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The role of clock genes in sleep, stress and memory

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Abstract

Circadian clock genes serve as the molecular basis for animals' ~24-h internal timekeeping. Clock gene expression inside and outside of the mammalian brain's circadian pacemaker (*i.e.* the SCN) integrates temporal information into a wealth of physiological processes. Ample data suggests that in addition to canonical cellular timekeeping functions, clock proteins also interact with proteins involved in cellular processes not related to timekeeping, including protein regulation and the interaction with other signaling mechanisms not directly linked to the regulation of circadian rhythms. Indeed, recent data suggests that clock genes outside the SCN are involved in fundamental brain processes such as sleep/wakefulness, stress and memory. The role of clock genes in these brain processes are complex and divers, influencing many molecular pathways and phenotypes. In this review, we will discuss recent work on the involvement of clock genes in sleep, stress, and memory. Moreover, we raise the controversial possibility that these functions may be under certain circumstances independent of their circadian timekeeping function.

1. Introduction

Clock genes serve as the basis of an intracellular timekeeping system, present throughout the body, which generates approximately 24-hour rhythms in physiology and behavior. Transcripts and protein products of these genes show near-24-hour oscillations in expression [18,74,79]. In mammals, interactions between the proteins circadian locomotor output cycles kaput (*Clock)* (or neuronal pas domain protein 2, *Npas2*), brain-and-muscle arnt-like protein 1 (*Bmal1*), period (*Per1, Per2 and Per3*), and cryptochrome (*Cry1* and *Cry2*) are responsible for precisely-timed circadian oscillations in the cells of many tissues [79]. Briefly, BMAL1 and CLOCK (or NPAS2) proteins are basic helix-loop-helix (bHLH)- Per-Arnt-Sim (PAS)

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transcription factors [35], which form heterodimer complexes through their PAS domains. As heterodimer complexes they interact with promoter region E-box sites via their bHLH domain, initiating the transcription of *Per1, Per2, Cry1*, and *Cry2* genes [30,79]. PER1, PER2, CRY1, and CRY2 proteins in turn form heterodimers which ultimately inhibit the transcription of their own genes by suppressing *Bmal1*- and *Clock*-mediated transcription activation. This inhibition is gradually relieved as the protein levels of PER1, PER2, CRY1, and CRY2 decrease through ubiquitin-mediated degradation [16,45], resulting in the initiation of a new cycle. A period of approximately 24 h is achieved via a delay between peak transcription and peak translation, and via the accumulation and degradation of proteins. These processes are orchestrated by multiple post-translational regulatory processes including phosphorylation, acetylation, and ubiquitination [16,25].

Besides clock protein interactions with one another, some clock proteins also interact with a broad array of other proteins [46]; these interactions may modulate cellular processes including protein transcription, stabilization, and trafficking. This interaction with proteins not involved in circadian timekeeping has been proposed as a mechanism for integrating time-of-day information into basic cellular processes [3,70]. Here, we highlight the involvement of clock genes in complex behaviors and brain functions, particular their role in sleep, stress responses, and learning and memory. To examine the contribution of clock genes to these processes, we will review how these genes are regulated, how they modulate intracellular processes, and how they affect behavior and physiology. Moreover, we also provide a perspective on additional non-clock related function of clock genes, and how they could be studied in future experiments.

2. Interactions between clock genes and sleep (or sleep loss)

2.1. Regulation of clock genes by sleep loss.

Sleep is thought to be a homeostatically regulated process. This homeostatic mechanism tracks the build-up of sleep-need as function of time spent awake, which, in turn, activates compensatory mechanisms during the next sleep episode [17,19,26]. Based on SCN lesion studies and forced desynchrony experiments, the homeostatic regulation of sleep and the build-up of sleep need during wakefulness appears to be largely independent of internal clock mechanisms [20,52,82]. Interestingly, the expression levels of certain clock genes are regulated by sleep need, and this regulation can lead to clock gene expression patterns that are deviating from their circadian oscillations, as we will discuss below.

