Altered sleep and behavioral activity phenotypes in PER3-deficient mice

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ABSTRACT

Sleep homeostasis and circadian rhythmicity interact to determine the timing of behavioral activity. Circadian clock genes contribute to circadian rhythmicity centrally and in the periphery, but some also have roles within sleep regulation. The clock gene Period3 (Per3) has a redundant function within the circadian system and is associated with sleep homeostasis in humans. This study investigated the role of PER3 in sleep/wake activity and sleep homeostasis in mice by recording wheel running activity under baseline conditions in wild-type (WT; n = 54) and in PER3-deficient (Per3-/-; n = 53) mice, as well as EEG-assessed sleep before and after 6 hours of sleep deprivation in WT (n = 7) and Per3-/- (n = 8) mice. Whereas total activity and vigilance states did not differ between the genotypes, the temporal distribution of wheel running activity, vigilance states, and EEG delta activity was affected by genotype. In Per3-/- mice, running wheel activity was increased and REM sleep and NREM sleep were reduced in the middle of the dark phase, and delta activity was enhanced at the end of the dark phase. At the beginning of
the baseline light period, there was less wakefulness and more REM and NREM in \textit{Per3}^- mice. \textit{Per3}^- mice spent less time in wakefulness and more time in NREM sleep in the light period immediately after sleep deprivation and REM sleep accumulated more slowly during the recovery dark phase. These data confirm a role for PER3 in sleep/wake timing and sleep homeostasis.

\textbf{Keywords:} circadian, sleep homeostasis, sleep deprivation, EEG, wheel-running activity

\textbf{Running title:} Sleep and behavioral activity in PER3-deficient mice

\section*{INTRODUCTION}

Sleep and circadian rhythms are intrinsically related. Sleep is modulated according to the hypothetical two-process model of sleep regulation (7), whereby a homeostatic process tracks the build-up of sleep pressure during wakefulness and its dissipation during non-rapid eye movement (NREM) sleep, while a circadian process modulates sleep timing, synchronizing sleep-wake to light-dark and social cycles (12). In the absence of external time cues in constant dim light or total darkness, the sleep-wake cycle is desynchronized from the normal 24h day. In this ‘free-running’ condition, circadian rhythmicity of the sleep-wake cycle persists, but with a period that is slightly longer than 24hr in humans (9, 40). In mammals, the master circadian clock is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (27, 32), where ‘clock genes’ and their protein products interact by transcriptional
feedback inhibition to create near-24h oscillations in output pathways that synchronize the daily rhythms of the whole organism (21, 22). The core molecular mechanism of the cellular circadian clock includes two transcription factors, CLOCK and BMAL1, which form a heterodimer that binds to the promoters of the negative feedback regulators, \textit{Period1 – 3} (\textit{Per1 – 3}) and \textit{Cryptochrome1 & 2} (\textit{Cry1 & 2}), inducing their expression. PER and CRY accumulate in the cytoplasm, where they are targeted for phosphorylation-dependent proteosomal degradation, prior to forming stable, multimeric complexes that translocate to the nucleus and inhibit the positive induction by CLOCK:BMAL1 (25).

While much is known about the molecular components of the circadian oscillator, much less is known about genes underlying sleep homeostasis (for review see Ref. 2). However, it is now becoming apparent that in addition to affecting sleep and wake timing, certain circadian clock genes also have a role to play in sleep structure and homeostasis (14, 25). Both heterozygous and homozygous \textit{Clock} mutant mice show a decrease in sleep duration in entrained baseline light/dark conditions, and during free-running conditions in constant darkness. They also have a reduced compensatory rebound in rapid eye movement (REM) sleep after 6h of sleep deprivation (SD) (28). When the \textit{Clock} paralog, \textit{Npas2}, is absent in knockout mice, electroencephalographic (EEG) activity in the spindle frequency range (10 – 15 Hz) was reduced in NREM sleep, and activity shifted to higher frequencies in the delta range (1 – 4 Hz), a marker for sleep pressure (15). In \textit{Cry1} and \textit{Cry2} double knockout mice, during baseline and constant dark conditions, as well as after SD, there was an increased NREM sleep bout duration and increased NREM delta.
power, both indicators of higher sleep drive (41). Mice lacking the clock-controlled gene albumin D-box binding protein (Dbp) gene, also show a reduced REM sleep rebound after SD, and less NREM sleep, which was accompanied by a decrease of the NREM sleep EEG delta power (16).

