phosphomimetic mutation on subcellular localization of mCRY1 protein**. GFP-tagged mCRY1 (WT or mutant) proteins were transiently overexpressed in HEK293 cells, and the subcellular distribution pattern of mCRY1 protein was analyzed. **(A)** Representative images of GFP-mCRY1 (WT or mutant) were detected by GFP fluorescence (green), and the nuclei were stained with Hoechst (blue). **(B)** Percentage of cells showing nuclear (N), nuclear–cytoplasmic (N + C), and cytoplasmic (C) staining as indicated in the plots. The ratio of cells with subcellular localization to the total transfected cells was analyzed by counting 100 cells three times in each experiment. **(C)** Percentage of colocalization of GFP-mCRY1 (WT or mutant) with nuclei. The 50 GFP-mCRY1 (WT or mutant)-expressing cells were analyzed by Image J software (Version 1.37c, NIH, USA). Mean and error bars (SEM) are shown ($n = 3$ for each experiment). Two additional experiments gave similar results (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, ANOVA). **(D)** Location of the important phosphorylated sites on the liner protein functional regions of mCRY1. The FAD binding domain, which contains phosphate binding loop, protrusion loop, C-terminal lid (Lid), and nuclear localization signal (NLS), has been indicated in this schematic diagram. The diagram was constructed using Illustrator for Biological Sequences (IBS) software (24).

TABLE 2 | Phosphorylation regulating the ratio of intracellular mCRY1 determines the circadian period length by different mechanisms.

Phosphomimetic mutants	Period phenotype	Repression activity	Protein stability	Nuclear colocalization	Potential mechanism
S71D	AR	↓	↑	↓	The mutant cannot enter the nucleus, as the binding to PER2 is too weak
S404D		↑	↑	N	The degradation is weak, as the binding to FBXL3 is weak
S158D	S	N	N	N	Introduction of negative charge to the surface region alters the interaction with other proteins
S247D		↓	—	—	The mutants enforce the phosphate-binding conformation to tune FAD, ultimately leading to change in mCRY1 protein function
T249D		↓	N	N	
Y266D		↓	↑	↓	Lower nuclear localization efficiency may be due to weak interaction with FBXL3 and PER2
Y273D		↓	↑	↓	
Y432D		↓	↑	↓	
S280D	L	N	N	N	The binding to FBXL3 is weak, as S280 is located in protrusion loop, which interacts with FBXL3 and constricts access to FAD
S588D		N	↑	↓	The ratio of nuclear protein is decreased. This may be due to S588 nearing the NLS sequence

AR, arrhythmicity; S, short period; L, long period; ↑, increase; ↓, decrease; N, no obvious change; —, no result.

In this study, we identified mutations that alter repression activity, protein stability, and cellular localization, suggesting that distinct mechanisms regulate each phenotype.

Previously, we have demonstrated that the proper ratio of intercellular CRY proteins determines the normal clock period length (17). In this study, we further determined that the ratio of functional CRY1 protein is regulated by phosphorylation in cells and the ratio imbalance disrupts circadian rhythmicity, although the mechanisms are different (**Table 2**). The S280D mutation displayed a long period, similar to that of S588D [previously reported in Ref. (12)]. We found that phosphorylation at S588 decreased nuclear protein localization and weakened interactions with FBXL3, increasing the protein stability. This may be due to the position of the S588 residue, which is near the NLS sequence of the C-terminal tail of mCRY1. The crystal structure of mCRY1 (19) shows that FBXL3, but not PER2, binds across the protrusion loop (S280) and the phosphate-binding loop (S247 and T249) (**Figure 4D**). Consistently, the S280D and T249D mutations reduced FBXL3 binding but did not affect PER2 binding (**Figure 3C**). In addition, the phosphate-binding and protrusion loops, with conformational flexibility, constrict the approach to FAD, which is critical to CRY1's functions. Phospho-Ser-mimicking mutations at this region may enforce the phosphate-binding conformation to tune FAD, ultimately leading to a change in mCRY1 protein function (8). Introduction of negative charge (Asp) to site S158, located in the surface region (19), may restructure and/or disorder the structural conformation between the phosphorylation site and nearby amino acid residues, affecting mCRY1's clock function. The Y266D, Y273D, and Y432D mutations displayed similar phenotype (short period, **Figure 1D**), weaker interactions with FBXL3 and PER2, and lower nuclear colocalization efficiency compared with WT-mCRY1. Interestingly, although the S71D mutant and S404D mutant are arrhythmic, their molecular character is very different. The S71D mutant displayed almost no interactions with FBXL3 and PER2 and high colocalization efficiency with the cytoplasm. Regardless of a subtle difference with previous reports (11), in which the S71D mutation increased interaction with FBXL3, phosphorylation at S71 is crucial for regulating circadian period. In addition, phosphorylation at these sites weakens the binding with PER2, slowing the rate of nuclear translocation and decreasing the concentration of functional protein (3, 29). Nuclear transport of the PER/CRY complex is reported to be one of the most important mechanisms for period regulation, as shown in the recent report on nuclear importin KPNB1 (30). In contrast, the phosphomimetic mutation of S404, located in the C-terminal lid, did not rescue DKO cells with hyper-repression activity (**Figures 2A,B**) and hypo-interactions with FBXL3 increasing protein stability. Neither did it affect binding to PER2 nor nuclear colocalization. We hypothesized that the S404D mutant binding to FBXL3 was very weak, thus slowing degradation, prolonging interactions with the BMAL1/CLOCK complex, and ultimately preventing the relief of inhibition and initiation of the next circadian cycle (31).

