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## Altered Circadian and Homeostatic Sleep Regulation in Prokineticin 2-Deficient Mice

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### Abstract

**Study Objectives**—Sleep is regulated by circadian and homeostatic processes. Recent studies with mutant mice have indicated that circadian-related genes regulate sleep amount, as well as the timing of sleep. Thus a direct link between circadian and homeostatic regulation of sleep may exist, at least at the molecular level. Prokineticin 2 (PK2), which oscillates daily with high amplitude in the suprachiasmatic nuclei (SCN), has been postulated to be an SCN output molecule. In particular, mice lacking the PK2 gene (PK2<sup>-/-</sup>) have been shown to display significantly reduced rhythmicity for a variety of circadian physiological and behavioral parameters. We investigated the role of PK2 in sleep regulation.

**Design**—EEG/EMG sleep-wake patterns were recorded in PK2<sup>-/-</sup> mice and their wild-type littermate controls under baseline and challenged conditions.

**Measurements and Results**—PK2<sup>-/-</sup> mice exhibited reduced total sleep time under entrained light-dark and constant darkness conditions. The reduced sleep time in PK2<sup>-/-</sup> mice occurred predominantly during the light period and was entirely due to a decrease in non-rapid eye movement (NREM) sleep time. However, PK2<sup>-/-</sup> mice showed increased rapid eye movement (REM) sleep time in both light and dark periods. After sleep deprivation, compensatory rebound in NREM sleep, REM sleep, and EEG delta power was attenuated in PK2<sup>-/-</sup> mice. In addition, PK2<sup>-/-</sup> mice had an impaired response to sleep disturbance caused by cage change in the light phase.

**Conclusions**—These results indicate that PK2 plays roles in both circadian and homeostatic regulation of sleep. PK2 may also be involved in maintaining the awake state in the presence of behavioral challenges.

### Keywords

Sleep; prokineticin 2; sleep homeostasis; circadian genes; EEG; behavioral challenge

### INTRODUCTION

Homeostatic and circadian processes have been postulated to regulate sleep.<sup>1</sup> The homeostatic process determines the duration and intensity of sleep, whereas the circadian process, driven by the suprachiasmatic nuclei (SCN) of the hypothalamus in mammals, determines the timing of sleep.<sup>2</sup> Recently, studies of circadian gene mutant mice have revealed that circadian genes

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affect not only circadian sleep distribution, but also the homeostatic regulation of sleep. Clock gene mutant mice sleep 2 h less than wild-type mice daily,<sup>3</sup> whereas *Bmal1/Mop3* deficient mice and double *Cry1* and *Cry2* deficient mice display 1.5 h and 1.8 h increases in total daily sleep amount,<sup>4,5</sup> respectively. In wild-type mice, sleep deprivation (SD) is followed by a compensatory sleep increase. Mice lacking both *Cry1* and *Cry2* did not exhibit rebound in non-rapid eye movement (NREM) and rapid eye movement (REM) sleep time,<sup>5</sup> while mice lacking *Clock* or *Bmal1/Mop3* have a reduced REM sleep rebound in response to SD.<sup>3,4</sup> Mutation of the *DBP* gene, encoding a clock-controlled transcription factor, also leads to a decrease in REM sleep amount, in addition to a reduction in circadian amplitude of NREM sleep amount.<sup>6</sup>

Prokineticin 2 (PK2) is a postulated SCN output molecule that regulates circadian rhythm.<sup>7–9</sup> PK2 oscillation in the SCN is regulated by *CLOCK* and *BMAL1* binding to E-boxes on the PK2 promoter.<sup>7</sup> PKR2, the G-protein coupled receptor activated by PK2, is expressed in the primary SCN output target regions, including the paraventricular and paratenial nuclei of the thalamus, the paraventricular nucleus of the hypothalamus, the dorsal medial nucleus of the hypothalamus, the lateral septal nucleus, and the bed nucleus of the stria terminalis.<sup>7,10</sup> Recently, PK2-deficient (*PK2*<sup>-/-</sup>) mice have been shown to display significantly reduced rhythmicity for a variety of physiological and behavioral parameters, including locomotor activity, body temperature, food intake, circulating glucocorticoid and glucose levels, and the expression of peripheral clock genes.<sup>11</sup> Thus, PK2 is critical for the maintenance of robust circadian rhythms, acting as an SCN output factor.

In this study, we investigated the sleep parameters of *PK2*<sup>-/-</sup> mice under both baseline and sleep deprived conditions, as well as sleep response to environmental challenges.

## MATERIALS AND METHODS

### Animals

*PK2*<sup>-/-</sup> mice and their littermate wild-type controls on a C57BL/6J : 129/Ola background were used for these experiments. The breeding and genotyping of *PK2*<sup>-/-</sup> mice were performed as described elsewhere.<sup>11</sup> All mice were 11 to 20 weeks old and weighed 22–28 g. All procedures regarding the care and use of animals were in accordance with institutional guidelines.

### Surgery Procedures

Mice were anesthetized with ketamine-xylazine (100 and 10 mg/kg, i.p.), and standard sterile surgical and stereotaxic procedures were employed for implant placements. The cranium was exposed, and 4 burr holes were drilled, anterior and posterior to bregma, bilaterally (AP 1.1, ML  $\pm$  1.45 and AP - 2.5, ML  $\pm$  1.45). Stainless steel screws were then inserted into these holes to record electro-encephalographic (EEG) signals. A pair of Teflon coated stainless steel wires with exposed ends were inserted into the dorsal neck muscles to record electromyogram (EMG) signals. Electrodes were cemented to the skull with dental acrylic. All electrodes were then connected to a plastic connector (Plastics One, Roanoke, VA) that was secured to the skull using dental acrylic. After surgery, animals were housed in individual cages (20  $\times$  20  $\times$  30 cm) with a 12 h : 12 h light : dark (LD) cycle (lights on at 07:00 and off at 19:00) and at a constant temperature (22–24°C). They had ad libitum access to food and water. They were allowed 10–14 days to recover, during which they were habituated to the recording conditions, with the home cage being the recording cage.

### Sleep Recording

The mice were connected to a wire tether / commutator system (Plastics One, Roanoke, VA) for the collection of EEG and EMG signals. This swivel system allowed the animal unrestricted movement throughout the recording cage. After at least 5 days of adaptation to the recording

environment, a 48-hour baseline EEG/EMG recording was collected on a LD cycle with lights on at 7:00 and off at 19:00. Mice were recorded concurrently in matched littermate pairs of PK2<sup>-/-</sup> and wild-type controls. EEG/EMG signals were amplified using a Grass Telefactor Model 15LT with 15A94 amplifier (Grass Instruments, West Warwick, RI) and filtered (EEG: 0.3–100 Hz, EMG: 30–300 Hz) before being digitized at a sampling rate of 128 Hz and stored on a computer.

