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## A role for *cryptochromes* in sleep regulation

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### Abstract

**Background:** The *cryptochrome* 1 and 2 genes (*cry1* and *cry2*) are necessary for the generation of circadian rhythms, as mice lacking both of these genes (*cry1,2*<sup>-/-</sup>) lack circadian rhythms. We studied sleep in *cry1,2*<sup>-/-</sup> mice under baseline conditions as well as under conditions of constant darkness and enforced wakefulness to determine whether *cryptochromes* influence sleep regulatory processes.

**Results:** Under all three conditions, *cry1,2*<sup>-/-</sup> mice exhibit the hallmarks of high non-REM sleep (NREMS) drive (i.e., increases in NREMS time, NREMS consolidation, and EEG delta power during NREMS). This unexpected phenotype was associated with elevated brain mRNA levels of *period 1* and 2 (*per1,2*), and *albumin d-binding protein (dbp)*, which are known to be transcriptionally inhibited by CRY1,2. To further examine the relationship between circadian genes and sleep homeostasis, we examined wild type mice and rats following sleep deprivation and found increased levels of *per1,2* mRNA and decreased levels of *dbp* mRNA specifically in the cerebral cortex; these changes subsided with recovery sleep. The expression of *per3*, *cry1,2*, *clock*, *npas2*, *bmall*, and *casein-kinase-1ε* did not change with sleep deprivation.

**Conclusions:** These results indicate that mice lacking *cryptochromes* are not simply a genetic model of circadian arrhythmicity in rodents and functionally implicate *cryptochromes* in the homeostatic regulation of sleep.

### Background

Sleep is regulated by both circadian and homeostatic mechanisms. As a consequence of a signal from the circadian clock, located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus in mammals [1], sleep is more likely to occur at certain times of the 24-h day than others, thereby determining the daily sleep-wake distribution [2].

Sleep is homeostatically regulated in the sense that sleep drive accumulates in the absence of sleep and decreases during sleep. Changes in sleep drive are thus driven by the sleep-wake history. Homeostatic and circadian mechanisms interact to determine the duration and quality of sleep and wakefulness [3–5]. Homeostatic regulation of sleep can still be observed in animals that lack circadian

rhythms after lesioning of the SCN [6–9], suggesting that circadian rhythms and sleep homeostasis are independent processes.

Homeostatic regulation of sleep can be quantified objectively after a period of enforced wakefulness (i.e., sleep deprivation), although a similar relationship between sleep parameters and spontaneous wakefulness can be quantified under baseline conditions as well [10,11]. The compensatory responses in time spent asleep, sleep consolidation (i.e., sleep bout duration; [12]), and/or sleep intensity observed after an extended period of wakefulness are all taken as evidence that sleep drive is increased during wakefulness and thus that sleep is homeostatically regulated. Non-REM sleep (NREMS) intensity, quantified as EEG power in the delta frequency range (1–4 Hz), and NREMS consolidation are in a quantitative and predictive relationship with sleep history: both variables increase with the duration of prior wakefulness and subsequently decline during NREMS [3,4,10,12,13]. These variables are thus quantitative markers of NREMS homeostasis and are presumed to reflect an underlying physiological drive for sleep [4].

The time constants describing the dynamics of the increasing NREMS drive during wakefulness and decreasing NREMS drive during NREMS [10,14] are compatible with a role for changes in gene expression in their regulation. Although past studies have shown that extended periods of wakefulness cause changes in gene expression (for review see [15]), no causal relationship between changes in gene expression and sleep homeostasis has been identified. A recent study in *Drosophila melanogaster* implicates transcriptional regulation by the circadian gene *cycle*, a homolog of the mammalian *bmal1* gene, in the homeostatic regulation of rest [16]. While rest in flies shares several features with sleep in mammals [17,18], it remains to be determined whether a similar role for BMAL1 or related transcriptional regulators is necessary for the homeostatic regulation of sleep.

In both flies and mammals, circadian rhythms are thought to be generated by transcriptional/translational feedback loops comprising a network of transcriptional regulators [19–21]. The core of this self-sustained molecular oscillation consists of positive and negative elements. In mammals, the positive elements are two basic helix-loop-helix (bHLH) PAS-domain-containing transcription factors, CLOCK and BMAL1, that form heterodimers that can drive the transcription of three *period* (*per*) genes *per1–3* and two *cryptochromes* (*cry1,2*). PER1,2 and CRY1,2 proteins suppress CLOCK:BMAL1-mediated transcription thereby forming the negative elements in the feedback loop. Consistent with their central role in circadian rhythm generation, genetic inactivation of both *cryptochromes*

(*cry1,2<sup>-/-</sup>*) results in circadian arrhythmicity in mice [22,23]. Given the widespread expression of the elements of the molecular clock in the brain (and elsewhere) and the large number of genes regulated by bHLH-PAS transcription factors (e.g. [24]), the role of *cryptochromes* may extend beyond circadian clock function. To determine whether transcriptional regulation by CRY1,2 influences the homeostatic regulation of sleep, we studied sleep in *cry1,2<sup>-/-</sup>* mice under baseline conditions, under conditions of constant darkness, and after sleep deprivation (SD). The expression of circadian genes that are regulated by *cryptochromes* were evaluated in the brain of *cry1,2<sup>-/-</sup>* mice and in sleep deprived wild type mice and rats.