Sleep and sleep deprivation significantly affect expression of clock genes outside of the SCN. Multiple rodent studies across laboratories have demonstrated the impact of sleep deprivation on the clock genes *Per1* and *Per2* mRNA; for example, 6 h of deprivation at the start of animals' rest phase increases expression of the period genes in whole brain or forebrain lysates [11,15,28,50,54,91,90], and even in midbrain structures such as the lateral habenula [98]. Based on studies of transgenic mice expressing a PER2-luciferase fusion protein, 6 h of sleep deprivation also increases PER2 protein levels, not only in the brain but also in the liver and kidney [14]. The forebrain PER2 increases were found to be stronger after 6 h sleep deprivation in the light phase compared to the dark phase, presumably because the loss of sleep was larger in the light phase, *i.e.* the main sleep

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phase of mice. These findings suggest that PER2 levels not only contain information about time of day but also keep track of the time spent awake [15]. Sleep loss also subtly impacts the expression of other clock genes. For example, 6 h of sleep deprivation has been reported to either increase [15], minimally impact, or decrease [91,90] *Cry1* mRNA levels in forebrain lysates, depending on the genetic background of the examined strains. A similar background-dependent alteration in *Cry2* mRNA levels after sleep deprivation has been observed [91]. Likewise, Wisor et al. [91] also reported strain-dependent increases in cortical *Bmal1* and *Clock* expression after sleep deprivation [91].

Some changes in clock gene expression after sleep deprivation are clearly brain region specific; for example, sleep deprivation increases *Npas2* in forebrain lysates [54], but not in cerebral cortical lysates [91]. Critically, however, clock gene expression in the SCN is unaffected by sleep deprivation [14]. While in humans the knowledge about the impact of sleep deprivation on clock gene expression in the brain is limited, for obvious reasons, effects on clock genes in peripheral tissues have been reported. A full night of sleep deprivation reduces expression of *BMAL1, CRY1*, and *PER2* in plasma leukocytes [1,38], and in skeletal muscle [7], but not adipose tissue [7]. This suggests that in humans as well as in rodents, there is tissue-specific regulation of clock genes in response to sleep deprivation. Thus, under specific circumstances, such as sleep deprivation, the expression levels of clock genes can be temporally uncoupled from their local circadian oscillations, while oscillations in the master clock (*i.e.* the SCN) are left undisturbed. A possible consequence of this diversity in regulatory mechanisms may be a tissue- or brain region-specific function of clock genes during or after sleep deprivation.

2.2. Mechanisms for regulation of clock genes by sleep loss.

The molecular mechanisms responsible for sleep deprivation–mediated clock gene expression changes are still being investigated (Fig. 1). It seems unlikely that in this context, increases in *Per1* and *Per2* are caused by the CLOCK::BMAL1 or NPAS2::BMAL1 complex (Fig. 1a). The binding of these complexes to E-boxes in the promoter region of *Per1* and *Per2* are either not enhanced, or are even decreased, during sleep deprivation [55]. Thus, one way to resolve this issue is to consider other (*i.e.*, non-E-box) promoter and repressor elements (and the transcription factors that bind them) in the regulatory regions of the clock genes themselves.

Transcription factor cAMP-responsive element binding (CREB) binds to cAMP-responsive elements (CREs), increasing transcription of numerous genes including *Per1 and Per2* (Fig. 1b). While *in vitro* analyses have shown that phosphorylated CREB (pCREB) can enhance expression of period genes [83], it is unclear whether CREB signaling drives increases in these genes across sleep deprivation. This is mainly due to the fact that there have been conflicting reports of pCREB levels being either increased [12], or attenuated [48,66,85], as a result of either brief (*e.g.*, 6-hour) or long-term (*e.g.*, 72-hour) sleep deprivation. Regardless of direction, which could be due to age and method of sleep deprivation [34], these effects of sleep deprivation have been reported in multiple forebrain brain areas, such as in the hippocampus, amygdala, and frontal cortex.