### Sleep Deprivation

After baseline recording, animals were sleep deprived for 6 hours during the last half of the light phase (13:00–19:00). For this purpose, animals and the EEG/EMG recordings for signs of sleep were continuously observed, and various objects (pieces of paper, plastic tubes) were introduced into the cage, or gentle handling was used to arouse the mouse as soon as it adapted a sleeping posture or showed EEG signs of sleep.<sup>12</sup> At dark onset (19:00), sleep deprivation was terminated and animals were left to sleep freely while being recorded for 24 h (from 19:00 to 19:00 on the next day).

### Cage Change

To examine the ability of mice to maintain wakefulness in a novel environment, we recorded the EEG after cage change.<sup>13,14</sup> A new set of animals were used for cage change studies. Mice were allowed to adapt to recording and baseline sleep data were collected. Without change of LD cycle, mice were then transferred from their habitual home cage to clean cages with fresh bedding and new nesting material at 13:00, and sleep-wake behaviors were assessed over the next 12 hours after the cage change (from 13:00 to 01:00 on the next day) with food and water freely available.

### Analysis of Sleep Data

After sleep data were collected, EEG/EMG records were scored semiautomatically by using the SleepSign scoring system (Kissei Comtec America, Irvine, CA) in 4-sec epochs scored as wake, REM sleep, and NREM sleep on the basis of standard criteria of rodent sleep.<sup>15–17</sup> This preliminary scoring was visually inspected and corrected when appropriate. The distribution and amount of the behavioral states were analyzed by expressing them as a percentage of recording time. Awake or sleep bouts were defined as a period of each state that was initiated by 2 consecutive 4-sec epochs and terminated by 2 consecutive 4-sec epochs classified as a different state from that of the bout. Mean duration of each state during the light or dark period was calculated by dividing the total time by the number of corresponding bouts. EEG spectral power was tallied in 0.5 Hz bins using Fast Fourier Transformation (FFT) of each 4-sec epoch. To account for individual differences in the EEG signal, power density value for each frequency bin was expressed as a proportion of the mean of total power across all frequency bins for NREM or REM sleep (e.g., the mean power of 0.5–25 Hz over a 24 hour period).<sup>18</sup> Delta power was determined by averaging the power density values from 1.0 to 4.0 Hz, and theta power was determined by averaging the power density values from 5.0 to 9.0 Hz. NREM and REM sleep latencies were calculated as the time from an intervention (cage change) to the first epoch of NREM or REM sleep. In addition, in order to determine differences from baseline sleep and sleep loss during sleep deprivation, we examined the percentage change relative to baseline value ( $100 \times [\text{treatment} - \text{baseline}] / \text{baseline}$ ) and the percentage sleep regained relative to the amount lost ( $100 \times [\text{recovery} - \text{baseline}] / \text{amount of sleep lost during sleep deprivation}$ ) for the recovery 12-h dark and recovery 12-h light periods after sleep deprivation (Figure 4D and E) and the 6-hr light period after cage change (Figure 5E).<sup>4,14</sup>

## Statistical Analysis

All results were expressed as means  $\pm$  s.e.m. All main effects of the factors “genotype” (PK2<sup>-/-</sup> vs WT), “time” (baseline vs recovery, or baseline vs cage change) were analyzed by 2-way ANOVA for repeated measures. When main effects were present, post hoc Bonferroni tests were performed to evaluate further differences between genotypes. In a few instances, comparisons between WT and PK2<sup>-/-</sup> mice were determined using unpaired, two-tailed Student’s *t* tests.

## RESULTS

### Baseline Sleep-Wake Patterns under LD Cycles

Sleep-wake patterns of PK2<sup>-/-</sup> mice differed from those of WT mice over the 24-hr baseline LD condition. PK2<sup>-/-</sup> mice slept about 83.5 min less than WT mice per day (genotype effect,  $F = 10.73$ ,  $P < 0.01$ ). The reduced total sleep time in PK2<sup>-/-</sup> mice resulted from changes in the 12-h light phase ( $-80.5$  min; genotype effect,  $F = 21.85$ ,  $P < 0.0001$ ), but not in the 12-h dark phase ( $-3.0$  min; genotype effect,  $F = 0.03$ ,  $P = 0.87$ ; Figure 1A). This altered sleep distribution resulted in a smaller light : dark ratio of sleep quantity in PK2<sup>-/-</sup> mice (Figure 1, left panels: A, B). The reduction in the total amount of sleep was entirely attributed to a reduction of the NREM sleep. PK2<sup>-/-</sup> mice had reduced NREM sleep time compared to WT mice in the 12-h light phase ( $-101.9$  min; genotype effect,  $F = 48.97$ ,  $P < 0.0001$ ), while in the 12 h-dark phase, PK2<sup>-/-</sup> mice had NREM sleep time similar to WT mice ( $-16.5$  min; genotype effect,  $F = 0.96$ ,  $P = 0.33$ ; Figure 1B). In contrast, PK2<sup>-/-</sup> mice had increased REM sleep time in both the 12-h light phase ( $+21.4$  min; genotype effect,  $F = 12.62$ ,  $P < 0.0001$ ) and the 12-h dark phase ( $+13.5$  min; genotype effect,  $F = 26.24$ ,  $P < 0.0001$ ; Figure 1C). Thus, deficiency of the PK2 gene affected NREM and REM sleep quantities in opposite directions. The REM / NREM sleep ratio was significantly higher in PK2<sup>-/-</sup> mice, during the 12-h light period, 12-h dark period, or the entire 24-h LD period (unpaired *t*-test,  $P < 0.05$ ,  $0.01$ , and  $0.01$ , respectively; Figure 1D).

We further analyzed the sleep architecture of PK2<sup>-/-</sup> mice. The reduction of NREM sleep in PK2<sup>-/-</sup> mice in the 12-h light phase was mainly due to shorter mean duration of NREM sleep bouts (PK2<sup>-/-</sup>,  $1.27 \pm 0.13$  min; WT,  $1.66 \pm 0.10$  min; unpaired *t*-test,  $P < 0.05$ ), without any change to the number of NREM sleep bouts (PK2<sup>-/-</sup>,  $270.2 \pm 20.7$ ; WT,  $264.8 \pm 11.0$ ). The mean duration (PK2<sup>-/-</sup>,  $1.13 \pm 0.13$  min; WT,  $1.11 \pm 0.05$  min) and the number (PK2<sup>-/-</sup>,  $203.1 \pm 32.4$ ; WT,  $201.3 \pm 20.4$ ) of NREM sleep bouts did not differ between genotypes in the 12-h dark phase, and there was no difference in the amount of NREM sleep in the dark phase (Figure 2). The increased wakefulness in PK2<sup>-/-</sup> mice in the 12-h light phase was due to a longer mean duration of bouts (PK2<sup>-/-</sup>,  $1.87 \pm 0.13$  min; WT,  $1.47 \pm 0.10$  min; unpaired *t*-test,  $P < 0.05$ ), whereas the number of wake bouts was not significantly different (PK2<sup>-/-</sup>,  $189.9 \pm 14.9$ ; WT,  $177.6 \pm 9.2$ ). The increased REM sleep in PK2<sup>-/-</sup> mice in the 12-h light phase may be due to increase of mean duration of REM sleep bouts (PK2<sup>-/-</sup>,  $0.47 \pm 0.08$  min; WT,  $0.31 \pm 0.03$  min; unpaired *t*-test,  $P = 0.08$ ). The number of REM sleep bouts during light phase was similar between genotypes (PK2<sup>-/-</sup>,  $107.3 \pm 8.4$ ; WT,  $114.4 \pm 12.8$ ; unpaired *t*-test,  $P = 0.64$ ). In the 12-h dark phase, PK2<sup>-/-</sup> mice showed trends of increase in both the number (PK2<sup>-/-</sup>,  $62.9 \pm 10.1$ ; WT,  $43.1 \pm 5.0$ ; unpaired *t*-test,  $P = 0.09$ ) and the mean duration (PK2<sup>-/-</sup>,  $0.39 \pm 0.05$  min; WT,  $0.30 \pm 0.03$  min; unpaired *t*-test,  $P = 0.15$ ) of REM sleep bouts.