In summary, we identified critical CRY1 sites where mutations disrupted circadian rhythmicity. Although some enzymes correspond to specific modifications in mCRY1, such as MAPK at S247 and AMPK at S71 and S280 (9, 11), the enzymes that modify the other sites are unknown. In addition, the effect of modifiers on rhythmicity varies after blocking (28), but how the enzymes work on the circadian clock components remains unclear. Our data indicate that key modifiers of CRY1 directly regulate the ratio of functional CRY1 protein by distinct mechanisms that determine the circadian rhythmicity, providing new insights on regulation of the circadian period.

AUTHOR CONTRIBUTIONS

NL and EZ conceived the study, designed the experiments, and analyzed the data. NL performed the experiments. NL and EZ wrote the manuscript.

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REVIEW
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Clock-Enhancing Small Molecules and Potential Applications in Chronic Diseases and Aging

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Normal physiological functions require a robust biological timer called the circadian clock. When clocks are dysregulated, misaligned, or dampened, pathological consequences ensue, leading to chronic diseases and accelerated aging. An emerging research area is the development of clock-targeting compounds that may serve as drug candidates to correct dysregulated rhythms and hence mitigate disease symptoms and age-related decline. In this review, we first present a concise view of the circadian oscillator, physiological networks, and regulatory mechanisms of circadian amplitude. Given a close association of circadian amplitude dampening and disease progression, clock-enhancing small molecules (CEMs) are of particular interest as candidate chronotherapeutics. A recent proof-of-principle study illustrated that the natural polymethoxylated flavonoid nobiletin directly targets the circadian oscillator and elicits robust metabolic improvements in mice. We describe mood disorders and aging as potential therapeutic targets of CEMs. Future studies of CEMs will shed important insight into the regulation and disease relevance of circadian clocks.

Keywords: circadian clock, small molecules, amplitude, metabolic disease, mood disorder, aging

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INTRODUCTION

The circadian clock is an intrinsic biological timing device operative in evolutionarily divergent species, ranging from microorganisms to human (1, 2). The clock drives daily oscillations of important molecular and physiological processes to anticipate and respond to the changing environment imposed by the rotation of the Earth. Consistent with its adaptive function, normal clock functions are required for organisms to survive and thrive. Coculture of cyanobacteria with varying period lengths demonstrated competitive growth advantage when inherent periodicity aligned with external light/dark rhythms (3), in accordance with findings from plant experiments (4). Likewise, circadian patterns of foraging and predator avoidance are well documented for animals in their natural habitats. For example, chipmunks whose central pacemaker, the hypothalamic suprachiasmatic nuclei (SCN), had been surgically removed suffered significantly higher mortality rate in the wild than those with fully functional clocks (5). The clock has also been postulated to protect early eukaryotes from irradiation during the day (6, 7). Despite the lack of acute lethality from genetic disruption of clock genes in laboratory animals, there exists a strong correlation, and in some cases causative relationship, between malfunctioning clocks and chronic diseases as well as aging (8, 9).

As we extend the list of clock-associated pathologies and probe for greater mechanistic understanding, the outstanding question remains whether and how to target the clock to combat disease and physiological decline (10–12). Except in the case of jet-lag, targeting the clock for health benefits will likely entail chronic intervention and gradual and systemic improvement of phenotypes and symptoms. Here, we highlight clock-associated metabolic disease, mood disorder, and aging as clock-associated processes characterized by dampened amplitude of circadian oscillation (13). Small-molecule enhancers of the circadian clock may strengthen the clock and clock-driven gene expression and physiology, retarding pathological deterioration. While this review will mainly focus on circadian amplitude enhancement, clock modulators capable of circadian phase and/or period modulation can show clinical utility in diseases states that are accompanied by circadian phase misalignment or abnormal periodicity (10, 14).

MAMMALIAN CIRCADIAN CLOCK

In the canonical mammalian clock, the molecular oscillator is the functional unit present in every cell of the body (15, 16). Comprised of interlocked feedback loops (**Figure 1**), molecular oscillators in individual tissues coordinate to govern highly tissue-specific expression programs of clock-controlled genes (CCGs). While 43% of genes have been shown to oscillate in at least one tissue in mice (17), indicating prevalent circadian gene regulation, the overlap of CCGs between tissues was found to be approximately 10% (18). At the system level, various tissue clocks are orchestrated by the SCN master pacemaker, a pair of neuron clusters bilaterally located in the anterior of the hypothalamus (19). The SCN displays tight coupling among its neurons (20) and functions to respond to photic signals to synchronize tissue and cellular clocks throughout the body *via* neural and hormonal signals.