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Another factor that can modulate clock gene expression and is affected by sleep deprivation is tumor necrosis factor (TNFα). *TNFα* is enhanced during acute and chronic sleep deprivation [99]. However, *in vitro* studies using primary cultured human rheumatoid synovial cells and fibroblasts cells indicated that TNFα treatment enhanced *Bmal1*, and *Cry1* expression, while it decreased *Per2* and *Per1* levels [6,96]. Because the direction of change for *Per2* and *Per1* caused by TNFα is opposite of that observed during sleep deprivation, this seems like an unlikely candidate pathway to mediate those effects.

DBP (D-site binding protein) binding to the D-box region promoter region of *Per1* and *Per2* enhances the transcription of *Per1* and *Per2* [95] (Fig. 1c). However, it seems unlikely that DBP is responsible for the observed sleep deprivation-enhanced levels of *Per1* and *Per2*, since DBP levels in the forebrain levels are attenuated after 6 h of sleep deprivation [15]. Contrary to DBP, binding of E4 promoter-binding protein 4 (E4BP4), also known as nuclear factor interleukin-3 (NIFL3), to the D-box promoter suppresses *Per1* and *Per2* transcription (Fig. 1c) [53]. After 6 h of sleep deprivation *E4BP4* mRNA levels are upregulated [54]. It seems to be unlikely that D-box binding of DPB and E4BP4 is responsible for the sleep deprivation-enhanced levels *Per1* and *Per2*. Altogether, the molecular mechanisms via which clock genes are regulated during sleep deprivation seems to deviate from the classical clock gene- clock gene interactions. However, which molecular mechanisms are responsible for the tissue- or brain- region specific regulation of clock genes during sleep deprivation is yet unclear.

2.3. Role of clock genes in sleep regulation

What function (or functions) could the induction of clock gene expression with sleep deprivation serve? Sleep loss activates compensatory mechanisms in the brain, resulting into more intense and longer subsequent sleep (known as the homeostatic sleep response or sleep rebound) [20]. This so called “recovery” sleep is expressed as increased non-REM sleep time, greater continuity of non-REM sleep periods, and greater EEG delta power (thalamocortical network activity of 1–4 Hz) immediately following sleep deprivation [19,81]. Accumulating evidence has demonstrated that clock genes may regulate these homeostatic changes. Following 6 h of sleep deprivation, *Bmal1*−/− mice and *Cry1*/*Cry2* double knockout mice show increased delta power relative to wild-type mice during recovery sleep [24,44,90]. Surprisingly, when *Bmal1* expression is rescued selectively in the brain of *Bmal1*−/− mice, the homeostatic sleep response remains altered, while skeletal muscle-targeted *Bmal1* rescue reverses the response phenotype [24]. This intriguing observation suggests that *Bmal1* acting in muscle, rather than brain, may regulate EEG changes in the brain after sleep deprivation. The tissue- and cell-level mechanisms mediating this effect are yet to be defined. In contrast to *Bmal1*−/− mice, *Per1* and *Per2* mutants show relatively subtle effects on post- sleep deprivation EEG delta power [40], and *Npas2*−/− animals show significantly decreased (rather than increased) EEG delta power after 6 h of sleep deprivation [27]. Unfortunately, these global knock-out animals are accompanied by arrhythmicity of both the master clock and local clocks [44,49,90], and can, therefore, not exclude the possibility that the observed impairments are secondary to distortion of local clocks. On the other hand, removal of the SCN (*i.e.*, master clock) also leads to arrhythmicity of some local clocks like in the hippocampus [13,92,93], and is not

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accompanied by alterations in homeostatic sleep processes [52]. This latter observation suggests that alterations in homeostatic sleep response may not be caused by local clock distortions, but might rather be a consequence of clock gene interactions with homeostatic sleep processes irrespective from their circadian oscillations (possible non-clock function).