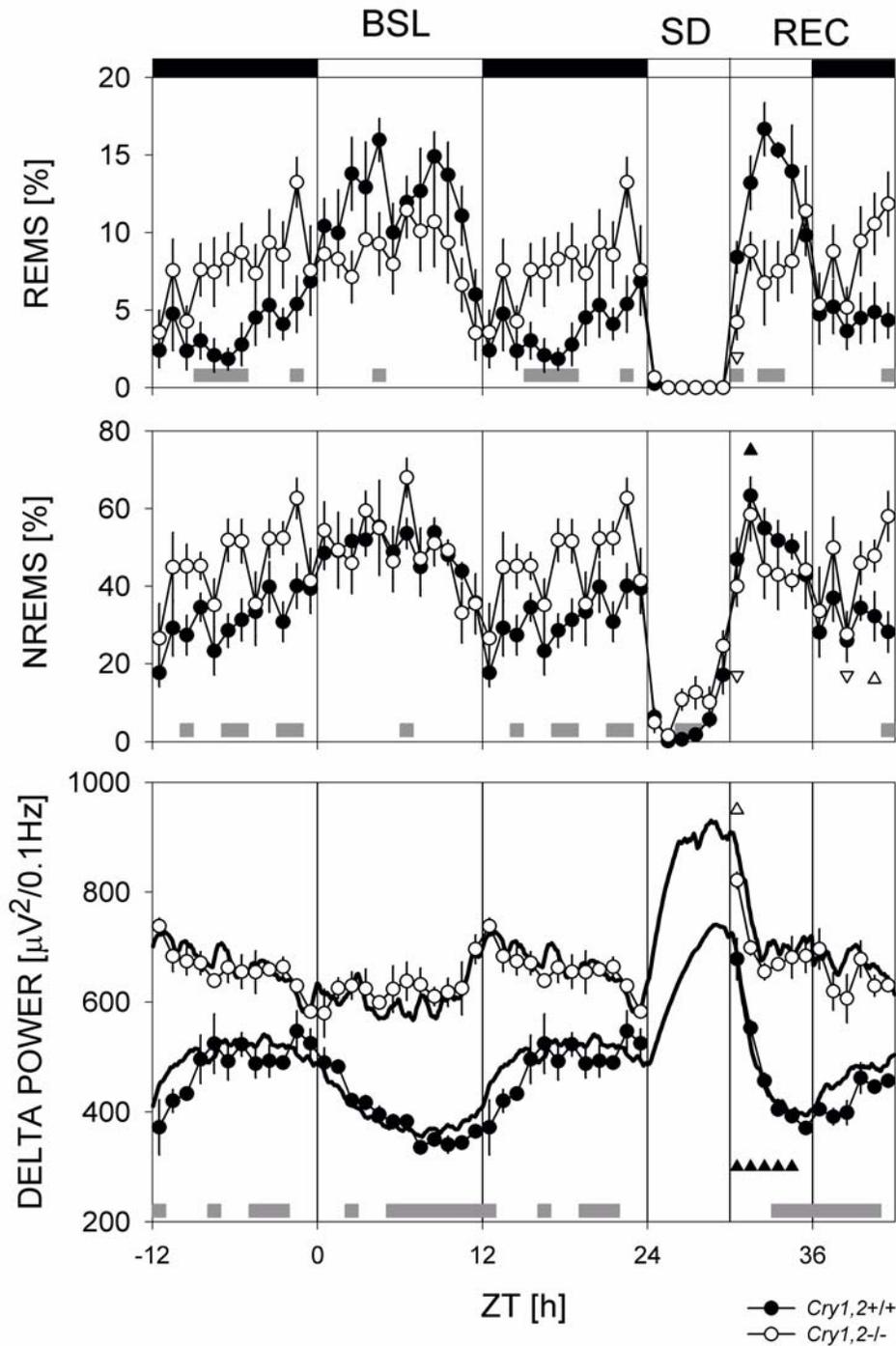
## Results

### Sleep regulation in *cry1,2<sup>-/-</sup>* mice

In baseline conditions, wild type mice showed the sleep-wake distribution typical for this nocturnal species with high values of sleep in the light period and low values in the dark period (Figure 1, Table 1). The sleep-wake distribution in *cry1,2<sup>-/-</sup>* mice was distinct from that of wild type controls (Figure 1) in that sleep variables did not differ between the 12-h light (L) and dark (D) periods (Table 1). Nevertheless, as in wild type controls, the light-to-dark transition was accompanied by a pronounced decrease in sleep time in *cry1,2<sup>-/-</sup>* mice (Figure 1).

During baseline, total daily NREMS time was significantly greater in *cry1,2<sup>-/-</sup>* mice than in wild type mice ( $683 \pm 25$  vs.  $576 \pm 12$  min,  $P < 0.002$ , unpaired t-test), whereas the daily time spent in REM sleep did not differ ( $118 \pm 7$  vs.  $113 \pm 10$  min). The difference between genotypes in NREMS time was present only in the dark period and not in the light (Table 1; Figure 1). Average NREMS bout duration, a measure of NREMS consolidation that is positively correlated with a high homeostatic sleep pressure [13], was greater by 34% in *cry1,2<sup>-/-</sup>* mice relative to wild type mice during the light period, and by 41% during the dark period (Table 1). Consistent with this increase in sleep consolidation, NREMS delta power was significantly higher in *cry1,2<sup>-/-</sup>* than in *cry1,2<sup>+/+</sup>* mice during most of baseline (Figure 1). This difference in EEG delta power was specific for NREMS and did not extend to other EEG frequencies (Figure 2A), indicating that the increase in delta power is not due to a difference in EEG amplification. This state-specific EEG difference in delta power was especially evident at the wake-to-NREMS transition. *cry1,2<sup>-/-</sup>* mice exhibited higher delta power values than *cry1,2<sup>+/+</sup>* immediately after the onset of NREMS (Figure 2B).

The same genotypic differences in sleep were observed in constant dark (DD) conditions (Figure 3). Mice lacking *cry1,2* spent more time in NREMS ( $719 \pm 29$  vs.  $629 \pm 29$



**Figure 1**

Time course of sleep (upper panels) and NREMS EEG delta power (lower panel). Data from baseline (BSL), sleep deprivation (SD), and recovery (REC) are shown. Open (*Cry1,2<sup>-/-</sup>*) and closed (*Cry1,2<sup>+/+</sup>*) symbols designate mean hourly values  $\pm$  1 SEM. In the lower panel, thicker lines connect mean predicted delta power values based on the sleep-wake distribution in individual mice; (see Methods). Triangles mark intervals in which recovery values differed from corresponding baseline values within each genotype (triangle orientation designates direction of deviation;  $P < 0.05$ , *post-hoc* paired t-tests). Gray bars at the bottom of each panel mark intervals with significant genotype differences ( $P < 0.05$ , *post-hoc* t-tests). The baseline dark period was depicted twice to illustrate the changes at the dark-to-light transition.

**Table 1: Sleep in 12-h light and dark periods under baseline conditions in *cry1,2<sup>+/+</sup>* and *cry1,2<sup>-/-</sup>* mice.**

NREMS amount [%]			
	light	dark	Difference
<i>cry1,2<sup>+/+</sup></i>	48.8 ± 1.9#	31.3 ± 2.3	17.5 ± 3.9
<i>cry1,2<sup>-/-</sup></i>	49.5 ± 1.9	45.4 ± 1.9*	4.2 ± 1.6*
NREMS bout duration [min]			
	light	dark	difference
<i>cry1,2<sup>+/+</sup></i>	2.9 ± 0.2	2.9 ± 0.2	0.1 ± 0.2
<i>cry1,2<sup>-/-</sup></i>	3.9 ± 0.2*	4.1 ± 0.4*	-0.2 ± 0.2
REMS amount [%]			
	light	dark	Difference
<i>cry1,2<sup>+/+</sup></i>	11.9 ± 1.1#	3.8 ± 1.0	8.2 ± 1.5
<i>cry1,2<sup>-/-</sup></i>	8.5 ± 0.5*	7.8 ± 0.7*	0.8 ± 0.8*