The molecular oscillator is composed of intersecting negative feedback loops to drive ~24-h gene expression rhythms (1). In the core loop, the positive arm consists of three bHLH-PAS transcription factors, including paralogous CLOCK/NPAS2 and their heterodimeric partner BMAL1 (**Figure 1**). CLOCK or NPAS2 each interacts with BMAL1 through the PAS and bHLH domains. After dimerization, CLOCK/BMAL1 and NPAS2/BMAL1 activate expression of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes *via* E-box promoter elements. PER and CRY proteins themselves heterodimerize and translocate into the nucleus to inhibit transcriptional activities of CLOCK/BMAL1 and hence their own transcription. CRYs belong to the photosensing photolyase protein family that functions in DNA damage repair in bacteria and in circadian photic entrainment in flies (21). However, the mammalian CRY proteins appear to have lost the photosensing ability yet acquired function as the major transcriptional repressor in the circadian core loop. Crystal structure studies showed that CLOCK and BMAL1 interact *via* bHLH and two PAS domains in an asymmetrical fashion, characterized by a β-sheet/α-helix interaction involving respective BMAL1 and CLOCK PAS-B domains (22). On the β-sheet surface of CLOCK PAS-B, mutagenesis screen identified several residues whose mutations attenuated CRY inhibition of CLOCK/BMAL1 transactivation, suggesting CLOCK/CRY interactions. Several crystal structures of CRY proteins have been reported. The FAD-binding domain of CRY proteins appears to be a key nodal point recognized by both a CRY-stabilizing small molecule (23) or an CRY-degrading E3 ligase (24), and that PER binding to CRY precludes access for the E3 ligase FBXL3 (25), thus stabilizing CRY. Future structural studies of core clock complex formation on promoter DNA will advance our understanding of circadian oscillator function.

Several other feedback loops have been shown to stabilize and/or modulate the core feedback loop (**Figure 1**). In the principal stabilization loop, CLOCK/BMAL1 and NPAS2/BMAL1 activate highly cyclic expression of genes encoding the nuclear hormone receptors REV-ERBα/β (26). REV-ERBs and their antagonistic receptors RORα/β/γ compete for binding to shared consensus elements (RORE and RevDR2) on the promoter of

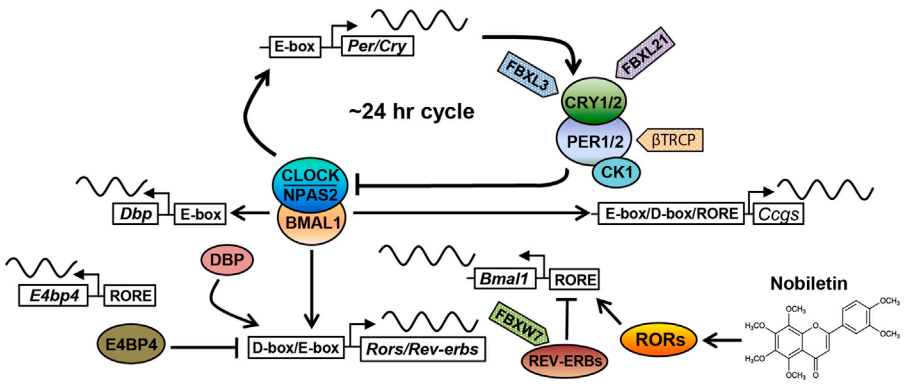

FIGURE 1 | The core circadian oscillator and regulatory molecules. The circadian clock oscillator is comprised of a network of transcriptional–translational feedback loops including the core loop (BMAL1/CLOCK/NPAS2 and PERs/CRYs), the stabilization loop (BMAL1/CLOCK, REV-ERBs, and RORs), and the auxiliary loop (DBP, E4BP4, REV-ERBs, and RORs). Various protein regulators (F-box-containing E3 ligases are shown as examples) and small-molecule modulators (nobiletin is shown) have been identified to target core clock components, regulating circadian periodicity and amplitude. See the main text for details.

Bmal1/*Npas2* and other target genes throughout the genome to promote robust oscillatory gene expression (27–29). In another auxiliary loop (30), CLOCK/BMAL1 activates expression of genes encoding the PAR-bZip transcription factor DBP (D-box binding protein), which in turn drives *Ror* gene expression *via* their D-box promoter elements. In addition to *Bmal1*, REV-ERBs and RORs also govern the expression of the *Nfil3* gene, which encodes a transcriptional suppressor (also known as E4BP4) that binds to the D-box to antagonize DBP transcriptional activity. Apart from these transcriptional feedback loops, other feedback mechanisms are also important, including a post-translational loop involving the NAD+-dependent sirtuin (SIRT) 1 deacetylase (31). CLOCK/BMAL1 activates the *Nampt* gene, which encodes the rate-limiting enzyme for NAD+ biosynthesis. The NAD+ level directly correlates with SIRT1 activity, which directly target core clock proteins including BMAL1 and PER2 (32, 33).

Degradation of core clock components has emerged as a key regulatory mode for circadian functions. Casein kinase 1 has been shown to phosphorylate PERs, thereby facilitating their proteasomal degradation by the F-box proteins β–TRCP1/2 (34). Likewise, the AMPK kinase phosphorylates CRYs to promote CRY degradation (35), mainly mediated by the F-box protein FBXL3 (36–38). FBXL21, a close homolog of FBXL3, was found to antagonize FBXL3 to decelerate CRY degradation in the nucleus, on the other hand, also accelerate CRY turnover in the cytoplasm (39, 40). Mice harboring hypomorphic mutations in *Fbxl3* and *Fbxl21* showed opposite effects on circadian period length, highlighting an important circadian function for ubiquitin-mediated proteasomal degradation. Autophagy is another major protein degradation mechanism, involving lysosomal degradation of protein cargo delivered *via* autophagosome (41). It was recently found that BMAL1 undergoes dual degradation by proteasome- and autophagosome-dependent pathways, and attenuation of both in *Clock*Δ*19*/+ heterozygous mice improves glucose homeostasis (42). Overall, the circadian clock system is regulated by an exceedingly complex array of molecular mechanisms encompassing all levels of gene expression, together ensuring temporal precision (~24 h) and oscillatory robustness (see below).