Together, these findings show that clock genes may not only regulate the circadian timing aspects of sleep which initiated by clock genes in the SCN. Outside the SCN, clock genes may also serve additional roles in regulating sleep intensity and sleep homeostasis, and these additional processes are unrelated to their clock mechanism. The precise mechanistic roles of these clock genes in the regulation of sleep homeostasis (and in sleep-dependent physiological functions), however, remains to be determined.

3. Interactions between clock genes and stress

3.1. Regulation of clock genes by stress.

Physiological stress occurs when environmental demands exceed the natural regulatory capacity of an organism - particularly in situations where these demands are unpredictable and uncontrollable [39]. The timing of a stressor can follow both a circadian pattern (when stressors occur always at a specific timepoint of the day), or a non-circadian pattern (when the occurrence of stressors are not following a 24-h pattern). To support animals' behavioral response to a stressor, the hypothalamic–pituitaryadrenal (HPA) axis and the sympathoadrenal system increase release of glucocorticoids and catecholamines into the circulation [84]. It seems that this molecular response towards the (circadian or non-circadian) occurrence of a stressor regulates many biological processes, including clock gene levels.

Model paradigms of acute and chronic stress have been used to study physiological effects of stressors. In both types of stress models, brain clock gene expression levels are altered. For example, subjecting animals to an acute stressor in the form of forced swimming or restraining significantly increases *Per1* mRNA and PER1 protein levels in multiple cortical areas, hippocampus, and basolateral amygdala; these changes can be detected within an hour of stressor termination [2,9,80]. The same acute stressors simultaneously decrease PER1 protein in the central amygdala and stria terminalis [2]. In contrast, acute stress induces *Per2* mRNA only in a few brain structures, including the ventral orbital cortex and paraventricular nucleus [9]. Interestingly, Takahashi et al. [80] showed that the regulation of *Per1* levels in the paraventrical nucleus depends on the time of day. As such, *Per1* levels were only affected (*e.g.* elevated) by a stressor when mice were subjected to a force swim test during the day, while *Per1* levels were not affected after a forced swim test during the night. Together, these findings suggest that acute stress responses may selectively alter *Per1* expression, with comparatively modest and brain area-specific effects on other clock genes. In addition, it seems that these stressor-dependent responses are depending on the time of the day. Clock gene expression in the SCN appears resilient to the effects of acute stressors - as is true for brief sleep deprivation [2,9,14,51].

In chronic models, animals are subjected to multiple stressors over a time course of multiple days. When chronic stressors were applied during 3 consecutive days at the beginning of the

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light phase, it led to a phase advance in *Per2* levels in peripheral and brain tissue including in the hippocampus and cortex. This phase shift was depending on the onset time of the stressor [78]. Also unpredictable chronic stress models led to alterations in clock gene levels and phase shifts of their circadian expression patterns across several brain areas [8,101]. In more detail, *Per1* and *Per2* transcript levels in the hippocampus were subsequently elevated (across the 24-h rest/activity cycle), while *BMAL1* levels were either not affected, or decreased [8]. Surprisingly, no clock gene changes occurred in the neocortex following this chronic stress exposure [8]. Another study, however, reported a significant reduction in BMAL1 and CLOCK protein levels in the prefrontal cortex, which was paralleled by the down regulation of PER and CRY levels [102]. Similar to the response to acute stressors, clock gene oscillations in the SCN are not affected by chronic stress [63,78]. Altogether, it is clear that stressful experiences are regulating clock genes in a brain are specific manner, and that the nature of the timing of the stressor determines the amplitude of clock gene regulation.