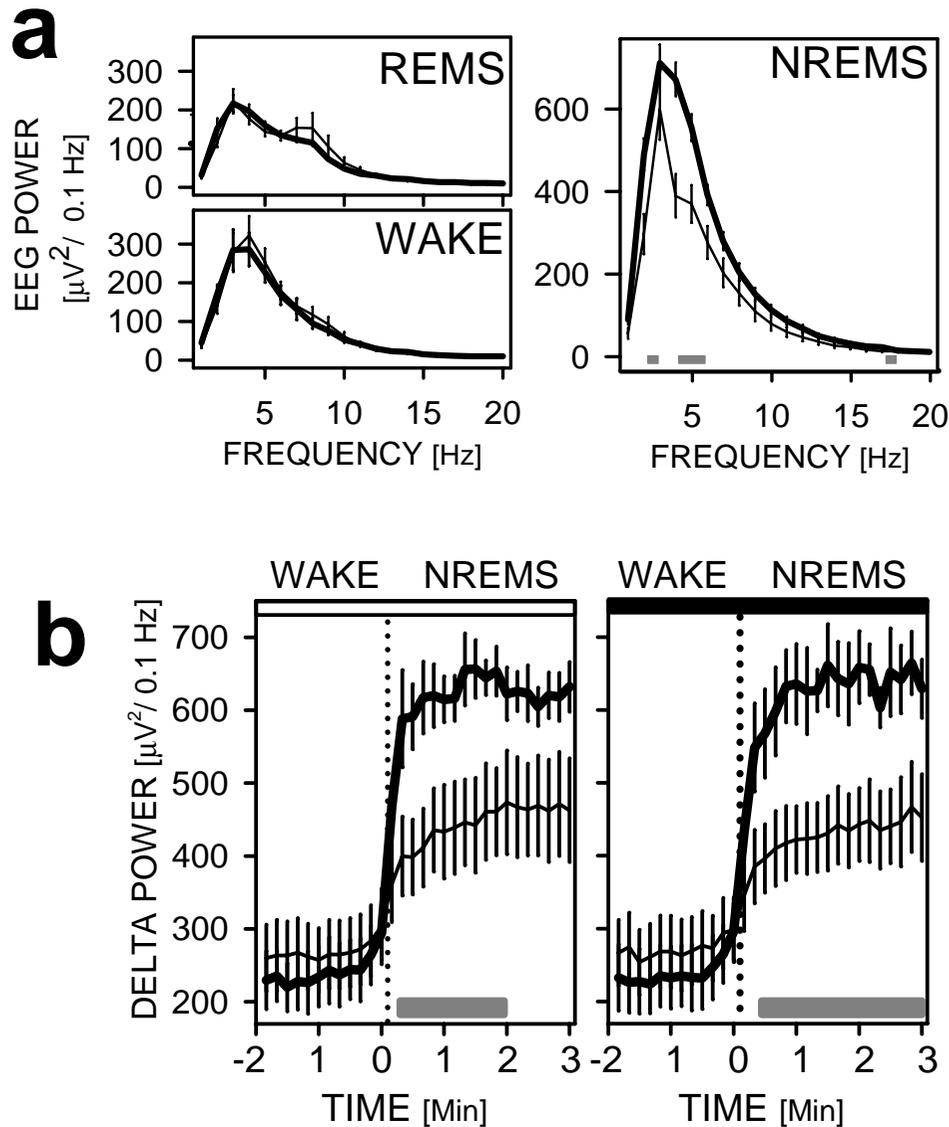
Post-hoc comparisons: \*  $P < 0.05$  vs. wild type, unpaired t-tests; #  $P < 0.05$  vs. dark period, within same genotype, paired t-tests. Differences represent the mean of individual light-dark differences.

min,  $P = 0.053$ , unpaired t-test), NREMS bouts were longer ( $3.8 \pm 0.3$  vs.  $2.6 \pm 0.1$  min,  $P < 0.001$ , unpaired t-test), and EEG delta power in NREMS was higher in *cry1,2<sup>-/-</sup>* compared to *cry1,2<sup>+/+</sup>* controls ( $594 \pm 21$  vs.  $448 \pm 52 \mu V^2/0.1$  Hz,  $P < 0.04$ , unpaired t-test; Figure 3). The decrease in sleep time prior to the onset of the dark period (Figure 1) was still present in DD in *cry1,2<sup>+/+</sup>* mice; sleep time decreased in the latter half of the subjective day and minimum average sleep time was reached immediately after the onset of the subjective night (Figure 3). The anticipatory decrease in sleep time that occurred prior to the onset of dark in *cry1,2<sup>-/-</sup>* mice in LD did not occur under DD conditions (Figure 3). Under DD conditions, less REMS was present in *cry1,2<sup>-/-</sup>* mice than in *cry1,2<sup>+/+</sup>* mice ( $119 \pm 7$  vs.  $149 \pm 9$  min,  $P < 0.03$ , unpaired t-test) due to an increase in REMS in *cry1,2<sup>+/+</sup>* mice in DD ( $149 \pm 9$  min) relative to LD ( $113 \pm 10$  min,  $P < 0.03$ , paired t-test).

To further investigate the homeostatic regulation of sleep in *cry1,2<sup>-/-</sup>* mice, we assayed the compensatory response to a 6 h SD (see Figure 1). After SD, wild type mice displayed the typical increase in REMS and NREMS time, NREMS bout duration, and EEG delta power above baseline levels ([10,13] Table 2, Figure 1). In contrast, *cry1,2<sup>-/-</sup>* mice did not exhibit significant increases in REMS, NREMS time or NREMS bout duration after SD; only a brief increase in NREMS delta power was observed (lasting 1 h in *cry1,2<sup>-/-</sup>* vs. 5 h in wild type mice; Figure 1). The initial increase in delta power (relative to baseline), measured over the first recovery hour, was significantly smaller in *cry1,2<sup>-/-</sup>* than the increase observed in wild type mice (Table 2). Delta power (Figure 1, Table 2) and NREMS bout duration (Ta-