EEG spectral power in the delta frequency range (1 – 4 Hz) is often used as an indicator for sleep drive or intensity.<sup>19</sup> We thus quantified the EEG power in the PK2<sup>-/-</sup> and WT mice. Although PK2<sup>-/-</sup> mice had a decrease in the amount of total sleep, the distribution of NREM sleep delta power was not different between PK2<sup>-/-</sup> and WT mice in both 12-h light (genotype effect,  $F = 1.15$ ,  $P = 0.29$ ) and 12-h dark (genotype effect,  $F = 0.001$ ,  $P = 0.97$ ) phases (Figure

4C). REM sleep theta power is decreased in PK2<sup>-/-</sup> mice in both 12-h light phase (genotype effect,  $F = 33.05$ ,  $P < 0.0001$ ) and 12-h dark phase (genotype effect,  $F = 22.35$ ,  $P < 0.0001$ ), which is consistent with increased REM sleep time in PK2<sup>-/-</sup> mice.

### Sleep in Constant Dark Conditions

Following 2 days under LD conditions, animals were continually recorded for 4 more days under constant dark (DD) conditions. As shown in Table 1, PK2<sup>-/-</sup> mice spent less time in total sleep and NREM sleep than WT mice during the light phase under LD and the subjective light phase under DD. As under the LD condition, PK2<sup>-/-</sup> mice spent more time in REM sleep during the dark phase under DD (Table 1). Distributions of total sleep, NREM, and REM sleep under the 2 days of LD and 4 days of DD were shown in Figure 3. ANOVA tests revealed there were significant genotype differences between PK2<sup>-/-</sup> and WT mice under DD condition in total sleep (genotype effect,  $F = 10.48$ ,  $P < 0.001$ ), NREM sleep (genotype effect,  $F = 36.43$ ,  $P < 0.001$ ), and REM sleep (genotype effect,  $F = 45.27$ ,  $P < 0.001$ ).

### Sleep Deprivation and Recovery

Animals were sleep deprived for 6 hours in the second half of the light phase. Although brief NREM sleep bouts could not be prevented, especially toward the end of SD, the total amount of residual NREM sleep during the SD period were similar in PK2<sup>-/-</sup> and WT mice. The recovery period began at the onset of the dark phase and persisted throughout the 12-h dark period and the subsequent 12-h light period (Figure 4). Compared with the same period under baseline LD conditions, NREM sleep quantity was significantly increased in the recovery 12-h dark period immediately after SD in both WT (condition effect,  $F = 30.94$ ,  $P < 0.0001$ ) and PK2<sup>-/-</sup> mice (condition effect,  $F = 18.81$ ,  $P < 0.0001$ ; Figure 4A). Similarly, REM sleep time was also significantly increased in the recovery period in WT (condition effect,  $F = 33.16$ ,  $P < 0.0001$ ) and PK2<sup>-/-</sup> mice (condition effect,  $F = 13.30$ ,  $P < 0.001$ ; Figure 4B). Post hoc tests revealed significant increases in both NREM and REM sleep amount during hours 1 to 4 and 7 to 8 of the 12-h recovery dark period in WT mice. However, an increased NREM sleep quantity was observed only during hours 1 and 2 of the 12-h recovery dark period in PK2<sup>-/-</sup> mice, and no significant increase in REM sleep time was found in any of the 2-h intervals. In the recovery 12-h light period, neither NREM nor REM sleep amount differed from the corresponding baseline levels in WT or PK2<sup>-/-</sup> mice (Figures 4A and B).

Direct comparison of recovery sleep between the two genotypes was complicated by different baseline levels and the different amounts of sleep lost during SD in PK2<sup>-/-</sup> and WT mice. For this reason, we analyzed the recovery sleep response by two different criteria for the recovery 12 h dark period as described.<sup>4</sup> For criterion 1, we determined the percentage change in recovery sleep relative to the baseline level ( $100 \times [\text{recovery} - \text{baseline}] / \text{baseline}$ ). As shown in Figure 4D, PK2<sup>-/-</sup> mice had a significantly smaller change in NREM sleep amount relative to the baseline value than WT mice in the recovery 12-h dark period (PK2<sup>-/-</sup>,  $31.55\% \pm 7.16\%$ ; WT,  $57.23\% \pm 9.61\%$ ; unpaired t-test,  $P < 0.05$ ), as was for REM sleep (PK2<sup>-/-</sup>,  $31.23\% \pm 11.48\%$ ; WT,  $88.92\% \pm 14.30\%$ ; unpaired t-test,  $P < 0.01$ ). For criterion 2, we calculated the percentage of regained sleep relative to the lost sleep amount during the 6-h period of SD ( $100 \times [\text{recovery} - \text{baseline}] / \text{amount of sleep lost during SD}$ ). REM sleep rebound in PK2<sup>-/-</sup> mice was reduced ( $21.74\% \pm 8.41\%$ ) compared with WT mice ( $50.39\% \pm 8.42\%$ ; unpaired t-test,  $P < 0.05$ ), while there was no significant difference between genotypes in the amount of regained NREM sleep (PK2<sup>-/-</sup>,  $39.61\% \pm 8.55\%$ ; WT,  $46.63\% \pm 12.57\%$ ; unpaired t-test,  $P > 0.05$ ) during the recovery 12-h dark period (Figure 4E). During the recovery 12-h light period after SD, however, no significant differences were found in NREM and REM sleep amount between WT and PK2<sup>-/-</sup> mice, according to both criteria (Figure 4D, E). Taken together, these results revealed that the sleep rebound response of PK2<sup>-/-</sup> mice to sleep deprivation was attenuated.



We next examined the delta power change in response to sleep deprivation. As shown in Figure 4C, NREM sleep delta power was significantly increased in both the PK2<sup>-/-</sup> and WT mice during the immediate recovery period after SD, indicating an increase in the drive for sleep. Interestingly, post hoc tests revealed that the increase in NREM delta power was restricted to the first 2 hours of the recovery period in PK2<sup>-/-</sup> mice, whereas in WT mice the increase lasted for 6 hours. Compared with the respective baseline values, the percentage of NREM sleep delta power increase in PK2<sup>-/-</sup> mice was also significantly smaller than that of WT mice (genotype effect,  $F = 9.85$ ,  $P < 0.01$ ) during the recovery 12-h dark period after SD. These results indicate that PK2<sup>-/-</sup> mice displayed attenuated rebound in NREM sleep delta power to sleep deprivation.