3.2. Mechanisms for regulation of clock genes by stress

Circulating glucocorticoids are a major end-product of stress-induced HPA axis activation and are able to exert a wide array of genomic effects, including effects on clock genes [61]. Plasma glucocorticoid levels peak within 30–60 min after a stressor, a time frame that overlaps with *Per1* and *Per2* increases after acute stress exposure [9]. Glucocorticoid binding to intracellular receptors permits their binding to glucocorticoid-response-elements (GREs) within the promoter sequence of targets genes (including *Per1* and *Per2*), leading to transcriptional enhancement (Fig. 1d) [4]. In cultured fibroblasts, applied glucocorticoids increase *Per1* and *Per2* levels [4]. A handful studies have examined the impact of stress exposure on *in vivo* clock gene expression in rodents that were either adrenalectomized or treated with a glucocorticoid receptor antagonist. Under these conditions, cortical *Per1* increases following restraint stress was unaffected, while increases in other brain structures were blunted [2,9]. Critically, however, circadian *Per1* and *Per2* oscillations in some brain areas appear to be directly driven by daily fluctuations in glucocorticoid levels [71,92]. In prefrontal cortex, bed nucleus of the stria terminalis, and central nucleus of the amygdala, adrenalectomy leads to a loss of *Per1* and *Per2* rhythmicity, which can be restored by daily timed corticosterone injections [92]. When mice are exposed daily to either restraint stress or fox odor, oscillations in PER1 and PER2 protein were shifted in the basolateral amygdala (BLA), hippocampus, and piriform cortex, in a circadian phase-dependent manner [64,78]. In contrast, clock gene expression patterns in the SCN of the same mice were unaffected, regardless of stressor timing. The latter might be explained by the fact that during adulthood the SCN does not have glucocorticoid receptors [69].

3.3. Functions of stress-induced clock gene expression.

It is hypothesized that stress-induced clock gene changes constitute an adaptive mechanism to prepare for future stressors, in case these are recurring events [77]. This is supported by studies that demonstrated that the phase of local clocks can be shifted after the exposure to daily timed recurring stress full experiences [64,78]. Next to this phase shift, more support for this adaptive function comes from the fact that some clock genes, in turn, can mediate suppression of glucocorticoid signaling. For example, CRY1, CRY2,

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REV-ERBα and CHRONO clock proteins interact with glucocorticoid receptors in a ligand-dependent fashion, to suppress GRE-mediated transcriptional activation [31,42,62]. In addition, clock protein can function as a histone acetyltransferase at GREs in the promoter region of a number of genes [21], and is able, in combination with its partner *Bmal1*, to repress glucocorticoid receptor-mediated transcriptional activity by inhibiting glucocorticoid receptor binding [60]. Thus, it appears that many clock genes (*i.e. Cry1/2*, *Bmal1*, *Clock*, *Chrono*, *Rev-erbα*) can suppress glucocorticoid signaling - which normally acts to enhance expression of clock genes such as *Per1* and *Per2*. It is likely that the efficacy of this feedback loop depends on the phase of the oscillation (time of the day), providing a gating-like mechanism that imposes daily rhythmicity onto the stress response [77]. Indeed, glucocorticoid biogenesis is gated by the clock gene expression in the adrenal cortex [103]. However, it remains to be determined whether the function of clock genes in non-SCN brain areas are providing similar gating like mechanisms within specific brain areas, in which the timing of a stressor will determine the magnitude of the stress response. A possible approach to investigate this latter scenario would be the investigation of the glucocorticoid-mediated stress response when only local clocks in specific brain regions are disrupted. Are the observed stress-dependent clock gene regulations and protein functions deviating when local clocks are disturbed or not? More specific disruption of clock genes within specific brain regions might be challenging, but not impossible due to the development of new techniques as we will discuss in more detail in the discussion section.