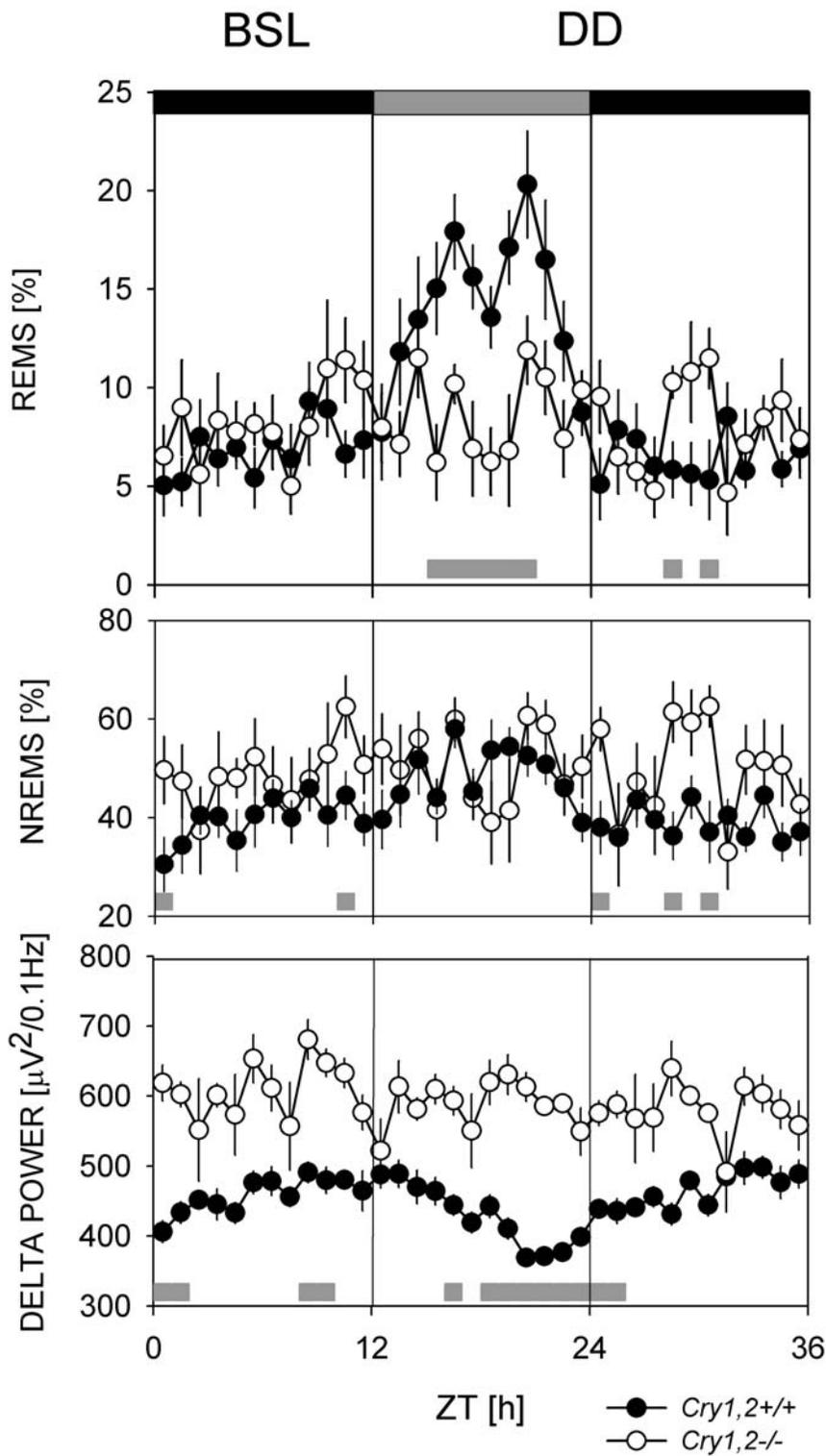
bles 1, 2), in *cry1,2<sup>-/-</sup>* mice is maintained at a level only attained by the wild type mice when their sleep pressure was highest, i.e., at the end of the active or dark period and after SD.

In wild type mice, EEG delta power still varied as a function of the sleep-wake history with high values at the end of the baseline dark period and after the SD and low values at the end of the light or major rest period (Figure 1). Presumably due to the altered sleep-wake distribution, the daily range of EEG delta power values was smaller in *cry1,2<sup>-/-</sup>* mice than in wild type mice (Table 1, Figure 1). We tested the assumption of a relationship between delta power and the sleep-wake history by using a mathematical method that predicts the level of EEG delta power occurring in individual NREMS bouts based on the 42 h sequence of 10-sec behavioral state scores for individual animals [10]. With this analytical tool, the time constants of the increasing delta power during wakefulness and its decrease during NREMS are estimated. For both genotypes, delta power in both baseline and recovery from SD could be reliably predicted on the basis of sleep-wake history (Figure 1), which is underscored by the highly significant correlations between empirical and simulated data ( $r = 0.91$  and  $0.87$  for *cry1,2<sup>+/+</sup>* and *cry1,2<sup>-/-</sup>*, respectively,  $P < 0.0001$  for both genotypes). Thus, in the absence of *cryptochromes*, NREMS delta power varied as a function of the prior sleep-wake history. However, in *cry1,2<sup>-/-</sup>* mice, a significantly shorter time constant for the increase (i.e., a faster build-up) of delta power was obtained ( $\tau_{\text{increase}}: 5.0 \pm 0.3$  h in *cry1,2<sup>+/+</sup>* vs.  $3.5 \pm 0.5$  h in *cry1,2<sup>-/-</sup>*;  $P < 0.05$ ; un-



**Figure 2**

EEG power in the 1–20 Hz range for NREMS, REM sleep (REMS) and wake during baseline. **(A)** EEG spectral power in *cry1,2<sup>-/-</sup>* (thick lines) and wild type mice (thin lines). Differences between the genotypes are limited to NREMS delta power. **(B)** Delta power (1–4 Hz) during wake-to-NREMS transitions in the baseline light (left) and dark (right) period. Gray horizontal bars underneath the curves indicate significant genotype differences ( $P < 0.05$ ; *post-hoc* t-tests). Error bars span  $\pm 1$  SEM.



**Figure 3**

Time course of sleep and NREMS EEG delta power during constant dark conditions (DD). Layout and symbols are same as in Figure 1. Gray bars at the bottom of each panel mark intervals with significant genotype differences ( $P < 0.05$ , *post-hoc* t-tests). The subjective day is marked with a gray horizontal bar at the top of the upper panel. The first 12-h represents the last dark-period under baseline (BSL) light-dark conditions.

**Table 2: Effect of 6 h SD on sleep time and delta power in *cry1,2<sup>+/+</sup>* and *cry1,2<sup>-/-</sup>* mice.**

NREMS amount [min]			
	baseline	post-SD	difference
<i>cry1,2<sup>+/+</sup></i>	264 ± 9	297 ± 15#	33 ± 8
<i>cry1,2<sup>-/-</sup></i>	312 ± 9*	318 ± 9	5 ± 4*
NREMS bout duration [min]			
	baseline	post-SD	difference
<i>cry1,2<sup>+/+</sup></i>	2.6 ± 0.1	3.3 ± 0.3#	0.7 ± 0.3
<i>cry1,2<sup>-/-</sup></i>	4.2 ± 0.4*	3.7 ± 0.2	-0.4 ± 0.3*
NREMS Delta power [ $\mu\text{V}^2/0.1 \text{ Hz}$ ]			
	baseline	post-SD	difference [%]
<i>cry1,2<sup>+/+</sup></i>	407 ± 59	704 ± 125#	170 ± 14
<i>cry1,2<sup>-/-</sup></i>	619 ± 67*	805 ± 40#	134 ± 8*
REMS amount [min]			
	baseline	post-SD	difference
<i>cry1,2<sup>+/+</sup></i>	52 ± 6	63 ± 3#	11 ± 7
<i>cry1,2<sup>-/-</sup></i>	53 ± 4	58 ± 1	5 ± 3

Post-hoc comparisons: \*  $P < 0.05$  vs. wild type, unpaired t-test; #  $P < 0.05$ , vs. baseline day, within same genotype, paired t-test. Delta power was calculated over the first hour after the SD (ZT6-ZT7). REMS and NREMS amount and bout duration were calculated over 12 hours (ZT6-ZT18). Differences indicate mean individual post-SD – baseline differences except for delta power which is calculated as percentage of baseline.

paired t-test), whereas the time constant describing the decline of delta power during NREMS did not differ ( $\tau_{\text{decrease}}$ :  $1.7 \pm 0.2$  h in *cry1,2<sup>+/+</sup>* vs.  $1.8 \pm 0.3$  h in *cry1,2<sup>-/-</sup>*).