### Sleep after Cage Change

Over the course of animal handling, we noticed a faster return of the PK2<sup>-/-</sup> mice to rest during the light period in response to cage changes. We thus quantified this observation by direct sleep measurement. When placed into new cages with clean bedding during the light phase, WT and PK2<sup>-/-</sup> mice showed increases in wakefulness and decreases in NREM and REM sleep amounts (Figure 5A, B, and C). During the 6-h period after cage change, considerable changes in wakefulness (condition effect,  $F = 57.71$ ,  $P < 0.0001$ ), NREM sleep (condition effect,  $F = 55.97$ ,  $P < 0.0001$ ), and REM sleep amount (condition effect,  $F = 58.52$ ,  $P < 0.0001$ ) were observed in WT mice, while only NREM (condition effect,  $F = 5.18$ ,  $P < 0.05$ ) and REM sleep (condition effect,  $F = 14.23$ ,  $P < 0.01$ ) quantities changed significantly in PK2<sup>-/-</sup> mice. Wakefulness increase by cage change was only evident in the first hour in PK2<sup>-/-</sup> mice, however, ANOVA analysis indicated no significant differences within the total 6-h period after cage change (condition effect,  $F = 1.63$ ,  $P = 0.21$ ). Compared to their respective baseline levels, WT mice showed a significant increase in wakefulness and decreases in NREM and REM sleep amount from 1 to 4 hours after cage change; however, PK2<sup>-/-</sup> mice only showed such changes for the first hour after cage change.

Comparing the percentage change in sleep measures relative to baseline levels during the first 6-h period after cage change ( $100 \times [\text{treatment} - \text{baseline}] / \text{baseline}$ ), PK2<sup>-/-</sup> mice showed significantly smaller reductions in total sleep (PK2<sup>-/-</sup>,  $15.8\% \pm 7.2\%$ ; WT,  $56.2\% \pm 7.5\%$ ; unpaired t-test,  $P < 0.01$ ), NREM sleep (PK2<sup>-/-</sup>,  $14.5\% \pm 6.2\%$ ; WT,  $53.7\% \pm 8.6\%$ ; unpaired t-test,  $P < 0.01$ ) and REM sleep (PK2<sup>-/-</sup>,  $30.9\% \pm 10.6\%$ ; WT,  $63.7\% \pm 8.1\%$ ; unpaired t-test,  $P < 0.05$ ; Figure 5E). Consistent with these data, NREM sleep delta power significantly increased in WT mice during the light period after cage change (condition effect,  $F = 53.34$ ,  $P < 0.0001$ ); however, no such difference was observed in PK2<sup>-/-</sup> mice (condition effect,  $F = 1.14$ ,  $P = 0.29$ ; Figure 5D). In addition, we also analyzed the time from cage change to the first epoch of sleep (i.e., sleep latency). As shown in Figure 5F, PK2<sup>-/-</sup> mice had shorter NREM and REM sleep latencies after cage change than did WT mice. These results indicated that PK2<sup>-/-</sup> mice had impaired abilities of maintaining wakefulness in response to novel environments.

## DISCUSSION

### PK2 Deficiency Affects Amount of Sleep under Both LD and DD Conditions

The most striking finding of this work is that PK2<sup>-/-</sup> mice slept less per day than WT mice under a LD cycle. The reduced sleep time in PK2<sup>-/-</sup> mice occurred mainly during the light period and was entirely attributed to a reduction of NREM sleep time and a shorter duration of NREM sleep bouts. Interestingly, REM sleep was increased in PK2<sup>-/-</sup> mice during both light and dark periods, even though the amount of total sleep was decreased. The alterations in total sleep, NREM and REM sleep in PK2<sup>-/-</sup> mice were preserved under DD condition, indicating that these alterations were not caused by masking effects of the LD cycle. Thus, the

mutation of the PK2 gene influences both the distribution and the amount of sleep, implying that PK2 is a molecule that regulates both circadian and homeostatic processes of sleep.

It has been proposed that the SCN is a component of the wake-regulatory pathway and that the efferent signals from the SCN have arousal-promoting effects in diurnal animals.<sup>20</sup> The reduced NREM sleep in PK2<sup>-/-</sup> mice occurred primarily in the light phase but not in the dark phase, and this phase-dependent effect is consistent with rhythmic expression of PK2 mRNA in the SCN.<sup>7</sup> This result implies the involvement of PK2 in sleep-wake regulation is dependent on its circadian effect of PK2. A correlation between increased daytime locomotor activity and reduction of the molecular rhythm of PK2 in the SCN has been observed in transgenic mice expressing a mutant Huntington's gene.<sup>21</sup> The SCN is connected to hypothalamic and brainstem nuclei having important roles in sleep-wake regulation.<sup>22</sup> The dorsomedial hypothalamus, a major SCN target nucleus, may integrate circadian signals from the SCN and other neural inputs while projecting to other sleep-wake regulatory regions, such as the dorsal tuberomammillary nucleus, the locus coeruleus, the ventrolateral preoptic nucleus and the median preoptic nucleus.<sup>23-25</sup> PKR2, which encodes the receptor for PK2, is expressed at high levels in the dorsomedial hypothalamus,<sup>10</sup> consistent with a possible involvement of PK2 in sleep-wake regulation via this relay nucleus.

REM sleep was increased in PK2<sup>-/-</sup> mice during both light and dark periods, and PK2<sup>-/-</sup> mice had different homeostatic control of sleep during the recovery dark period after sleep deprivation, suggesting that at least some function of PK2 in sleep regulation might be independent of SCN circadian clock function. Besides the SCN, PK2 is also expressed in other brain regions,<sup>10</sup> such as in the medial preoptic nucleus, the amygdala and nucleus accumbens that have been implicated in sleep control,<sup>26-28</sup> and therefore may contribute to sleep changes in PK2<sup>-/-</sup> mice. In addition, PKR2 is also expressed in the nuclei that are not SCN target areas but that are important for sleep-wake regulation,<sup>10</sup> such as the lateral hypothalamus, the perifornical region of the hypothalamus and the dorsal raphe nucleus.<sup>29-31</sup> It is possible that the changes of sleep regulation in PK2<sup>-/-</sup> mice, such as those affecting REM sleep, may be due to the functional changes of these areas in the PK2<sup>-/-</sup> mice.