CIRCADIAN AMPLITUDE REGULATION

Amplitude denotes the robustness of circadian oscillation, measured by the difference between peak and trough of the circadian cycle. Whereas dampened circadian amplitude has been shown to closely correlate with chronic diseases and aging (10, 12, 43), the molecular and physiological mechanisms underlying circadian amplitude regulation are not well understood. Within the core oscillator, multiple lines of evidence indicated the importance of balancing positive vs. negative activities. For example, in mouse MEF cells, CLOCK/BMAL1 (positive factors) are in higher abundance than PER/CRY (the negative arm); as a result, overexpressing PER and CRY, but not CLOCK or BMAL1, strongly enhanced circadian amplitude (44). Such functional balance is further illustrated by the antagonistic transcriptional function of REV-ERBs and RORs in the secondary loop. Whereas ROR levels cycle only weakly, REV-ERB mRNA and protein levels are highly oscillatory. By directly competing for binding to promoter elements, they together govern a significant fraction of genome-wide circadian gene expression (29, 45). The clock is inherently a self-limiting, rhythmic machinery, namely, a limit cycle. Maintaining the "Yin–Yang" balance may lead to sustained oscillation, whereas brute force beyond a homeostatic range will dampen the overall amplitude of the following cycles. In other studies, CLOCK overexpression was found to enhance amplitude (46, 47), yet it remains unclear whether the primary mechanism involved is simply the greater level and activity of the positive transcription factor or an optimized functional balance.

More recent studies have provided insight into the functional complexity and dexterity of core clock components in amplitude regulation. In one study, REV-ERBα was found to be phosphorylated by cyclin-dependent kinase 1 (CDK1) at T275, a site not conserved in REV-ERBβ (48). Phosphorylated REV-ERBα was subsequently recognized by the F-box protein FBXW7 for proteasome degradation. Knockdown of CDK1 or FBXW7 reduced the amplitude of a circadian reporter in a dose-dependent manner, suggesting this REV-ERBα degradation pathway plays an important role in circadian amplitude. Another study described a "facilitated recruitment" mechanism where REV-ERBs are recruited to open chromatin following a rate-limiting step mediated by ROR/BMAL1 and transcription cofactors SRC-2/PBAF (49). It was posited that recruitment of the REV-ERB repressors by the activators ROR/BMAL1 ensures efficient and timely transcriptional shutdown, resulting in robust amplitude in target gene expression.

At intercellular and physiological/behavioral levels, oscillator coupling is of paramount importance to maintaining robust oscillation (50). The SCN rhythm is known to be exceptionally refractory to genetic perturbation compared with peripheral cells due to the tight coupling between SCN neurons (20). For example, several clock genes, including *Per1* and *Cry1*, are required for sustained PER2:LUC reporter rhythms in dissociated fibroblast cells and SCN neurons. At the tissue level, whereas lung explants remained arrhythmic, SCN slices showed robust cycling of the PER2:LUC reporter. In accordance, *Per1*-null mice displayed clear rhythmic locomotor behavior, albeit with a short period length (51). These studies together indicate that intercellular synchronization between SCN neurons, likely involving vasoactive intestinal polypeptide (VIP) (48), strengthens system amplitude. Such coupling-induced rhythm stabilization can also be observed in peripheral cells, where single-cell reporter rhythms were less robust or stable compared with those in tissue slices (16, 52). Besides genetic perturbation, intercellular coupling can also confer protection against pharmacological disturbance and stochastic noise (53). Reciprocally, intercellular coupling can also facilitate noise-generated stochastic rhythm. While dispersed SCN neurons from *Bmal1*−/− mice showed no circadian rhythmicity, *Bmal1*−/− SCN slices displayed shorter and highly variable circadian rhythms (54). Such unstable rhythms were shown to be abolished by tetrodotoxin-induced uncoupling in the SCN slices, further indicating that intercellular coupling augments rhythmic stability and robustness.

CLOCK-ENHANCING SMALL MOLECULES (CEMs) AND EFFICACIES IN METABOLIC DISEASE MODELS

More than half of top-selling drugs act on protein targets encoded by cyclically expressed genes (17), and xenobiotic metabolism is subjected to circadian regulation (55). These findings indicate a close circadian regulation of pharmacodynamics and pharmacokinetics (56–58). On the other hand, rather than aligning the timing of chronotherapy with intrinsic rhythms, a distinct strategy is to manipulate the clock or clock components to alleviate clock-regulated disease symptoms (10–12, 14). Behavioral or dietary manipulations have been shown to modulate circadian rhythms, such as light exposure (59–61), exercise (62) as well as feeding/fasting regimens (63). For example, a series of studies have shown that time-restricted feeding (TRF) can improve sleep and metabolic homeostasis and delay cardiac aging in *Drosophila* (13, 64) At the molecular level, TRF activates genes involved in circadian rhythms and mitochondrial electron transport chain complexes. Similarly, timed caloric restriction (CR) led to highly consolidated food intake, which enhanced the expression and amplitude of core clock genes and improved lipid homeostasis, eventually contributing to life span extension (63, 65). Finally, bright light and melatonin, both major circadian synchronizers that strengthen rhythms, have been shown to improve cognition and mood in the elderly (66). These studies exemplify the beneficial effects of enhancing the molecular and physiological rhythms on physiology and behavior.