4. Interactions between clock genes and memory processing

4.1. Regulation of clock genes during learning and memory storage

Not many studies have investigated the regulatory effects of learning and memory processes on clock genes. Only a few studies revealed that a brief training on hippocampus-dependent learning tasks leads to a rapid increase in hippocampal *Per1* expression in rodents [22,41,86]. The mechanisms mediating this effect are still largely unknown, however, *Per1* is epigenetically controlled at its CRE promoter site by a repressive histone deacetylase, HDAC3. Under baseline conditions, HDAC3 suppresses CRE-dependent transcriptional activity. When an animal is exposed to a learning task, HDAC3 releases its suppressive effects, allowing CREB to bind to CRE, resulting in transcriptional enhancement of *Per1* [41]. Another plausible mechanism for learning-associated regulation of *Per1* is the cyclic adenosine monophosphate (cAMP)/ mitogen-activated protein kinase (MAPK)-signaling cascade. This pathway is essential for the consolidation of long-term memories [33,37], and influences gene expression via CRE promoter elements (*e. g.*, *Per1* and *Per2*) [83].

4.2. Roles of clock genes in memory storage.

It is currently unclear how changes in hippocampal clock gene expression affect memory storage. However, reducing *Per1* mRNA levels in the hippocampus by 30% using siRNA prior to training impairs hippocampal learning [41]. Conversely, overexpression of *Per1* in the hippocampus of aged mice prevents aged-related hippocampal memory deficits [41], suggesting that PER1 serves an essential and causal role in hippocampal memory processes. PER1 might affect the function of circuits like the hippocampus through regulating CREB-mediated transcription by promoting the nuclear translocation of CREB

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kinase (pMAPK-activated ribosomal S6 kinase), which is essential to the CRE-mediated transcription of numerously plasticity related genes. Loss of *Per1* disrupts both CREB-dependent gene expression and long-term hippocampal memory formation [41,68,67]. Together, this suggests that upregulation of PER1 following learning contributes to the subsequent formation of hippocampal long-term memories [68].

Other clock genes contribute similarly to memory related processes. For example, *Per2*−/− mice display deficits in long-term trace fear conditioning (with normal performance on other hippocampus-dependent tasks such as contextual fear conditioning and Morris water maze), accompanied by a decrease in both hippocampal CREB phosphorylation and long-term potentiation [87,100]. These data suggest that specific aspects of hippocampal learning are affected by the loss of PER2 protein. PER2 protein appears to have cellular functions that could contribute to hippocampal memory processes. For example, by inhibiting protein synthesis via the mTOR pathway, *i.e.*, by binding to mTORC1 and recruiting the suppressor Tsc1 (tuberous sclerosis complex 1). This complex also regulates a number of basic cellular processes, including autophagy and cell proliferation [94].

Bmal1−/− mice, *Clock* mutant mice, and *Npas2*−/− mice also exhibit impairments on hippocampus dependent cognitive tasks, including contextual fear memory and Morris water maze [29,75,89], and in the case of *Npas2*−/− mice, also amygdala-dependent cued fear memory [29]. BMAL1 and CLOCK/NPAS2 proteins influence many cellular processes, and thus may affect neuronal and synaptic function at many levels. Together, they promote E-box-mediated transcription of numerous genes [35], including an essential E-box dependent role in regulating neuroligin 1 (NLGN1), which encodes a post-synaptic adhesion molecule critical for synaptic plasticity and memory [47]. BMAL1 (when phosphorylated by S6 kinase) directly interacts with translating ribosomes to promote translation [46]. The role of these clock-regulated transcriptional and translational processes in memory processes remains to be determined. However, because *de novo* transcription and translation are both essential for hippocampal memory storage, these clock-mediated pathways likely influence the cellular mechanisms of memory consolidation. Lastly, $Cry1^{-/-}Cry2^{-/-}$ double knockout mice also exhibit memory impairments - specifically in the object recognition task [5], and time-place learning [56,97].