The other components of the circadian negative feedback limb, the PER proteins, have also been associated with sleep homeostasis. Like Cry, single *Per1* and *Per2* knockouts have altered sleep/wake timing (circadian) phenotypes, but no disruption of homeostatic sleep regulation, although both mutants showed a lower initial delta power increase during NREM recovery sleep after SD (24). Similarly, another study reported that single *Per* mutants (including *Per3*) and *Per1* and *Per2* double mutants did not show any overt differences in sleep regulation (31). However, the data did indicate higher EEG theta levels in REM sleep in *Per1* and *Per3* knockout animals, and enhanced EEG delta power in *Per1* knockouts and *Per1/2* double knockouts after SD, compared to wild-type (WT) mice.

These data, while not conclusive for *Per* genes, show that circadian clock genes contribute towards sleep homeostasis in rodents. In humans, some clock gene variations have been associated with sleep timing and duration (e.g. 1, 3, 4, 8, 23, 35), but only one clock gene polymorphism, the *PER3* variable number tandem repeat (VNTR), has been found to be associated with phenotypic differences in sleep homeostasis, while showing no differences in circadian markers such as hormones and clock gene expression (5, 10, 19, 39).

In 129/sv mice, the absence of PER3 has only a subtle effect on circadian phenotype (30), and, unlike PER1 and PER2, PER3 is not required
to maintain circadian rhythmicity (6). However, recent data from C57BL/6J mice have shown that while disruption of Per3 does not overtly alter circadian phenotype, it does associate with light-dependent phenotypes such as free-running period in constant light, and negative masking (37). Thus, disruption to, and variation within PER3 leads to non-circadian phenotypes in both mice and humans. Therefore, the aim of this study was to compare the sleep/wake regulation and EEG activity of WT and Per3-/− mice on a C57BL/6J genetic background under baseline conditions and in response to 6h of SD. This was achieved by comparing the following established measures of sleep/wake regulation: 1) The duration and temporal pattern of locomotor activity, 2) The duration and temporal pattern of EEG-assessed vigilance states, 3) EEG power density in all vigilance states, 4) The consolidation of sleep and wakefulness, and 5) The response to sleep deprivation for all of these variables.

MATERIALS AND METHODS

Animals and housing conditions
All mice used in this study were on a C57BL/6J genetic background. Mice homozygous for a targeted disruption of the Per3 gene (Per3−/−), (29, 30) and their wild-type controls were bred in-house from heterozygous breeding pairs and genotyped as described previously (37). Mice were housed individually in cages equipped with a running-wheel (ClockLab, Actimetrics, Wilmette, IL) in light-tight, sound-attenuated cabinets. Food and water were available ad
libitum. Mice were maintained on a 12-h light: 12-h dark cycle (LD), with a light intensity of 800 ± 13.4 mW/m² at the level of the cage bottom during the light period, and kept at a controlled ambient temperature (20 – 22 °C) and relative humidity (55% ± 10%). All experimental procedures were approved by the University of Surrey Animal Ethics Committee and carried out under a UK Home Office License and in accordance with the Declaration of Helsinki.

**Duration and temporal pattern of locomotor activity**

Adult male mice (11.8 ± 0.3 weeks old; AVG ± SEM) were entrained to an LD 12:12 cycle for at least 14 days. Average profiles of running wheel activity were calculated over the last 10 days of the LD cycle (WT n = 54; Per3^-/- n = 53 mice). The total amount of active minutes per hour was calculated for the last day of activity recordings. Intensity of running-wheel activity (number of wheel revolutions) was expressed as average activity per active minute. As a measure of activity fragmentation, the duration of all episodes of consecutive minutes with running wheel activity or inactivity was calculated over the dark periods during the last 10 days.