#### Circadian gene expression in the brain of *cry1,2<sup>-/-</sup>* mice

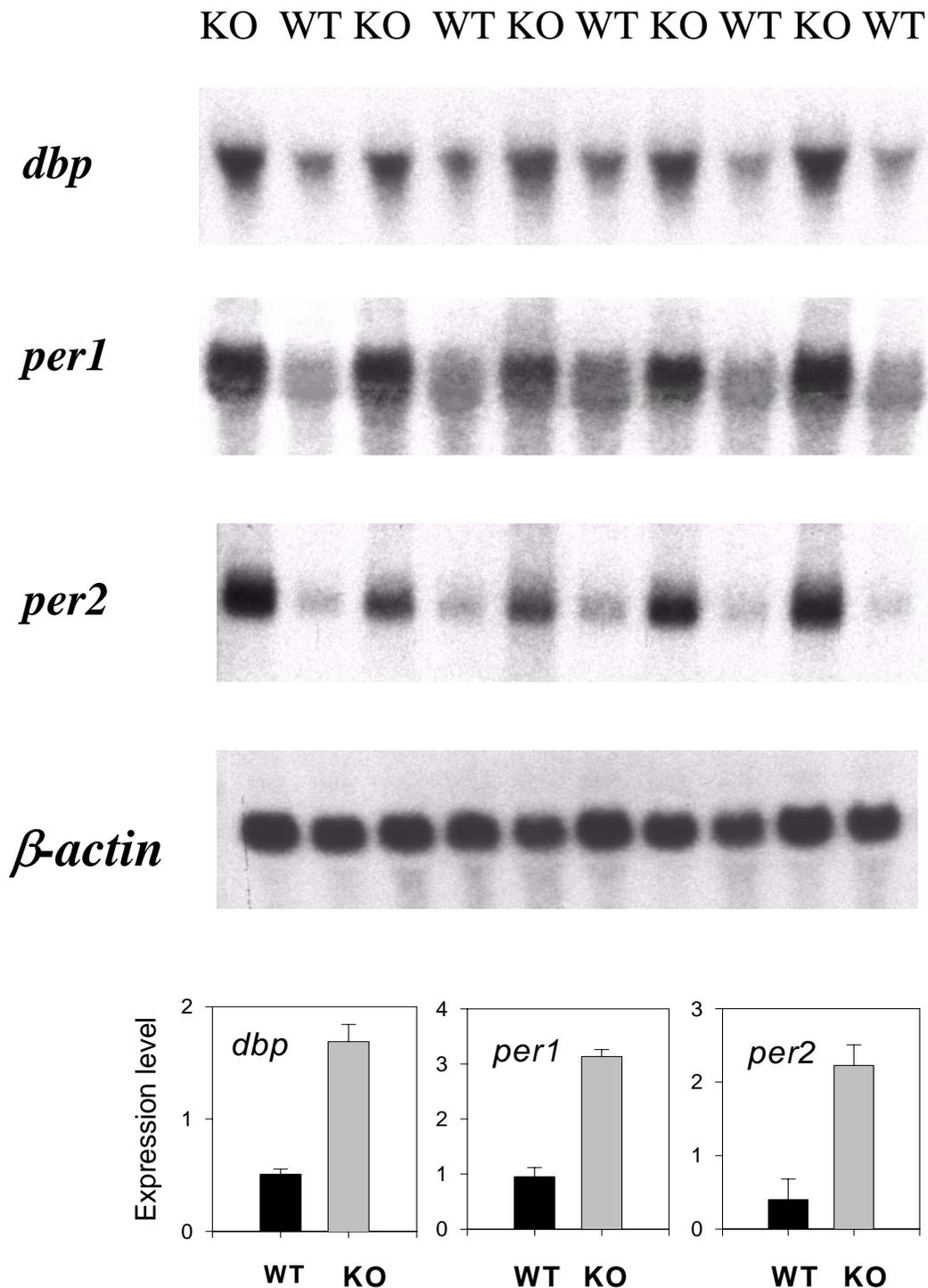
Deletion of the *cryptochromes* disinhibits the transcriptional activation of CLOCK:BMAL1 and NPAS2:BMAL1 target genes, resulting in increased levels of their transcripts in the SCN, liver, and retina [20,23,25]. We assayed the expression in the brain of three genes that are known targets of *cryptochrome* mediated transcriptional inhibition: *albumin D-binding protein (dbp)* [26], *period (per)1*, and *per2* [20,23,27], in the middle of the daily light period. Confirming the earlier studies in other tissues, mRNA levels for all three genes were higher in the brains of *cry1,2<sup>-/-</sup>* mice compared to wild type mice (Figure 4). This relative increase was highly significant for all three genes (*dbp*, *per1*: 3.3-fold; *per2*: 5.6-fold;  $P < 0.0003$ , unpaired t-tests,  $n = 5/\text{genotype}$ ).