These knock-out studies underscore the importance of most of clock genes in memory processes, particularly those involving the hippocampus. However, based on these studies using mice lacking clock genes in the entire brain, it remains a challenge to define whether the observed impairments are caused by the disruption of complimentary clock gene functions or the disruption of local clocks. It is known, that the hippocampus, for example, exhibits circadian clock gene rhythm *in vivo* [10,32,36,43,59,87]; these may actually play a role in learning and memory storage, allowing integration of circadian/temporal information into memory processes. These hippocampal clock rhythms are entrained by the master clock, the SCN, and when the hippocampal circadian clock is disrupted animals displayed deficits in long-term novel object recognition [72], contextual fear conditioning and Morris water maze [65], suggesting that the memory impairments in most knock-out studies might be secondary to the disruption of forebrain clocks. Indeed, a forebrain-specific deletion of *BMAL1* also impairs hippocampus dependent learning tasks [76]. Interestingly, a CAMKII

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dependent *Bmal1* knock out did not result into a deficit in hippocampus dependent learning tasks [88], questioning the prominent role of role of hippocampal clocks in memory processes. Altogether, it seems that several clock genes are involved into memory processes, and that the strength of a memory depends on the timing of its acquisition and/or retrieval. However, how local clocks are directly contributing to memory processes remains to be determined.

5. Conclusions and future directions

Taken together, multiple lines of evidence show that clock gene expression outside of the SCN can be regulated at the transcriptional, translational and post-translational level by a variety of cellular processes related to sleep, stress and memory. Clock proteins can in turn modulate a wide array of molecular brain processes. The classical function of clock genes is to integrate temporal information inside vs. outside the body. Clock gene patterns in different brain and body structures may be used to alter the structures' own behavior and physiology in accordance with changes to their local environment, without affecting the master clock (*i.e.* SCN). These changes could be tissue specific and diverse, as roughly 80% of all protein coding genes can be diurnally expressed, and regulated by the intracellular clock mechanism [59]. Many seemingly tissue specific processes follow a diurnal pattern, including complex brain processes highlighted in this review.

As described in this review, under certain conditions clock genes are regulated by various processes, uncoupling them from their clock mechanics. A repetitive timed event, such as a stressor, adapts local clocks towards the timing of the reoccurring event [64,78]. However, what will happen when the occurrence of the same event is irregular and unpredictable? Would this also mean that the expression levels are deviating from their circadian rhythms, misaligning local clocks with the environment leading to less optimal clock genes functions? Another scenario would be that the function of clock genes in certain processes is not depending on their local clock mechanics. In this latter scenario, local clock play a role in making these processes more dynamic and perhaps, partially independent of the time of day. The study of these additional/ complementary "non-clock" roles can be challenging. As described above, the additional roles of clock genes are often heavily intertwined with local clocks and circadian input from the environment. Therefore, excluding that the observed effects are not secondary to the disruption of local clocks might sometimes be difficult.

How could timekeeping functions of clock proteins be disentangled from other (possibly time-independent) cellular functions? One possible approach is to investigate whether clock gene expression levels can be regulated by inputs *independent* of the time of day. When the regulation of clock genes by certain inputs is not depending on timing, this might indicate that clock genes have additional non-clock roles which are independent of their clock mechanic. On the other hand, when non-circadian regulation of clock genes is modulated by the time of the day, then clock genes might have a function which is more related to the integration of local clocks into non-circadian processes. As illustrated above, the regulation of clock genes by stressful experiences depends on the timing, providing a mechanism to integrate local clock information into the stress response.