**Duration and temporal pattern of EEG-assessed vigilance states**

Adult male mice (Per3^-/- n = 8; WT n = 7; 10.0 ± 0.3 weeks old [AVG ± SEM]) were implanted with a telemetric transmitter (weight = 3.9 g, volume = 1.9 cc; TL11M2-F20-EET, Data Sciences International, St. Paul, MN, USA.) connected to electrodes for continuous EEG and electromyogram (EMG) recordings. There was no significant difference in body weight between the genotypes (WT = 26.55 ± 0.95 g, Per3^-/- = 27.94 ± 0.45 g; [AVG ± SEM]).
Under anesthesia (isoflurane; induction 3.6%, maintenance 1.5 – 2.5%), two nickel-plated brass EEG electrodes (length of screw shaft: 2mm; outer diameter of screw thread: 1mm) were implanted epidurally over the right frontal and parietal cortex, as previously described (17). The EEG electrodes were covered with dental cement (GC Fuji PLUS; GC United Kingdom Ltd., Newport Pagnell, UK). Two EMG leads were inserted into the neck muscles approximately 5 mm apart and sutured into place. The telemetry transmitter was placed in a subcutaneous pocket and positioned along the left-dorsal flank of the mouse. Analgesia was administered at the onset of the surgery (Vetergesic®, 30 μg/kg, subcutaneous injection). After the surgery, animals were allowed to recover for 1.9 ± 0.2 weeks (AVG ± SEM) and baseline data collection started when the mice were 11.8 ± 0.2 weeks of age (AVG ± SEM).

Telemetric transmitters were activated on the day before the baseline day and EEG/EMG signals were then recorded continuously for 48-h using Data Sciences International hardware and Dataquest ART™ software (DSITM, St. Paul, MN, U.S.A.). The EEG and EMG signals were modulated with a high-pass (3 dB, 0.5 Hz) and a low-pass, anti-aliasing (50 Hz) analog filter. The data analyzed consisted of 48-h recordings including a 24-h baseline starting at light onset (Zeitgeber time [ZT] 0) and a subsequent 24-h period during which mice were subjected to sleep deprivation (SD) during the first 6 h of the light period (ZT 0 – 6). SD was performed by introducing novel objects into the cage, such as nesting material or cardboard, whenever the animal appeared to be drowsy, or by disturbing bedding and/or the nest, if necessary. Researchers were blind to genotype during this intervention and novel objects were introduced to animals in the same order. Hence, SD was carried out in a
similar way for both genotypes. Halfway through the SD, the cage was exchanged with a fresh one to provide additional stimulation (some soiled bedding from the old cage was transferred into the new one to minimize stress). Recovery sleep recordings were obtained during the 18 h following SD (ZT 7 – 24).

The telemetric EEG and EMG data were transmitted at 455 KHz to an RPC-1 receiver (DSi™) and sampled at 500 Hz. Vigilance states for consecutive 4-s epochs were classified by visual inspection of the EEG and EMG signals according to standard criteria (17) as follows: 1) wakefulness (high and variable EMG activity and a low-amplitude EEG signal), 2) non-rapid-eye movement sleep (NREM sleep; high EEG amplitude, dominated by slow waves, low EMG), and 3) REM sleep (low EEG amplitude, theta oscillations [5 – 9 Hz] and loss of muscle tone).

The time spent in each vigilance state was expressed as a percentage of total recording time over various time intervals (i.e., 1-h, 2-h, 3-h, 6-h, 12-h, or 24-h). Additionally, REM sleep was also expressed as a percentage of total sleep time (TST). The effects of SD on recovery sleep were assessed as accumulated differences from the baseline condition for consecutive 1-h intervals starting at light onset (ZT 0). The occurrence of bouts of wakefulness and total sleep (TS; i.e. NREM sleep + REM sleep) was assessed per hour of wakefulness or total sleep, respectively, over time bins (by counting the number of consecutive 4-s epochs) of logarithmically increasing size (as previously described (34)). Thus, the range of consecutive time bins was: < 4, 8 – 12, 16 – 28, 32 – 60, 64 – 124, 128 – 252, 256 – 508, 512 – 1020, and > 1024 seconds.
EEG power density

EEG power spectra were computed for consecutive 4-s epochs by a Fast-Fourier Transform (FFT) (frequency range: 0.00 – 249.76 Hz; resolution: 0.24 Hz; Hanning window function). One WT mouse was excluded from the spectral analysis due to artifacts in the EEG signal. For the mice included in spectral analyses (Per3−/−: n = 8; WT: n = 6), epochs containing EEG artifacts were discarded from EEG spectral analyses (% of recording time: 0.4 ± 0.09 %).