#### Sleep deprivation-induced differences in circadian gene expression

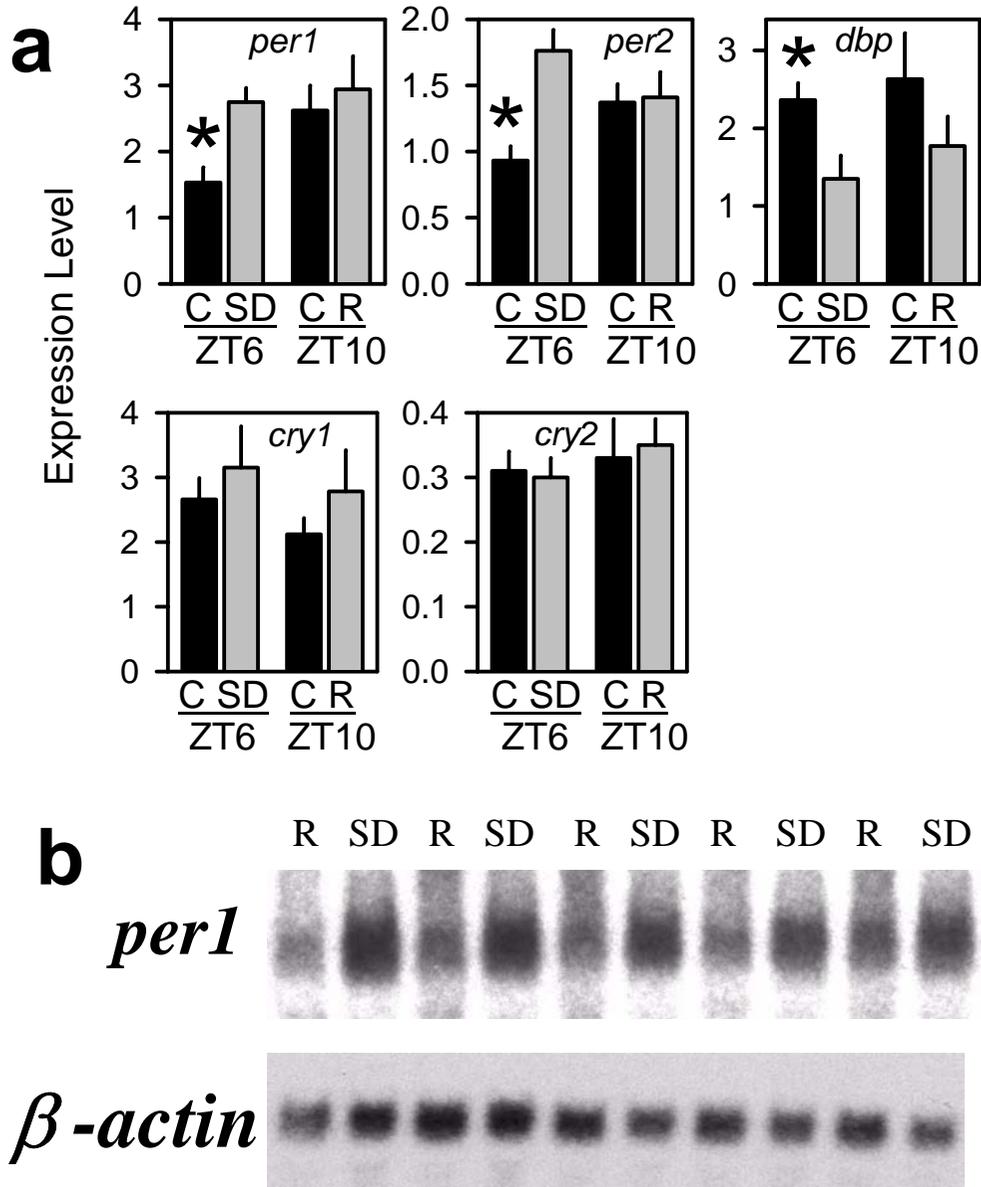
Messenger RNA levels for *per1,2*, *cry1,2*, and *dbp* were quantified by RT-PCR in three brain areas (cerebral cortex, basal forebrain, and hypothalamus) from C57BL/6 mice

that were sacrificed immediately after 6 h of SD or after 4 h of recovery sleep (ZT10). Significant differences in expression were observed only in the cortex (Figure 5A) and not in hypothalamus or basal forebrain (data not shown). Both *per1* and *per2* mRNA levels were higher immediately after SD compared to controls, *cry1,2* expression did not change, and *dbp* mRNA decreased significantly (Figure 5A). After 4 h of recovery sleep, *per1,2* and *dbp* expression returned to the normal levels for that time of day (ZT10; Figure 5A). We also measured the expression of five other circadian genes: *bmal1*, *clock*, *npas2*, *per3*, and *casein kinase-1-ε (csnk1ε)* in the cortex; the levels of these five mRNAs were not affected by SD (data not shown).

As confirmation of our results in the mouse, we determined the expression of *per2* and *per1* in the rat by RT-PCR and Northern analysis, respectively. In a similar experimental paradigm, rats were sleep deprived for 6 h and then either sacrificed immediately after the SD (ZT6), or after 2 h of recovery sleep (ZT8;  $n = 5/\text{group}$ ). The cortex-specific increase in *per2* mRNA was confirmed by the RT-PCR analysis and, as in the mouse, *per2* expression returned to basal levels after a period of recovery sleep (data not shown). Northern analysis confirmed that recovery sleep was associated with a decline in *per1* mRNA relative



**Figure 4**  
 Whole-brain mRNA levels for *dbp*, *per1*, and *per2* in *cry1,2<sup>-/-</sup>* and wild type mice. Levels of all three genes are elevated in *cry1,2<sup>-/-</sup>* mice (KO) relative to wild type (WT) controls at ZT6 when *dbp*, *per1*, and *per2* mRNAs are lowest in the forebrain of wild type mice [37]. In the lower three panels mean ( $\pm$  1 SEM) expression levels are depicted.  $\beta$ -actin expression was used as an internal standard.



**Figure 5**

Sleep deprivation alters mRNA levels of *per1*, *per2*, and *dbp*. **(A)** RT-PCR analysis of the expression of five 'clock'-genes in the mouse cortex across four experimental conditions [C = control; R = recovery sleep; SD = sleep deprived; ZT = Zeitgeber time (i.e., 6 or 10 h after light-onset)]. *g3pdh* expression was used as an internal standard. Bars depict mean  $\pm$  1 SEM. Asterisks denote significant differences ( $P < 0.05$ ) between the experimental and corresponding control group (Student-Newman-Keuls *post-hoc* tests; 1-way ANOVA factor 'condition':  $P < 0.05$ , for *per1*, *2*, and *dbp* only;  $n = 7$ /condition) **(B)** *per1* mRNA falls significantly in rat cortex during a 2-h recovery period (R) subsequent to 6 h SD ending at ZT6 ( $P < 0.05$ , t-test). Northern analysis was performed on cortex of five sleep-deprived rats and five rats that were allowed 2 h of recovery sleep (ZT8).

to the level of expression reached at the end of the SD (Figure 5B).

## Discussion

Upon release into constant darkness *cry1,2<sup>-/-</sup>* mice immediately become arrhythmic at the behavioral level [22,23], at the level of SCN electrophysiology [28], and at the cellular/molecular level [23]. Of the available mouse models for circadian dysfunction, only *per1,2* double mutant mice [29], *bmal1* knockout mice [30], and mice with an ablation of the SCN [31] show a similarly dramatic phenotype. Thus, *cry1,2<sup>-/-</sup>* mice appear to be a suitable model for studies of the regulation of sleep in the absence of an intact circadian clock.

Under light / dark (LD) conditions, running wheel activity patterns and, as we show here, the distribution of sleep in *cry1,2<sup>-/-</sup>* mice still exhibit diurnal variation. LD cycles can influence the expression of sleep by entraining the circadian pacemaker that drives the diurnal rhythm of sleep and/or by directly affecting the expression of sleep, thereby 'masking' the influence of the pacemaker on sleep. Masking seems to be the mechanism by which light drives these rhythms under LD conditions in *cry1,2<sup>-/-</sup>* mice [22], since the daily modulation of NREMS that occurs in *cry1,2<sup>-/-</sup>* mice under a light/dark cycle immediately disappears upon placement in constant darkness ([22], Figure 3). At the molecular level, *per2* expression (but not that of *per1*) is rhythmic in the SCN of *cry1,2<sup>-/-</sup>* mice under LD conditions. Upon release into constant dark conditions, *per2* rhythmicity disappears concomitant with the immediate loss of behavioral rhythmicity, suggesting a role for 'light-driven' *per2* expression in generating behavioral rhythms [23].