Various chemical compounds capable of manipulating clocks have been discovered *via* either unbiased phenotypic screens or targeted approaches focusing on particular clock components (67–72). As described above, the clock is a self-limiting machine with a myriad of check-and-balance mechanisms governing its periodicity and robustness. Excessive functional manipulation, either stimulatory or inhibitory, of a specific clock protein may compromise the inherent balance within the clock, eventually diminishing or even abrogating the intended effects. Therefore, when searching for small molecules capable of enhancing circadian robustness, it is important to evaluate the sustained effects on reporter rhythms rather than assaying only the molecular function of individual clock components. Below, we describe our recent efforts to utilize phenotypic screening to identify chemical modifiers that enhance circadian amplitude.

In two separate screens using cell-based phenotypic assays, we reported a group of clock amplitude-enhancing small molecules dubbed CEMs. The first screen of 200,000, largely synthetic, compounds identified 4 CEMs that potentiated cellular and tissue reporter rhythms in both WT and *Clock*Δ*19*/+ heterozygous mutant backgrounds (73). In contrast to *Clock*Δ*19*/+ heterozygous cells that displayed attenuated but sustained circadian rhythms, *Clock*Δ*19*/Δ*19* homozygous or *Bmal1*-null cells where the oscillators are essentially broken were refractory to CEM (14). CEM3, a benzimidazole compound, was uniquely able to further potentiate the robust reporter rhythms of the SCN pacemaker. In a second, smaller screen, a natural flavonoid compound called nobiletin (NOB) was identified as a novel CEM, along with its close analog tangeretin (74). NOB showed strong enhancing activities in circadian reporter cells, with an EC50 in the low micromolar range. NOB is a major polymethoxylated flavone found in citrus peels and exhibits a favorable pharmacokinetic profile devoid of significant toxicity (75). Previous studies have reported diverse biological activities against metabolic syndrome, oxidative stress, inflammation, and cancer (76–80); however, its molecular mechanism of action and direct protein targets were unknown.

A potential metabolic efficacy of NOB is intriguing and provides a focal point of connecting circadian manipulation and metabolic fitness. Previous research has established a regulatory role of the circadian clock in metabolic homeostasis (31). For example, the *Clock*Δ*19*/Δ*19* mutant mice showed a broad array of metabolic dysfunctions, including blunted feeding rhythms, hyperphagia, exaggerated obesity risk under high-fat diet (HFD) feeding or at older ages, elevated blood glucose levels and hypoinsulinemia (81). Reciprocally, metabolism and/or nutrition also modulate our internal clocks (82, 83). For example, under *ad libitum* HFD feeding, mice showed a slight increase in the free-running period length (~23.8 h) compared with regular chow-fed animals (~23.6 h), and importantly a marked decrease in amplitude of circadian rhythms, including both clock gene oscillation in the periphery and feeding rhythms (82, 84). Both examples showed a correlation of circadian amplitude reduction and metabolic dysfunction, consistent with human studies where blunted insulin secretion rhythm associates with increased risk for diabetes (85).

We therefore examined the efficacy of NOB in two mouse metabolic disease models, namely the HFD-induced obese mice and *db/db* diabetic mice. Metabolic characterization illustrated that NOB effectively mitigated body weight gain without altering food intake, stimulated energy expenditure (EE) and circadian activity, enhanced glucose and insulin tolerance, and diminished lipid content in circulation and in liver (74). The alleviated liver steatosis phenotype was accompanied by restored oscillation of core clock components in mouse liver. In addition to energy homeostasis, NOB was also found to reduce serum ammonia levels in different diets and appeared to enhance urea cycle gene expression and function under HFD feeding (86). *Clock*Δ*19*/Δ*19* homozygous mutant mice showed no or much diminished response to NOB, indicating clock requirement for NOB effects. Microarray analysis using mouse liver showed extensive remodeling of energy metabolic pathways including lipid metabolism and mitochondrial respiration. Together, these findings support the notion that clock enhancement by NOB contributes to metabolic improvement (87).