EEG delta power during NREM sleep was computed by averaging the EEG power in the frequencies ranging from 0.977 to 3.906 Hz. In order to maximize the number of data points contributing to each bin, mean values were calculated per 3-h and individually normalized to the mean delta power in NREM sleep for the entire 24-h baseline period on day one, during which the total delta power did not differ between genotypes. Accumulated delta power during NREM sleep was represented as hourly cumulative values of delta energy (i.e., summed delta power over all epochs) and expressed as a percentage of the total delta energy during the last 18 h of baseline day (ZT 6 – 24).

Genotypic differences in EEG power spectra were determined for NREM sleep and REM sleep during the 12-h baseline light period. EEG power spectra were expressed as a percentage of total EEG power (frequency range: 0.24 – 25.15 Hz; resolution: 0.244 Hz). The theta-peak frequency (TPF) in REM sleep was determined by taking the frequency at which EEG power density was maximal.
Statistics
Data were analyzed using SAS (v9.2, SAS Institute, Cary, NC, USA) PROC MIXED for analysis of variance followed by the LSMEANS (least square means) statement for post-hoc multiple pair-wise comparisons, and for non-parametric analyses PROC NPAR1WAY (Kruskal-Wallis test) and PROC FREQ (Chi Square test), which was followed by PROC TTEST for post-hoc multiple pair-wise comparisons. Statistical tests have not been corrected for multiple comparisons, which would be overly conservative for these types of analyses.

RESULTS

Duration and temporal pattern of locomotor activity
While there was no overall difference in locomotor running wheel activity levels between WT and Per3<sup>−/−</sup> mice (PROC MIXED, P > 0.05), there were significant contrasts at different times of day (PROC MIXED - LSMEANS). Specifically, the total number of active running wheel minutes per hour was higher in Per3<sup>−/−</sup> mice than in WT mice during the dark period at ZT 14 – 15 and 18 – 20 (P < 0.05 and P < 0.005 respectively; PROC MIXED – LSMEANS, Fig. 1A). The intensity of running wheel activity (revolutions/minute) was also significantly higher in Per3<sup>−/−</sup> mice at ZT 18 – 19 (P < 0.05; proc MIXED – LSMEANS, Fig. 1B). Distributions of the episode
lengths of running activity and inactivity episodes in the dark period indicated that \( \text{Per3}^{-/} \) mice showed a decreased number of episodes of activity with durations of 1 min \((P < 0.005; \text{PROC MIXED – LSMEANS, Fig. 1C})\). No differences in the number of inactivity episodes were observed (Fig. 1D). During the experiment, body temperature data were also collected simultaneously by telemetry (data not shown). The overall temperature profiles were not significantly different between genotypes (both absolute data and z-scored data), with significant time-point-specific differences limited to baseline ZT 21 – 22 \((P < 0.05; \text{PROC MIXED – LSMEANS})\) and recovery ZT 20 – 21 \((P < 0.005)\), where \( \text{Per3}^{-/} \) temperature was higher, and recovery ZT 6 – 8 \((P < 0.05)\), where \( \text{Per3}^{-/} \) temperature was lower. The former, higher temperatures are in close proximity to where we observed higher running wheel activity in \( \text{Per3}^{-/} \) mice (Fig.1A), and the latter, lower temperatures correspond to where we observed reduced wakefulness in these mice (Fig.3).

**Duration and temporal pattern of EEG-assessed vigilance states at baseline**

The distribution of bout lengths of wakefulness and total sleep (TS) showed minor differences between the genotypes. \( \text{Per3}^{-/} \) mice had more bouts of wakefulness with a duration of 32 – 60 s during the 12-h light period, and fewer bouts of TS with a duration of 32 – 60 seconds during the 12-h dark period, suggesting less fragmented sleep in \( \text{Per3}^{-/} \) during the 12-h dark period compared to WT mice (PROC FREQ, \( P[\chi^2] < 0.005; \text{PROC TTEST}, P < 0.05; \) Fig. 2).
During the baseline day, the 24-h averages of time spent in wakefulness, NREM and REM sleep were not different between the genotypes (Table 1). The average time spent in REM sleep as a percentage of TST during the baseline dark period was decreased in Per3^-/- mice (8.69% vs. 12.86% for WT, PROC MIXED, \( P < 0.05 \); table 1). This was primarily related to less REM sleep during the interval ZT 20 – 22 (Fig. 3, PROC MIXED – LSMEANS, \( P < 0.005 \)). During this interval, there was also less NREM sleep and wakefulness was increased in Per3^-/- mice (Fig. 3, PROC MIXED – LSMEANS, \( P = 0.0005 \) and \( P < 0.0005 \), respectively). At the beginning of the baseline light period (ZT 0 – 2), Per3^-/- showed more NREM sleep and REM sleep amount and reduced wakefulness (Fig. 3, PROC MIXED – LSMEANS, \( P = 0.005 \), \( P < 0.01 \), and \( P < 0.005 \), respectively).