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A second indication of potential non-clock roles of clock genes might come from studies showing that the consequences of clock gene/protein manipulations are not secondary to the disruption/modulation of cellular/systemic internal clocks, but are a direct consequence of physical interaction of clock gene proteins with pathways not related to core clock functions. Through the comparison of the effects/phenotypes of body-wide clock gene modulation (*i.e.* the use of classic knock-out models) versus the effects when local clocks are modulated/disrupted, studies might provide indications about possible non-clock functions in specific brain areas. In more detail, a knock-out of a clock gene can lead to a change in a certain phenotype due to the involvement of that specific clock gene. If this change in phenotype is related to the local clock function in a specific brain area, then the disruption of local clocks will lead to a similar change in phenotype. However, when the disruption of local clocks are not revealing any changes in phenotype then local clock genes might fulfil a non-clock role in the examined phenotype. Disruption of local clocks (*i.e.* clock gene oscillations) can be done via, for example, manipulating the master clock (*i.e.* SCN), which is responsible for the entrainment and coordination of widely distributed cellular- and tissue-level circadian clocks [23,70,79]. However, local clocks can be self-sustaining and not always need input from the SCN for their oscillatory cycles. Therefore, disruption of these self-sustaining clocks can be more challenging and requires a different approach.

More localized disruption of local clocks are essential to investigate possible additional non-clock functions. Localized knock-out studies already provide more spatial information since the knock-out can be specific for certain brain regions instead of the whole brain. However, localized knock-out studies are still accompanied by developmental problems due to the importance of clock genes in developmental processes [73]. Therefore, to study non-clock functions of clock genes we should make use of more advanced techniques that allows you to control clock genes in a specific brain area at an controlled time point. By using optogenetics, for example, clock gene function can be determined on tissue or brain region specific level [57,58]. Moreover, these approaches allow you to manipulate clock genes only during a relatively short amount of time (*i.e.* seconds to minutes), providing the unique opportunity to investigate the effects of temporally deviating expression levels (from oscillations) on local clocks. Altogether, this might contribute to the dissection of the complicated and diverse functions of clock genes in the brain.

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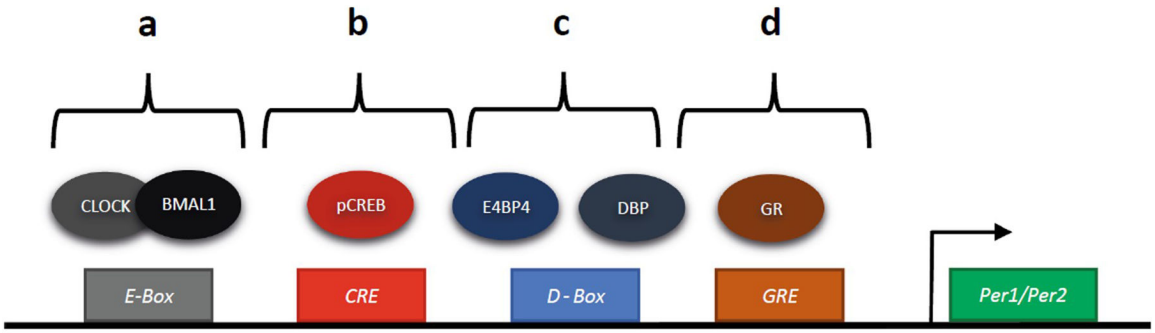

Fig. 1. Overview of possible molecular mechanisms responsible for the bidirectional regulation of Per1 and Per2.

Multiple proteins are able to regulate the transcription of Per1 and Per2 via binding with the Per1/Per2 promoter elements. (a) When CLOCK/BMAL1 complex binds to an E-box, transcription of Per1/2 is attenuated. (b) Interaction between pCREB and its response element CRE enhances the transcription of Per1/Per2. (c) The binding of E4BP4 to the D-box attenuates transcription of Per1/Per2, while the binding of DBP to the same D-box enhances transcription of Per1/Per2. (d) GR and GRE leads to an increase in Per1/Per2 transcription. Abbreviations: E4BP4, nuclear factor, interleukin 3; DBP, D-box binding protein; GR, glucocorticoid receptor; GRE, glucocorticoid response element; pCREB, phosphorylated cAMP response element-binding protein; CRE, CREB response element.