The most striking and unexpected finding of the current study is that, under baseline conditions, *cry1,2<sup>-/-</sup>* mice exhibit all the hallmarks of high NREMS pressure, including more consolidated NREMS, increased NREMS time, and higher levels of EEG delta power relative to wild type mice that were attained immediately after NREMS onset. The failure of *cry1,2<sup>-/-</sup>* mice to exhibit a robust increase in any of these measures after 6 h SD is consistent with the interpretation that these mice are already under high NREMS pressure during baseline conditions. Determination of the time constants that most accurately describe the dynamics of NREMS delta power revealed that during wake, the propensity for high NREMS delta power increases during wake in *cry1,2<sup>-/-</sup>* mice at a faster rate than in wild type mice. This could help explain why NREMS delta power is chronically high in *cry1,2<sup>-/-</sup>* mice. The coincidence of high NREMS time and chronically high delta power in *cry1,2<sup>-/-</sup>* mice is all the more striking when one considers that during NREMS, the drive for NREMS should dissipate and result in lower delta power [14,32].

These findings in *cry1,2<sup>-/-</sup>* mice contrast with the findings of sleep studies in animals that are rendered arrhythmic by lesioning of the SCN. In nocturnal rodents, lesioning the SCN results in more fragmented sleep, with lower EEG delta power, but leaving the daily sleep time unchanged [6–8,31]. Lesioning the SCN in a diurnal primate, the squirrel monkey, did result in an increase in NREMS time, but sleep was more fragmented, with a higher proportion of 'light' NREMS [5]; i.e., with lower overall levels of EEG delta power. Furthermore, the homeostatic response to sleep deprivation does not seem to be altered in SCN-lesioned rodents [6–8]. Thus, the sleep characteristics of *cry1,2<sup>-/-</sup>* mice do not support the concept of *cry1,2<sup>-/-</sup>* mice as simply a genetic model for ablation of the circadian clock in the SCN. Together, these unexpected results are compatible with a role for *cryptochromes* in the homeostatic regulation of sleep in addition to their role in generating circadian rhythms.

Recent observations, including the current report, suggest a complex interrelationship between homeostatic and circadian influences on sleep at the molecular level. Deletion of the *cycle* gene in *Drosophila* produces flies that have an exaggerated homeostatic response to rest deprivation in addition to their lack of circadian rhythmicity [16]. In a striking parallel to our current results, flies with a mutation in the *cryptochrome* gene also exhibit increased rest time as well as a reduced compensatory response to rest deprivation (P. Shaw, personal communication). The *clock* mutation in mice, which has a profound effect on circadian rhythmicity [33], decreases NREMS time and consolidation under baseline conditions [34]. The *clock* sleep phenotype is the inverse of the sleep characteristics we report here for the *cry1,2<sup>-/-</sup>* mice and is thus consistent with CLOCK and CRY1,2 being positive and negative transcriptional regulators, respectively. *Albumin D-binding protein (Dbp)* is a transcription factor whose expression is under the direct transcriptional control of CLOCK:BMAL1 [26]. Deletion of the *dbp* gene, which results in a shortening of the circadian period [35], also results in decreased sleep consolidation and NREMS delta power [36].

We assume that the effects on sleep we observed in *cry1,2<sup>-/-</sup>* mice are a result of a lack of *cryptochrome*-dependent inhibition of the transcriptional activation provided by the bHLH-PAS heterodimers CLOCK:BMAL1 and NPAS2:BMAL1 [19,20,37], although *cryptochromes* also play a role in stabilizing and nuclear sequestration of PER proteins [38], and in photoreception [39]. Lack of *cryptochromes* results in increased mRNA levels of CLOCK/NPAS2:BMAL1 target genes, including the circadian genes *per1* and *per2* [20,23]. The expression of these two genes is viewed as a state variable of the molecular circadian clock or a marker of CLOCK/NPAS2:BMAL1-induced transcription, although at least *per1* transcription can also be (rap-

idly) induced by light [40,41], through a CREB-dependent signaling pathway [42–44]. The observation of high brain levels of *per1,2* transcripts under baseline conditions in *cry1,2*<sup>-/-</sup> mice raises the possibility that these or other CLOCK/NPAS2:BMAL1 target genes are involved in the homeostatic regulation of sleep. The observation of elevated *per* gene expression in the cortex of sleep-deprived rats and mice (Figure 5) supports this hypothesis.

The increase in *per* expression after the sleep deprivation was specific to the cerebral cortex, although it cannot be ruled out, based on the present study, that circadian gene expression changes with sleep-wake history in specific nuclei within the other two regions examined; i.e., the hypothalamus and basal forebrain. A surprisingly small number (~0.5%) of the ~10,000 genes screened by mRNA differential display and cDNA microarrays in the cortex to date change their expression with sleep deprivation [45]. It is intriguing and encouraging that, of the initial genes we assayed, three changed their expression with sleep deprivation (*per1*, *per2*, *dbp*), none of which were identified in the aforementioned screens. *cry1,2* expression at the mRNA level did not change with sleep deprivation in wild type mice. This observation does not necessarily obviate a direct role for CRY proteins in mediating a response to sleep deprivation. CRY proteins may play a role at steady state levels or there may be post-translational changes in the functioning of CRY proteins in association with sleep deprivation, such as phosphorylation state, ubiquitination [38], or intracellular localization [46] of the protein, all of which are regulated dynamically, at least *in vitro*. In the liver, CRY protein oscillations are not necessary for circadian oscillations of target transcripts, as CRY proteins are present in excess of PER and oscillations in the latter produce rhythmicity [46]. A similar situation might exist in the cortex.

The high *per* levels in *cry1,2*<sup>-/-</sup> mice and the low *per* levels in *clock* mutant mice [26,47] correlate with their contrasting sleep phenotype (see above; [34]). In this context, the sleep abnormalities in *dbp*<sup>-/-</sup> mice might also be related to a reduction in *per* expression since, at least *in vitro*, DBP can amplify the CLOCK:BMAL1-induced transcription of *per* [48], but it is not known whether *per* transcript levels are altered in *dbp*<sup>-/-</sup> mice. Apart from the present observations in sleep-deprived rats and mice, several other reports confirm that cortical levels of *per* expression in wild type animals are high at times when sleep drive is high, irrespective of the phase at which the circadian expression of *per* peaks in the SCN. Thus, in both nocturnal and diurnal species, *per* expression in the cortex is maximal in conjunction with the major waking episode [37,49,50]. Under conditions where the phase (methamphetamine administration, restricted feeding) or distribution (circadian splitting) of locomotor activity is altered, *per* expres-

sion in the cortex parallels the overt rhythm of wakefulness, whereas the circadian oscillation of *per* gene expression in the SCN remains unaffected [49,51,52]. Thus, in contrast to its role in the SCN, PER protein in the cortex is not a component of a self-sustaining circadian oscillator [53]. Instead, *per* expression in the cortex seems to follow sleep-wake history, consistent with the hypothesis that it is related to homeostatic regulation of sleep. However, in the current study and those cited above, the expression of *per* genes was studied at the mRNA level. PER protein level may be affected differentially from that of *per* mRNA and levels of the PER protein are reduced in *cry1,2*<sup>-/-</sup> mice due to reduced stability of PER proteins in the absence of heterodimerizing CRY partners [19,38,46].