**Duration and temporal pattern of EEG-assessed vigilance states in response to SD**

*Comparison to Baseline*

In response to the 6 h sleep deprivation, both genotypes showed differences compared to baseline in the amount of time spent in each vigilance state. These differences were located in the light period during the 2 h immediately after SD and in the 2 h just before the dark period (Fig 3). As expected, there was reduced wakefulness in both genotypes immediately after SD (ZT 6 – 8 compared to baseline ZT 0 – 2; PROC TTEST [paired], \( P < 0.005 \) for WT, \( P < 0.05 \) for Per3^-/-), but also increased wakefulness in Per3^-/- just before the dark period (ZT 10 – 12 compared to baseline ZT 4 – 6; \( P < 0.05 \)). SD induced a significant increase in NREM sleep time at ZT 6 – 8 (compared to baseline ZT
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0 – 2) and a decrease at ZT 10 – 12 (compared to baseline ZT 4 – 6) in both WT (\( P < 0.005 \) and \( P < 0.05 \), respectively; Fig.3) and Per3\(^{-/-} \) mice (\( P < 0.05 \) for both). REM sleep time ‘rebound’ was observed only in WT mice (\( P < 0.05 \)), while a reduction relative to baseline was only observed in Per3\(^{-/-} \) mice at ZT 10 – 12 (compared to baseline ZT 4 – 6; \( P < 0.05 \); Fig.3). Because mice showed an expected anticipatory rise in wakefulness before the dark period, which is not present at ZT 4 – 6, we also compared vigilance states at ZT 10 – 12 in both baseline and recovery. For this comparison, WT mice showed reduced wakefulness (\( P < 0.05 \)) and increased NREM sleep (\( P < 0.05 \)).

**Comparison of Genotypes**

Per3\(^{-/-} \) mice did not show any significant differences compared to WT mice in the average time spent in each vigilance state during the 6-h light recovery and 12-h dark recovery periods (Table 2).

To further characterize the effects of sleep deprivation, we accumulated the hourly differences in sleep time between baseline and recovery days (Fig. 4). Negative slopes indicate loss in sleep time relative to baseline, null slopes indicate no loss or gain, and positive slopes indicate a gain in sleep time. There were no genotype differences in sleep time profiles during SD, except for one time point (ZT 4 – 5) where Per3\(^{-/-} \) mice lose more TST (PROC MIXED - LSMEANS, \( P < 0.05 \)). NREM sleep time recovery showed a similar profile in both genotypes. The effect of Per3 disruption was associated with a slower REM sleep recovery in Per3\(^{-/-} \) mice compared to WT mice (ZT 12 – 18 and ZT 19 – 21; PROC MIXED - LSMEANS, \( P < 0.05 \); Fig.
4). *Per3*<sup>−/−</sup> mice also showed reduced total sleep time accumulation after SD at ZT 20 – 21 (PROC MIXED – LSMEANS, *P* < 0.05).

**EEG power density**

The NREM sleep EEG power spectrum during the 12-h baseline light period did not reveal any significant differences between genotypes (Fig. 5). Delta frequencies in the range 1.22 to 1.95 Hz were higher in the *Per3*<sup>−/−</sup> mice in the REM sleep power spectra (Fig. 5). The REM sleep spectrum showed a shift of theta frequencies in *Per3*<sup>−/−</sup> mice towards faster frequencies, and a corresponding decrease of power density for the frequency bin of 6.35 Hz (Fig. 5). The theta peak frequency (TPF) showed a trend for a ‘faster theta’ in *Per3*<sup>−/−</sup> mice (TPF= 7.26 Hz vs. 6.96 Hz for WT, PROC TTEST, *P* = 0.06).