Importantly, NOB was found to directly activate ROR receptors *via* filter binding and functional studies including mammalian one-hybrid assays (74). This key finding highlights the role of RORs in circadian amplitude regulation and also sheds important insight on the functional complexity of NOB and ROR. First, despite the robust affinity of NOB–ROR interaction, the activation of ROR target genes, including core clock genes (e.g., *Bmal1*) and downstream output genes, was generally moderate (74). This observation is consistent with the limit cycle nature of the clock where the balance between positive and negative limbs is paramount to the overall amplitude. Second,

a large number of ROR inverse agonists and REV-ERB agonists have been identified (71, 88). Despite opposite molecular functions relative to NOB as an ROR agonist, several of these compounds have been shown to improve energy metabolism in metabolic disease models (89, 90). This apparent paradox illustrates a potential functional dexterity of ROR (and also REV-ERB). It is possible that specific ligands, either agonists or antagonists, of ROR/REV-ERB can promote metabolic health, likely *via* distinct compound-specific mechanisms. A recent study (91) showed that three antagonists of RORγt employed divergent molecular mechanisms to affect its promoter binding and target gene expression and exhibited different degrees of mimicry with genetic RORγt disruption. These studies highlight the importance of in-depth mechanistic understanding of CEMs in circadian rhythms and downstream physiology.

MOOD DISORDERS AND AGING AS POTENTIAL PATHOPHYSIOLOGICAL TARGETS OF CEMs

Below we highlight two potential targets of CEMs, namely mood disorders and aging, where accumulating evidence indicates a strong correlation between pathophysiology and clock amplitude decline.

Mood Disorders

Mood disorders and circadian dysfunction are closely associated. Various manifestations of major mood disorders such as major depressive disorder, bipolar disorder, and seasonal affective disorder (BPD and SAD, respectively) exhibit diurnal rhythms, with the most severe symptoms typically occurring in the morning or around sunset (92, 93). In an early study comparing depressed, recovered, and healthy subjects, the depressed group exhibited blunted circadian rhythms, with a significant correlation to scores on depression severity (94). Recovered participants following 3 weeks of antidepressant treatment showed restored circadian amplitude, suggesting that depression is closely linked to circadian rhythmicity. In SAD patients suffering from depression during winter months with shorter daytime (95), circadian rhythms in feeding, sleep, body temperature, cortisol, and melatonin release, neurotransmitter (serotonin, norepinephrine, and dopamine) have been shown to be disturbed or dampened (96, 97). Another mood disorder is Sundowning syndrome, also referred to as "nocturnal delirium" (93). Sundowning syndrome is characterized by a worsening of behavior (i.e., aggression, restlessness, delirium, and agitation) in the late afternoon or early evening, particularly in the elderly population suffering from dementia. Clinical and preclinical data suggest that disturbances in sleep, environmental entrainment cues, and the SCN pacemaker all contribute to Sundowning syndrome (93). Specifically, sleep disruptions including impaired NREM sleep consolidation, sleep fragmentation, daytime sleeping, and reduced sleep efficiency are common among both the elderly and demented (98), and circadian amplitude disturbances manifested as sleep disruptions listed above can contribute to mood imbalance (99).

Mouse studies have begun to supply evidence for a possible causal relationship between clock function and mood. For example, behavioral assays using the *Clock*Δ*19*/Δ*19* mice revealed manic-like behaviors similar to human bipolar mania (100), including hyperactivity, decreased sleep, hyperhedonia, and an increased preference for cocaine use. Disrupted circadian rhythms are also commonly found in human mania (94). More recently, the subcapsular cell hyperplasia associated with adrenal tissue remodeling was reported to enhance circadian amplitude of glucocorticoid rhythm, but not the total glucocorticoid levels (101). Interestingly, the enhanced stress hormone rhythm promotes anxiolytic function. It was postulated that the high-amplitude oscillation of the anxiogenic glucocorticoid, the descending phase in particular, endows a robust anxiolytic response to regulate mood balance.

Consistent with a close relationship between clock disruption and mood disorders, various treatment options are known to manipulate or enhance circadian and/or sleep cycles. Among the environmental therapies are bright light therapy, social rhythm therapy (SRT), and sleep deprivation. Bright light therapy is the treatment of choice for SAD and has also been applied to depression, bipolar disorder, and sleep–wake cycle disturbances (102). Bright light in the morning serves to advance the circadian phase to correct the phase delays commonly seen in SAD patients and may also function as a strong photic zeitgeber to improve daily rhythms. Likewise, SRT (103) entails social zeitgebers such as routine daily tasks to restore stability of biological rhythms in depression patients. Finally, a total sleep deprivation paradigm has also been developed to temporarily alleviate SAD symptoms. Its biological basis is not well understood, although it has been shown to impact neurotransmitter function and rapidly reset behavioral and circadian rhythms (104). Therefore, behavioral and environmental cues employed in these therapies reset and potentiate circadian rhythms, mainly at the behavioral levels, to counter the debilitating depressive tendency.

Various pharmacological agents have been used in mood disorders, including antidepressants, antimanic or mood-stabilizing drugs, and antipsychotics (**Table 1**). Lithium is a mood-stabilizing drug that has been used to treat bipolar disorder for more than 50 years. In addition to its mood-stabilizing effects, lithium has been reported to lengthen the free-running circadian period in mammals including hamsters and mice (105, 106). A potentially important target of lithium is GSK-3β (107), a kinase broadly acting in various signaling pathways. GSK-3β was previously shown to phosphorylate and stabilize REV-ERBα, and lithium treatment accelerated proteasomal degradation of REV-ERBα (108). More recently, lithium was found to activate *Per2* gene expression and enhance the circadian reporter amplitude in both SCN and periphery (106). Another pharmacological treatment that affects the circadian system is valproic acid or valproate. Valproate is traditionally an anti-epileptic drug but has been repurposed as a mood-stabilizing drug. Valproate has been shown to alter circadian period (109) and acute valproate treatment of PER2:LUC bioluminescence experiments in skin fibroblasts yielded amplitude enhancement and induced phase-shifts, depending on the relative level of PER2:LUC protein

expression (110). Previous mouse studies have also suggested antidepressive functions of NOB (111, 112) (**Table 2**). For example, NOB was found to improve mouse performance in forced swimming test and tail suspension tests, while pretreatments with drugs targeting monoaminergic systems disrupted the NOB effects (112). It will be interesting for future studies to investigate a role of circadian clocks in these NOB efficacies.