### **Mutation of the PK2 Gene Attenuated the Compensatory response to sleep deprivation**

Loss of circadian genes affects the homeostasis of sleep, as displayed by their altered response to sleep deprivation. Deletion of *Bmal1/Mop3* caused an attenuated compensatory response to sleep deprivation,<sup>4</sup> where as *Clock* mutant mice presented a decreased rebound after sleep deprivation in REM sleep but not NREM sleep.<sup>3</sup> Similar to *Clock* mutant mice, *DBP* knockout mice showed a decreased rebound in REM sleep after sleep deprivation, without any difference in NREM sleep rebound.<sup>6</sup> *Cry1* and *Cry2* double deficient mice showed no rebound in the amount of NREM and REM sleep after sleep deprivation.<sup>5</sup> *Per1* and *Per2*, negative elements of the molecular clock in the SCN, however, may not be required for homeostatic regulation of the daily amount of wakefulness, NREM sleep, or REM sleep.<sup>12</sup> In the present study, PK2<sup>-/-</sup> mice showed a reduced rebound response in REM sleep, NREM sleep, and NREM sleep delta power in response to sleep deprivation. PK2<sup>-/-</sup> mice generated compensatory increases in NREM and REM sleep times and NREM delta power during the recovery 12-h dark period, but the rebound sleep durations were shorter than in WT mice. Unlike WT mice, PK2<sup>-/-</sup> mice exhibited a smaller change relative to baseline values in NREM and REM sleep times and NREM delta power during recovery 12-h dark period. Moreover, during the recovery 12-h dark period, PK2<sup>-/-</sup> mice regained a smaller percentage of the total amount of REM sleep lost, although they regained a similar percentage of the lost NREM sleep. Thus, PK2<sup>-/-</sup> mice showed an attenuated compensatory response to sleep deprivation, further supporting the idea that PK2 has an influence on the homeostatic regulation of sleep.

EEG delta power is considered a prominent marker of NREM sleep homeostatic regulation, as it exhibits a predictive quantitative relationship with the duration of previous wakefulness.<sup>19</sup> EEG delta power is increased in mice lacking *Bmal1/Mop3*,<sup>4</sup> *Cry1* and *Cry2*,<sup>5</sup> whereas the daily amplitude of EEG delta power decreases in mice lacking the *DBP* gene.<sup>6</sup> No difference in NREM sleep delta power was found between *PK2*<sup>-/-</sup> and WT mice during both baseline light and dark periods, even though the *PK2*<sup>-/-</sup> mice slept less daily than did WT mice. A possible explanation is that mutation of the *PK2* gene slows the accumulation of homeostatic sleep parameters, e.g., sleep “need”. The same finding was reported in *Clock* mutant mice.<sup>3</sup> Immediately after sleep deprivation, although NREM sleep delta power increased dramatically in both *PK2*<sup>-/-</sup> and WT animals, the *PK2*<sup>-/-</sup> mice had a much shorter rebound duration and a smaller increase of NREM sleep delta power in the recovery 12-h dark period, indicating that NREM sleep delta power rebound is significantly attenuated in *PK2*<sup>-/-</sup> mice. For NREM sleep delta power, a decreased compensatory response following sleep deprivation was also seen in mice lacking *Cry1* and *Cry2*, *Per1* and *Per2*, and *Bmal1/Mop3*.<sup>4,5,32</sup> The altered response to sleep deprivation in these mutant mice of circadian genes appears to support the notion that the circadian and homeostatic processes underlying the regulation of sleep are linked, at least at the molecular level.

### **PK2<sup>-/-</sup> Mice Had Impaired Abilities of Maintaining Wakefulness in Response to Novel Environments**

Considerable evidence supports that environmental challenges can have significant effects on arousal states in animals, including prolonged sleep latencies and reduced total sleep time.<sup>13,14</sup> When confronted with behavioral challenges such as novel housing condition and starvation, it is important for an animal to maintain an elevated alertness state, at least for a short period of time. Such a wakefulness maintenance response may be required for proper adaptation of animals to these environmental challenges. In the present experiment, we show that the *PK2*<sup>-/-</sup> mice did not exhibit prolonged wakefulness in response to cage change. During the first 6-h period after cage change, the *PK2*<sup>-/-</sup> mice showed a substantially reduced increase in wakefulness. The enhancement of wakefulness by cage change was restricted to only the first hour in *PK2*<sup>-/-</sup> mice, whereas it lasted for 4 hours in WT mice. These results indicated that a null mutation of the *PK2* gene attenuates the arousal enhancement induced by cage change. This reduced response is unlikely to be due to a difference in the baseline sleep parameters, as *PK2*<sup>-/-</sup> mice actually had reduced total sleep amount during light phase. The neurobiological mechanism of *PK2* in mediating the arousal stimulation remains to be determined. It is well established that amygdala is a critical structure for stress responsiveness and emotional regulation.<sup>33</sup> The amygdala is heavily interconnected with hypothalamic and brainstem nuclei known to be involved in sleep control.<sup>34</sup> Therefore, the attenuated response of *PK2*<sup>-/-</sup> mice to novel environments may be linked to the expression of *PK2* in the amygdala.  
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In summary, mice lacking the *PK2* gene, which encodes a postulated SCN output molecule, exhibit altered baseline sleep parameters and an attenuated compensatory rebound in response to sleep deprivation, in addition to an altered circadian sleep distribution. *PK2*<sup>-/-</sup> mice also exhibit impaired response to behavioral challenges. These findings indicate *PK2* is involved in both circadian and homeostatic regulation of sleep.

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#### **Disclosure Statement**