To further investigate whether the disruption of Per3 affects the homeostatic sleep regulation, the time course of EEG delta power (1 – 4 Hz) during NREM sleep (i.e., an indicator of sleep pressure) was computed. A characteristic decline during the light period and an increase during the dark period of EEG delta power were observed during the baseline day in both genotypes (Fig. 6). In addition, *Per3*<sup>−/−</sup> mice had higher delta power immediately after SD (ZT 6 – 9) compared to baseline (ZT 0 – 3; PROC TTEST [paired], *P* < 0.005). Compared to WT mice, *Per3*<sup>−/−</sup> mice showed higher EEG delta power at the beginning and the end of the baseline dark period compared to WT mice (PROC MIXED - LSMEANS, *P* < 0.005). Compared to WT, *Per3*<sup>−/−</sup> mice also had higher delta power during the last nine hours of the recovery dark period (PROC MIXED – LSMEANS, *P* < 0.05 for ZT 15 – 21, and *P* < 0.0005 for ZT 21 – 24).
To investigate the association between NREM sleep duration and EEG delta power during NREM sleep (i.e., an indicator of sleep pressure), delta energy was computed at a higher temporal resolution by accumulating the hour-by-hour delta energy values for the baseline day and for the recovery period. Delta energy was significantly higher in $\text{Per3}^{-/-}$ mice for the first baseline interval (ZT 0 – 1; PROC MIXED - LSMEANS, $P < 0.0001$; Fig. 7) due to a 3-fold increase of NREM sleep duration in $\text{Per3}^{-/-}$ within this first interval (30.8 min for $\text{Per3}^{-/-}$ vs. 9.7 min for WT; PROC TTEST, $P < 0.005$). Although there are no significant differences for accumulated delta power during the dark period for both baseline and recovery, accumulated delta power was higher in the $\text{Per3}^{-/-}$ mice for both dark periods, in agreement with data presented in Figure 6. The rate of accumulation of delta power was also higher during recovery after SD, reflecting the higher NREM sleep bouts observed in the $\text{Per3}^{-/-}$ mice immediately after SD (Fig. 3).

**DISCUSSION**

A previous study investigated sleep rhythmicity and homeostasis in mice with targeted disruption of each of the three $\text{Per}$ genes and for $\text{Per1/2}$ double mutants in baseline and recovery after 6 h of SD in the second half of the light period (31). The main results from that study were higher and longer-lasting delta power in $\text{Per1}^{-/-}$ mice after SD, and enhanced delta power in the $\text{Per1/2}$ double mutants after SD. No significant differences between the $\text{Per3}^{-/-}$ mice and WT mice were presented. The main conclusions of the study were that
Sleep homeostasis is intact in all Per mutants, and that sleep levels are maintained in arrhythmic Per1/2 double mutants.

By contrast, here, with Per3−/− mice on a C57BL/6J background, we present data for baseline and recovery after 6 h of SD in the first half of the light period that show differences in the time series and accumulation of vigilance states across baseline and recovery periods between genotypes after SD, differences in the REM sleep power spectra between genotypes, and differences in the amount and accumulation of delta power across baseline and recovery between genotypes. We also show robust differences in wheel-running activity levels between genotypes. Thus, we show significant Per3 genotype-dependent differences in all major established measures of sleep/wake regulation. Hence, while we confirm that sleep homeostasis is intact in Per3−/− mice, it is functionally altered.

We have shown that there are significant differences between Per3−/− and WT mice on a C57BL/6J background in the temporal distribution of sleep and waking activity, as measured independently by running-wheel activity and EEG. The significant difference that we observed in wheel-running activity at ZT 19 and 20 in the dark period between Per3−/− and WT mice is strikingly similar to the behavioral phenotype observed in Npas2−/− mice on the same genetic background (13). In both cases, the WT mice display a clear decrease in locomotor activity, which is consistent with other published data (26, 36) but less pronounced or absent in the Per3−/− and Npas2−/− mice, respectively. Dudley et al. conclude that this reduction in activity in the middle of the dark
phase therefore requires NPAS2, and the same could be argued, perhaps to a lesser extent, for PER3.