Aging

Gradual decline in metabolic, physiological, and behavioral functions with age leads to increasing risk of chronic disease and mortality (130). One physiological basis for such system-wide deterioration is age-related circadian attenuation (13, 43). Various clock-regulated physiological and behavioral processes are known to display reduced amplitude with age (43, 61, 131). For example, aging correlates with impaired rhythms in SCN firing rate, hormone secretion (e.g., cortisol and melatonin), and body temperature (132). Sleep fragmentation, characterized by multiple short periods of sleep episodes throughout the normal sleep phase and also sleep during the normal active phase, indicates amplitude dampening of the sleep/wake cycle and constitutes a well-documented characteristic of aging and various age-related diseases including Alzheimer's disease (133). At the molecular level, there is also broad dysregulation of

TABLE 1 | Pharmacological treatments for mood disorders targeting the circadian system.

Drug name	Therapeutic effect	Circadian target(s)	Circadian-related effect(s)	Reference
Lithium	Mood stabilizer	GSK-3β	Lengthened circadian period; enhanced PER2 protein expression; and oscillatory amplitude	(105, 106)
Valproate	Mood stabilizer	Dopamine-mediated, possibly PER2	Shortened circadian period of behavioral rhythms in DAT-KD mice and rhythms in suprachiasmatic nuclei explants from PER2:LUC mice	(109)
Quetiapine	Mood stabilizer; adjunctive antidepressant; antipsychotic	Per1/2, Bmal1	Enhanced Per1/2 mRNA at different ZTs in the mouse amygdala	(113)
Carbamazepine	Mood-stabilizer	Undetermined	Shortened length of locomotor activity; stabilized running activity	(114)
Fluoxetine	Antidepressant	Per2/3, Cry2, GSK-3β	Altered circadian period; enhanced hippocampal clock gene expression; altered phase re-entrainment	(115–117)
Agomelatine	Antidepressant	MT1/2 receptors	Accelerated resynchronization of circadian rhythms; improved rest–activity cycle more than common antidepressant; entrained circadian rhythms; induced phase-shifts	(118–123)
Ramelteon	Antidepressant	MT1/2 receptors	Phase advance	(124)
Tasimelteon	Antidepressant	MT1/2 receptors	Phase advance/delay	(125)

TABLE 2 | Antidepressive and neuroprotective roles of nobiletin.

Species	Treatment duration	Effect	Cellular effects	Reference
Mouse (despair model *via* FST and TST)	60 min prior to assay	Antidepressant	Monoamine upregulation	(112)
Mouse	11 days	Antidepressant; improved memory impairment	Activated ERK/MAP kinase-dependent signaling and increased CREB phosphorylation	(111)
Mouse AD (APP-SL 7-5 Tg mice)	4 months	Reduced Aβ plaque pathology; improved memory impairment	ERK phosphorylation; enhanced neprilysin activity	(126)
Mouse AD (3XTg-AD)	3 months	Improved cognitive impairment	Reduced soluble Aβ levels, reduced ROS levels in the hippocampus of WT and 3XTg-AD mice	(127)
Mouse (senescence-accelerated mouse prone 8, SAMP8)	2 months	Improved recognition and context-dependent fear memory	Restored decrease in GSH/GSSG ratio, increased antioxidant (GPx) enzyme activity, reversed tau phosphorylation at Ser202 and Thr231	(128)
MPTP-treated model mice	14 days	Improved motor and cognitive deficits	Increased levels of CaMKII autophosphorylation and phosphorylation of DARPP-32 in the striatum and hippocampus; restored CaMKII- and cAMP kinase-dependent TH phosphorylation; enhanced dopamine release in striatum and hippocampus	(129)

Future studies are required to delineate the role of circadian clock in these efficacies.
FST, forced swim test; TST, tail suspension test.

clock gene expression (61, 134, 135). Whereas peripheral clocks appear to suffer amplitude dampening (136, 137), the central clock neurons maintain robust molecular oscillation (135, 137). It is possible that cellular coupling and/or output pathways are compromised during aging, leading to systemic decline. In accordance, old age in both humans and mice is associated with delayed adaptation to phase shift cues (138, 139), suggesting that aging compromises circadian synchronization and weakens entraining response. Genetic studies have also provided evidence linking the clock and aging. The *Bmal1*-null mutant mice, exhibiting arrhythmic clock gene expression and defective clock-controlled physiological processes such as metabolism and activity (140, 141), suffered premature aging phenotypes such as sarcopenia, cataracts, and early mortality (142, 143). On the other hand, the αMUPA transgenic mice, as a long-living mouse model, displayed 24-h circadian periodicity regardless of age (144). These mice maintained robust behavioral and physiological rhythms, and core clock gene expression showed enhanced amplitude. Collectively, the evidence indicates that circadian robustness, involving both clock gene oscillation and systemic synchronization (145), may confer beneficial effects on life span and health span.