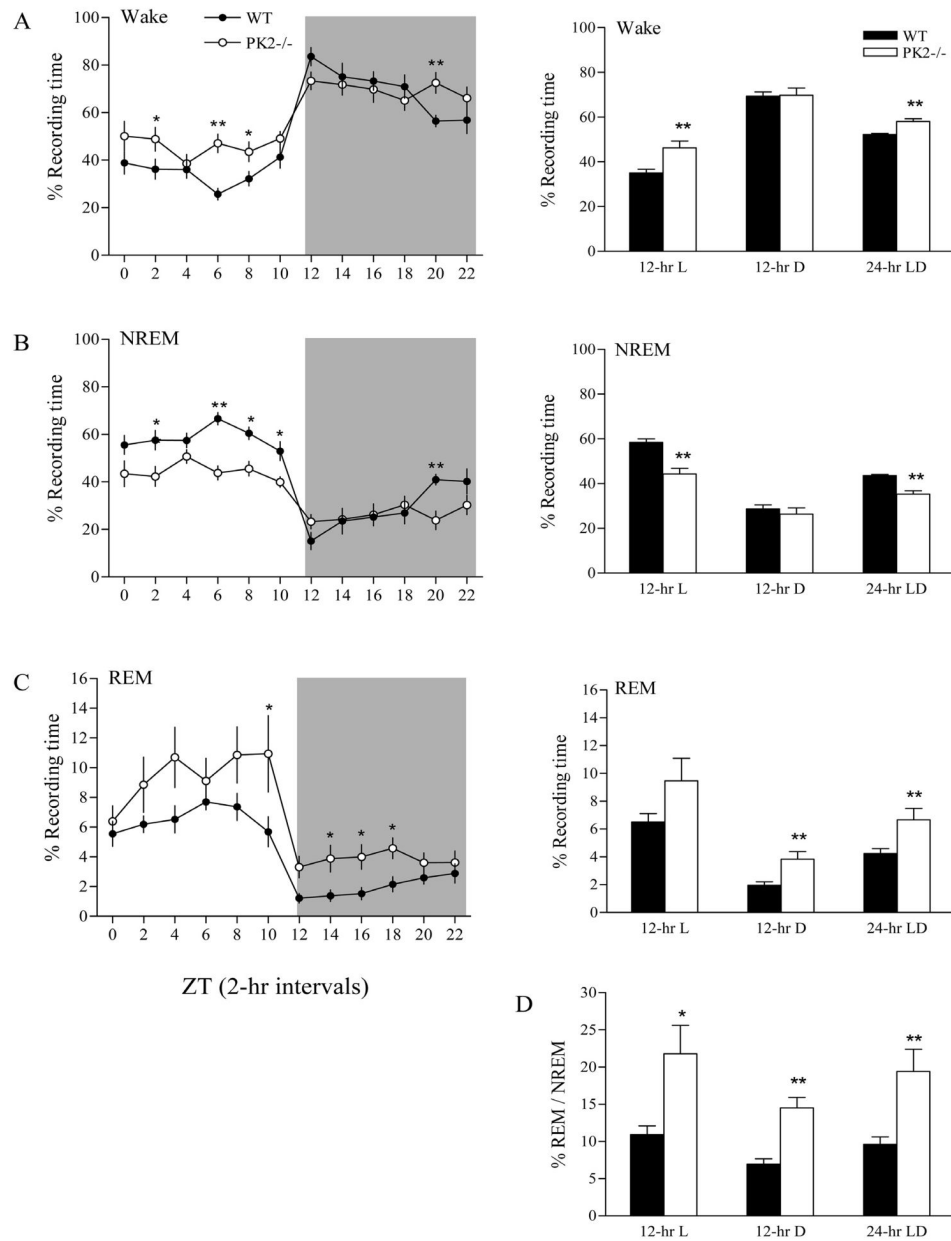


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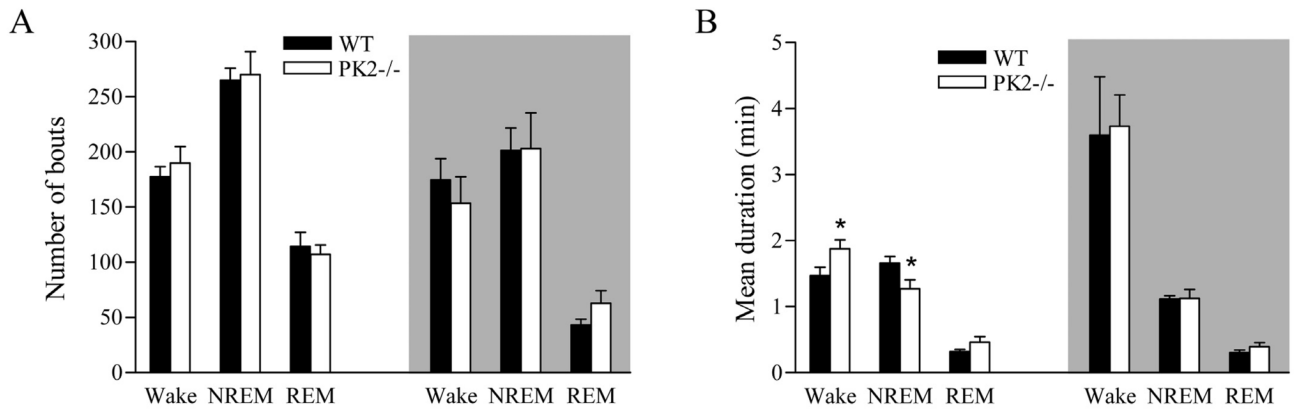
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**Figure 1.**

Baseline sleep-wake patterns in wild-type (WT) and PK2<sup>-/-</sup> mice. Left panels: Distribution of wake (A), non-rapid eye movement (NREM) sleep (B) and rapid eye movement (REM) sleep (C) in 2-hr intervals under a 12 h : 12 h light : dark (LD) cycle for WT (filled circles) and PK2<sup>-/-</sup> (open circles) mice. Sleep-wake values were expressed as percentage of recording time. Recordings were initiated at light onset (zeitgeber time, ZT0) and the dark phase is indicated by the shaded background. \*P < 0.05; \*\*P < 0.01, post hoc Bonferroni test. Right panels: Amounts of baseline sleep-wake states (A: wake; B: NREM; C: REM) and ratio (%) of REM / NREM (D) were averaged over the 12-h light, 12 h-dark and 24-hr intervals for each

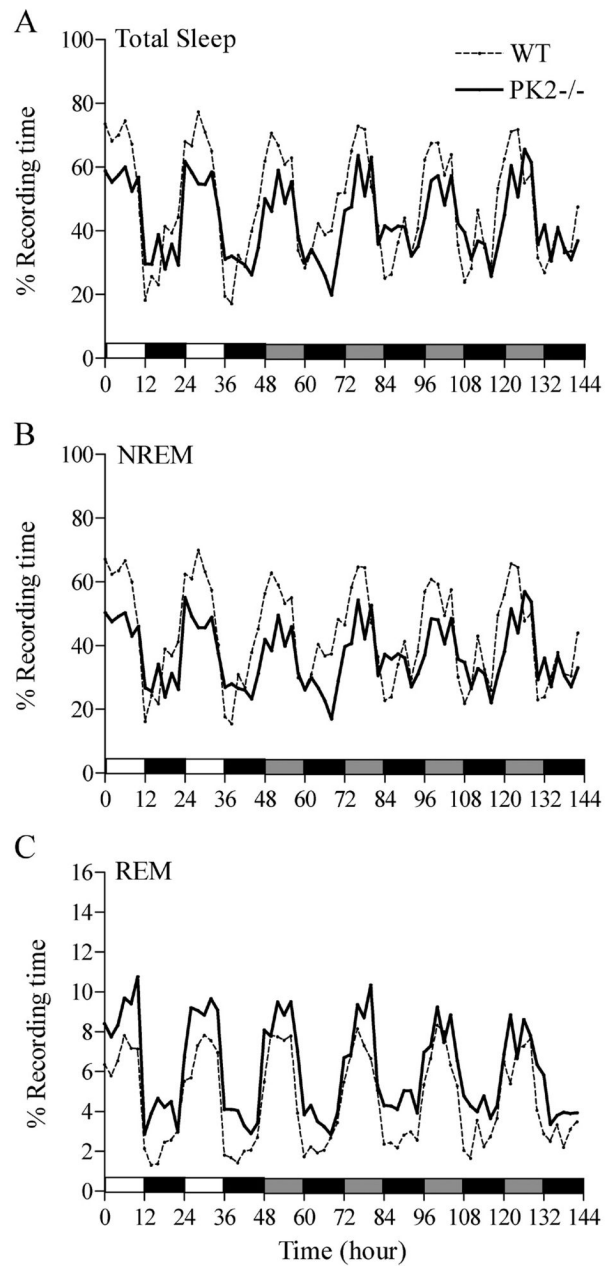
genotype. \*P < 0.05; \*\*P < 0.01, unpaired t-test. Values represent group means  $\pm$  s.e.m. n = 9 mice / per genotype