## Conclusions

In the discussion, we have focused on *per1,2* mRNAs as transcriptional targets of *cry1,2* because the expression patterns of these circadian genes have been widely described and because their transcriptional control by CRY proteins and by CLOCK/NPAS2:BMAL1 has been well established. At least 90 genes are regulated in a similar fashion by NPAS2:BMAL1 [37] and the identity of the target genes critical for the NREMS phenotype in *cry1,2*<sup>-/-</sup> mice remains to be determined. In the absence of *cry1,2*, the stability and overall level of PER proteins, particularly in the nucleus of the cell, is reduced [19,38,46]. It would therefore be interesting to observe sleep in *per1,2* single and double mutant mice, the latter of which have been subjected to behavioral observation [29] but not to sleep EEG studies. *per1* single knockout mice and mice expressing a non-functional PER2 protein both exhibit subtle differences from wild type in the homeostatic rebound after SD [54], an observation compatible with the hypothesis that these genes are correlates of sleep homeostasis. In addition, measurement of the effects of sleep deprivation on the expression of *cry1,2* and *per1,2* genes at the protein level will provide critical information. Finally, from a functional perspective, it is interesting that CLOCK/NPAS2:BMAL1 transcriptional activity is sensitive to redox state [24]. This transcriptional activity might thus provide a link between neuronal activity and an energy regulatory function for NREMS, as has been suggested previously [55].

## Methods

### Sleep studies

Mice were generated by mating *cry1* and *cry2* single knockout mice, both of mixed background (ca. 3/4 C57BL/6 – 1/4 129/Sv; [23]) to generate double heterozygotes that were interbred to generate double knockouts (*cry1,2*<sup>-/-</sup>) and wild type controls (*cry1,2*<sup>+/+</sup>). Eight wild type and 6 *cry1,2*<sup>-/-</sup> male mice were surgically prepared for EEG and electromyographic (EMG) recordings as described previously [56]. Following two weeks of post-surgical recovery,

mice were isolated for recordings in sound-attenuated chambers. The experiments were conducted under an LD12:12 cycle (lights-on; i.e., Zeitgeber Time ZT0, at 0600 h). Twenty-four hour baseline recordings, starting at ZT0, were followed by a 6 h sleep deprivation (SD) starting at ZT0. The sleep deprivations in this experiment and the other two experiments (see below) were performed by the introduction of novel objects into the cage or by gentle handling. In addition to the SD experiment, animals were subjected to one day of baseline recording in constant darkness separated from the SD experiment by 72 hours. All experimental procedures complied with institutional and NIH guidelines.

Digitized EEG and integrated EMG were stored in 10-s epochs and classified as NREMS, REM sleep (REMS), or wakefulness by visual inspection. The EEG was subjected to a Fast-Fourier-Transformation yielding power spectra between 0–20 Hz. Delta power was calculated as the average EEG power in the delta (1–4 Hz) frequencies for epochs scored as NREMS. For visual representation, hourly delta-power values (Figure 1) were expressed relative to an individual mean (i.e., 24-h baseline mean) before transforming back to absolute values to capture both the highly reproducible individual time course of delta power and the genotype differences in absolute values [10]. Statistical evaluation of genotype differences were based, however, on the absolute EEG values. Post-SD delta power was compared to baseline during the first hour of spontaneous sleep subsequent to SD, while NREMS amount and bout duration (Table 2) were compared over 12 hours (ZT6-ZT18), in accordance with the distinct dynamics of the compensatory responses of these variables to SD [57]. NREMS bouts were defined as periods of NREMS initiated by three consecutive 10-second epochs of NREMS and terminated by three consecutive epochs not classified as NREMS. EEG delta power changes at transitions between wakefulness and NREMS were measured during those transitions characterized by at least 12 consecutive 10-s epochs of wake followed by at least 18 consecutive 10-epochs of NREMS.

A mathematical algorithm was used to quantify the sleep-wake dependent dynamics of delta power during both baseline and recovery from SD [10]. In this algorithm, delta power decreases during NREMS and increases during wakefulness according to saturating exponential functions, the time constants of which are estimated by minimizing the square of the differences between empirical (delta power) data and the values produced by the mathematical functions. The two time constants (for the increase and decrease of delta power) that resulted in the smallest deviation from empirical values within each individual were used to statistically assess genotype effect on the dynamics of delta power [10].

### Northern analysis

Five *cry1,2*<sup>-/-</sup> mice and 5 wild type controls were sacrificed in the middle of the light period (ZT6). Brains were rapidly removed and frozen on dry ice. Total RNA was extracted and Northern analysis was performed with 10 µg whole brain total RNA as previously described [58].

Ten Wistar rats were sleep deprived for 6 h beginning at light-onset (ZT0). Five rats were sacrificed at ZT6 at the end of the SD and five were allowed to recover for 2 h following the SD and were sacrificed at ZT8. Rats were monitored by EEG/EMG throughout this period as described [59]. Brains were rapidly removed, dissected and frozen, and Northern analysis performed.

### Quantitative real-time Reverse-Transcriptase Polymerase-Chain-Reaction (RT-PCR) analyses

Four experimental groups of male C57BL/6J mice were studied: 1) sleep deprived from light-onset (ZT0) to ZT6; 2) control mice for the SD group; 3) 6 h SD (ZT0-ZT6) followed by 4-h recovery sleep (ZT6-ZT10); and 4) control mice for the recovery group (n = 7 mice/group). Animals were sacrificed by decapitation. Brains were rapidly removed and the cerebral cortex, basal forebrain, and hypothalamus were dissected. After a mid-sagittal cut, the entire cortical tissue was peeled off and separated from the hippocampus and underlying diencephalon. Striatal tissue was also removed. From each animal, quantitative real-time PCR determinations of 5 target genes (*cry1,2*, *per1,2*, and *dbp*) and a reference cDNA (glyceraldehyde-3-phosphate dehydrogenase, *g3pdh*) were made from the cortex, basal forebrain, and hypothalamus (primer/probe sequences available upon request). For five other targets (*bmal1*, *clock*, *npas2*, *per3*, and *csnk1e*), expression was quantified in the cortex only. To confirm the specificity of the nucleotide sequences chosen for the primers and probes and the absence of DNA polymorphisms, BLASTN searches were conducted against the dbEST and nonredundant set of Genbank, EMBL, and DDBJ databases. Dual color fluorescence was detected using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Corp., Foster City, CA). For each experimental sample, the amount of the target and *g3pdh* reference was determined from the standard curve (range of 0.2–200 ng total RNA) measured in the same assay. A normalized value was obtained by dividing the target cDNA amount by the *g3pdh* reference. For details see [59].

### Authors' contributions

CPS and AS generated the *cry1,2*<sup>-/-</sup> mice and provided some preliminary data on microarray gene expression profiles in the brains of these mice. JPW and DME conceived and implemented sleep studies in *cry1,2*<sup>-/-</sup> mice. PF analyzed sleep EEG data and conceived, with BFO, AT, and TSK, the molecular studies. BFO, AT, and TSK per-

formed gene expression assays on brain tissues from mice and rats. JPW and PF drafted the manuscript. All authors read and approved the manuscript.

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