An established circadian output marker is melatonin (146), a sleep-regulating hormone in humans whose synthesis pathway is governed by the clock (147). Aging dampens the circadian peak (and amplitude) and daily total secretion of melatonin (148–150), contributing to lower sleep quality including decreased rapid eye movement, slow wave sleep, and increased stage 2 non-REM sleep in the elderly (151, 152).

Aging is associated with prevalent metabolic deterioration (130). For example, total EE declines during aging, as the elderly display diminished EE and gross energy intake (EI) compared with young adults (153). Such age-related energy imbalance, with EI > EE in the elderly and EI < EE in young adults, causes exaggerated body mass index during aging (154). Body temperature is a circadian output that shows a diurnal pattern with a dip during sleep (146, 155, 156). Thermogenesis plays a significant role in energy homeostasis, and age-related deterioration in energy homeostasis impairs circadian body temperature rhythm. For example, despite largely comparable basal body temperature, phase and amplitude of body temperature rhythm have been shown to significantly differ between the elderly and young- or middle-aged subjects (155, 157, 158). Liver and muscle play important roles in body temperature regulation, and attenuated skeletal muscle mass and mitochondrial function significantly contribute to dampened energy homeostasis and thermogenesis during aging (62, 157).

Caloric restriction universally prolongs life span (159). CR depletes white adipose tissue, especially the pro-inflammatory and diabetogenic visceral fat that accumulates over age (160). Timed CR leads to highly consolidated food intake within a few hours, enhancing the amplitude of circadian metabolic rhythms (63, 161) and core clock gene oscillation (65). CR involves several nutrient-sensing pathways including AMPK, AKT, and mTORC1, all of which have been reported to functionally interact with the clock (31, 42, 160, 161). In particular, the NAD+-dependent deacetylase SIRT proteins play important roles at the interface of energy homeostasis, clock, and aging (161, 162). Mammals express seven SIRT proteins (SIRT1–7), several of which have been implicated in circadian regulation of metabolism (32, 33, 163, 164). For example, SIRT1 directly deacetylates core clock components including BMAL1 and PER2, regulating their molecular function and CCG expression (32, 33). More recently, SIRT1 was found to interact with PGC-1α to control *Clock* and *Bmal1* gene expression in the SCN, consequently regulating CLOCK/BMAL1 target genes (165). Various SIRT1-activating small molecules (e.g., resveratrol) have been shown to extend life span (166); resveratrol, in particular, has been shown to modulate physiological and behavioral rhythms and clock gene expression (167–169).

FUTURE DIRECTIONS AND CONCLUDING REMARKS

Circadian amplitude regulation and pharmacological modifiers are exciting research topics with promising translational potential. The list of CEMs will likely continue to grow, either from phenotypic screening, as in the case of NOB, or from targeted ligand development (14). On the other hand, pharmacological agents shown to target or mimic clock-enhancing pathways such as CR, TRF, and exercise are a rich venue for discovery of additional clock-targeting agents (63, 130, 161, 170). For example, a growing number of small molecules or drugs have been shown to extend life span and health span, including those deliberately designed to mimic CR and other manipulations (170, 171). Future studies should characterize their circadian clock effects and delineate molecular mechanisms.

Besides metabolic diseases, mood disorders, and aging, other chronic diseases such as neurodegenerative diseases (172, 173) have also been shown to correlate with dampened circadian amplitude or clock dysregulation and may represent new venues for studies of clock modifiers. In addition to antidepressive effects, several studies have shown neurological efficacies of NOB using transgenic disease models (**Table 2**). For example, 11-day oral administration of NOB resulted in an overall memory improvement in olfactory-bulbectomized (OBX) mice based on the step-through passive-avoidance task and the Y-maze test (111). OBX mice share clinical features with both human neurodegenerative diseases and major depression (174). The depression-like phenotype is thought to derive from pathological or compensatory mechanisms within the cortical–hippocampal–amygdala circuit, which typically involve deterioration of spine density and/or synaptic strength changes (175). Future studies are required to determine the specific role of circadian clocks and RORs in disease models.

Significant gaps of knowledge remain regarding circadian amplitude regulation, especially the mechanisms employed by CEMs. At the intracellular level, questions of particular interest include gene expression regulation, such as cofactor recruitment, epigenetic mechanisms, and chromosome dynamics (1). At the intercellular and system levels, other coupling molecules in addition to VIP and the communication between peripheral and central clocks are outstanding questions (50). It is conceivable that CEMs execute distinct mechanistic schemes to restore a robust

overall output under disease or aging conditions. Exemplified by the complex and divergent ROR mechanisms when bound by distinct ligands (74, 90, 91), a detailed mechanistic understanding is important to fully exploit the therapeutic potential of individual CEMs.

In conclusion, circadian clocks safeguard physiological health, and dysregulated and dampened clocks can serve as therapeutic targets to mitigate disease symptoms. Exciting functional and mechanistic studies await to develop CEMs as novel preventive and therapeutic agents.

AUTHOR CONTRIBUTIONS

GG, S-HY, and ZC contributed to manuscript preparation.

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