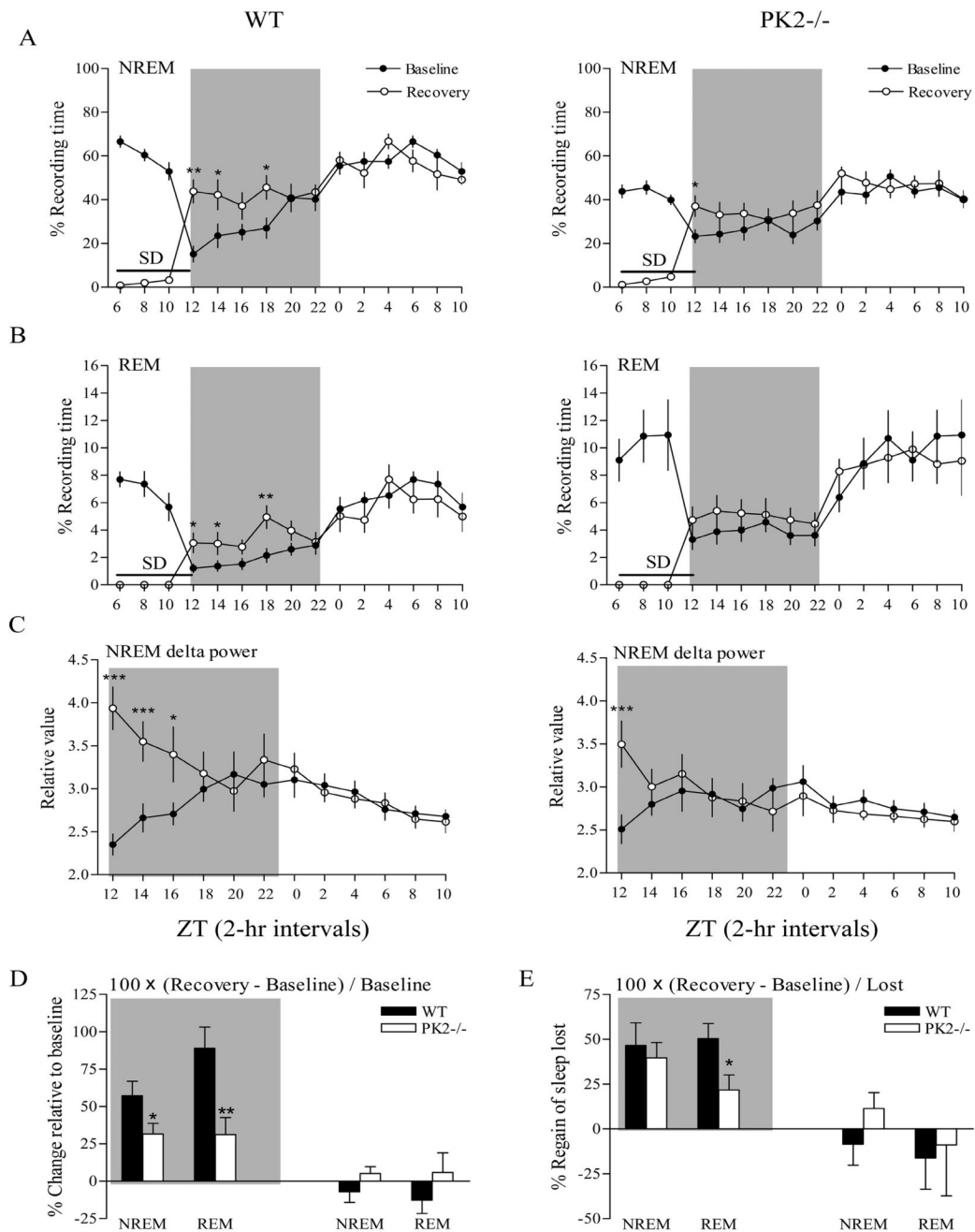
**Figure 2.**

Baseline sleep-wake fragmentation in wild-type (WT) and PK2<sup>-/-</sup> mice. The number (A) and the mean duration (B) of wake, NREM sleep and REM sleep bouts during the light and dark (indicated by the shaded background) phases. \*P < 0.05, unpaired t-test. n = 9 mice / per genotype.





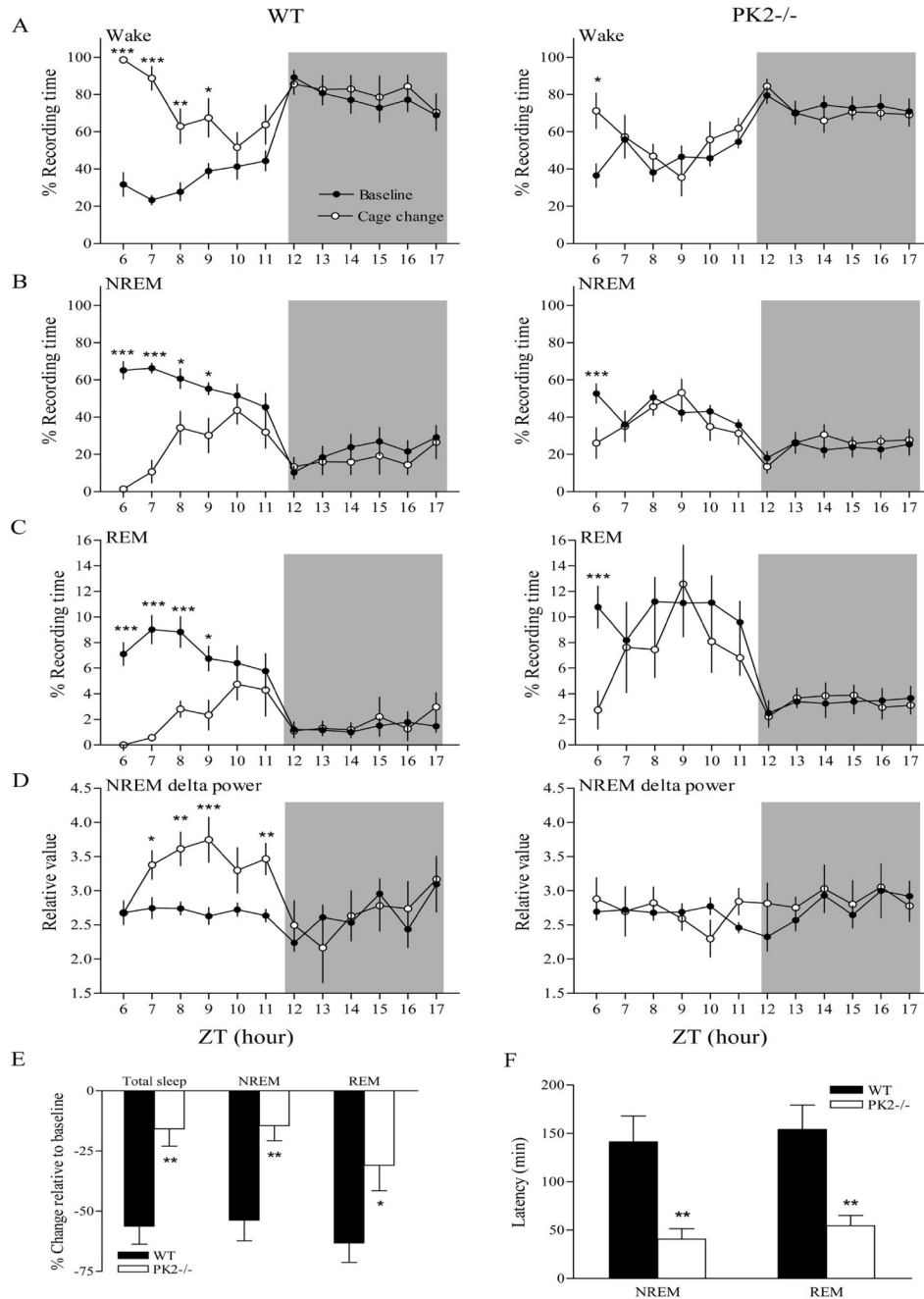
**Figure 3.** Time courses of total sleep (A), NREM sleep (B) and REM sleep (C) for 2 d baseline under 12 h : 12 h light : dark (LD) and followed by 4 d under constant darkness (DD) in wild-type (WT) and PK2<sup>-/-</sup> mice. Black horizontal bars indicate dark phase, and shaded bars indicate the subjective light phases under DD. n = 7 mice / per genotype.



**Figure 4.**

Recovery from sleep deprivation in wild-type (WT) and PK2<sup>-/-</sup> mice. Animals were sleep deprived (SD) in the last 6 h of the light phase, as indicated with the horizontal line. The recovery phase began at dark onset (zeitgeber time, ZT 12) and continued throughout the 12-h dark period (indicated by the shaded background) and the 12-h light period. Left panels: Baseline (filled circles) and recovery (open circles) patterns in WT mice for NREM sleep (A), REM sleep (B), and NREM delta power (C), graphed in 2-hr intervals. Right panels (A, B, C): Baseline (filled circles) and recovery (open circles) patterns in PK2<sup>-/-</sup> mice. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001, post hoc Bonferroni test. D, Percentage change relative to baseline value ( $100 \times [\text{recovery} - \text{baseline}] / \text{baseline}$ ) for NREM and REM sleep during the 12-h dark period

(indicated by the shaded background) and the 12-h light period after 6-h SD in WT and PK2<sup>-/-</sup> mice. \*P < 0.05; \*\*P < 0.01, unpaired t-test. E, Percentage sleep regained during recovery period relative to sleep lost during 6-h SD ( $100 \times [\text{recovery} - \text{baseline}] / \text{amount of sleep lost during SD}$ ) for NREM and REM sleep during the 12-h dark period (indicated by the shaded background) and the 12-h light period after 6-h SD in WT and PK2<sup>-/-</sup> mice. \*P < 0.05; unpaired t-test. n = 9 mice / per genotype.

**Figure 5.**

Sleep-wake patterns after cage change in wild-type (WT) and PK2<sup>-/-</sup> mice. Animals were placed in new and clean cages at ZT6. Recordings were made from the last 6 h of the light phase to the first 6 h of the dark phase (indicated by the shaded background). Distribution of wake (A), NREM sleep (B), REM sleep (C) after cage change in 1-hr intervals for WT (Left panels) and PK2<sup>-/-</sup> mice (Right panels). \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001, post hoc Bonferroni test. D, NREM delta power plotted hourly after cage change in WT (Left panels) and PK2<sup>-/-</sup> mice (Right panels). \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001, post hoc Bonferroni test. E, Percentage change relative to baseline value ( $100 \times [\text{treatment} - \text{baseline}] / \text{baseline}$ ) for total sleep, NREM, and REM sleep during the 6-h light period after cage change in WT

and PK2<sup>-/-</sup> mice. \*P < 0.05; \*\*P < 0.01, compared with WT mice, unpaired t-test. F, Latency of NREM sleep and REM sleep in minutes following cage change in WT and PK2<sup>-/-</sup> mice. \*\*P < 0.01, compared with WT mice, unpaired t-test. n = 7 mice / per genotype.



**Table 1**  
Sleep Amounts for 12-hr Interval in LD and DD Conditions

	Total Sleep			NREM			REM		
	WT	PK2-/-	P	WT	PK2-/-	P	WT	PK2-/-	P
LD1									
Light	70.7 ± 1.8	56.8 ± 3.7	<0.01	64.4 ± 2.0	47.9 ± 3.2	<0.01	6.3 ± 0.3	8.9 ± 1.4	0.09
Dark	33.5 ± 2.4	36.5 ± 2.4	0.37	31.0 ± 2.3	31.3 ± 1.9	0.81	2.4 ± 0.3	5.2 ± 1.0	<0.05
LD2									
Light	65.4 ± 2.4	52.9 ± 3.4	<0.01	59.3 ± 2.3	45.2 ± 2.9	<0.01	6.2 ± 0.4	7.8 ± 1.2	0.23
Dark	30.9 ± 1.6	32.7 ± 2.4	0.53	28.6 ± 1.4	28.1 ± 2.1	0.97	2.3 ± 0.3	4.6 ± 0.8	<0.05
DD1									
Subjective Light	61.9 ± 3.1	49.1 ± 4.9	<0.05	55.4 ± 2.9	41.2 ± 4.5	<0.05	6.5 ± 0.7	7.9 ± 0.8	0.21
Subjective Dark	35.8 ± 4.3	29.8 ± 5.7	0.38	33.7 ± 4.2	25.6 ± 5.0	0.23	2.0 ± 0.5	4.1 ± 0.8	<0.05
DD2									
Subjective Light	61.2 ± 2.3	50.7 ± 3.9	<0.05	54.9 ± 2.8	43.1 ± 4.3	<0.05	6.3 ± 0.9	7.7 ± 0.8	0.26
Subjective Dark	34.4 ± 5.1	38.8 ± 3.2	0.46	31.8 ± 4.9	34.1 ± 2.8	0.63	2.5 ± 0.6	4.7 ± 0.7	<0.05
DD3									
Subjective Light	60.9 ± 3.5	49.6 ± 3.2	<0.05	54.8 ± 2.8	42.3 ± 2.8	<0.01	6.2 ± 0.5	7.3 ± 1.3	0.46
Subjective Dark	32.9 ± 4.7	35.3 ± 5.2	0.74	30.4 ± 4.2	30.6 ± 4.2	0.92	2.5 ± 0.7	4.7 ± 1.2	0.14
DD4									
Subjective Light	61.9 ± 2.6	53.1 ± 3.8	0.08	55.7 ± 2.4	45.9 ± 3.6	<0.05	6.3 ± 0.6	7.2 ± 0.9	0.39
Subjective Dark	32.5 ± 4.6	35.7 ± 4.6	0.73	29.8 ± 4.1	31.2 ± 3.9	0.76	2.7 ± 0.8	4.5 ± 1.0	0.19

Data are presented as mean ± SEM of 12-hr intervals for 2 days under light / dark and 4 days of constant dark. P indicates the probability of the unpaired t tests comparing the genotypes. (n = 7 mice / per genotype)