In agreement with previous data from Per3−/− mice on a different genetic background (31), we observed no genotype difference in the percentage of total time spent in wakefulness, NREM sleep, and REM sleep during the 24-h baseline day. An overall genotype difference in vigilance states at baseline was found in the amount of REM sleep as a percentage of total sleep time (not computed in Shiromani et al.), where Per3−/− had less REM sleep during the baseline dark period. When vigilance states were analyzed with a 2-h resolution their temporal distributions at baseline were different between the genotypes, with Per3−/− mice showing less wakefulness, and more NREM and REM sleep at the beginning of the light period. The timing of the genotypic differences in relation to the dark-light transition is in line with our previously reported light-dependent behavioral phenotypes in Per3−/− mice (37). Per3−/− mice also showed more wakefulness and less NREM and REM sleep in the baseline dark period around ZT 20. These differences in the temporal distribution of vigilance states occurred at roughly the same time where running-wheel activity in an independent, much larger group of animals was greater in Per3−/− mice, indicating again a parallel between the expression of behavioral activity and sleep dynamics in these mice under baseline conditions.

Per3−/− mice exhibited higher NREM EEG delta power at the beginning and the end of the baseline dark period compared to WT mice (Fig.6), which agrees with the less fragmented sleep recorded in Per3−/− mice during the dark period (see bottom right panel of Fig.2). This shows that the Per3−/− mice
accumulated higher levels of delta power during the baseline dark period, compared to WT (see also Fig.7). While the absence of PER3 may disrupt other mechanisms that cause an increase in delta power in the Per3−/− mice, this is most likely associated with an increased homeostatic drive since higher NREM sleep in the Per3−/− mice immediately after lights on in baseline presumably reflects the dissipation of a higher build-up of delta power in the dark period during the preceding 24 h. Although not significant, this is reflected also in the tendency for higher NREM sleep observed in the Per3−/− mice immediately after SD (Fig.3), with a corresponding faster accumulation of delta power (Fig. 7). Consistent with this, Per3−/− mice also showed a slower REM sleep accumulation during the recovery period (Fig.4). Higher EEG delta power is also present in Per3−/− mice in the last 9 hours of the recovery dark period. This difference increases towards the end of the recovery dark period, as also confirmed in the rate of delta accumulation in Figure 7. In a previous study where SD was performed in the second half of the light period (i.e., shifted 6 h compared to here), there was a similar increase in delta power compared to WT during the last 3 h of recovery dark period in Per3−/− mice (31). There was no difference in EEG power density spectrum for NREM sleep between the genotypes, but the REM sleep spectrum showed significant differences in the delta (higher in Per3−/−) and theta frequency bands (lower in Per3−/− mice). Because of the way in which this analysis was carried out (% of total EEG power), it is possible that the reduced theta power is a direct consequence of the increased delta power. However, there is also evidence for a shift towards higher frequency for the theta peak in Per3−/− mice, which is not likely to be caused by an artifact of analysis and would
explain the observed reduction in amplitude at 6.35 Hz. Increased REM sleep theta power is a marker for sleep pressure in humans (11) and this change in the theta peak is of interest with regard to the increased REM sleep theta power observed in human \( PER3^{5/5} \) individuals compared to \( PER3^{4/4} \) (39). The genotype effects on delta power during the dark periods, when sleep pressure is higher in \( Per3^{-/-} \) mice, are in accordance with phenotypes that are associated with a VNTR polymorphism in \( PER3 \) reported in humans (39). While the human \( PER3 \) VNTR polymorphism has been linked with differences in sleep homeostasis (39), cognitive vulnerability to sleep loss (20), and differences in fMRI-assessed brain activity in response to sleep loss (38), none of these studies has shown any associations between the VNTR and circadian markers. This agrees with studies that show a redundant role of \( PER3 \) in the animal circadian clock where minimal (30) or zero (37) period differences are seen in \( Per3^{-/-} \) mice, and also the behavioral phenotypes that have been observed in the \( Per3^{-/-} \) mice that are light-dependent (reduced negative masking and shorter period in constant light) (37). It should also be noted that while polymorphisms in the \( PER3 \) promoter have been associated with reduced levels of reporter gene expression (3), there is no evidence to suggest that the \( PER3 \) VNTR is linked with reduced levels of \( PER3 \) expression.

It has been shown in \( Per3^{-/-} \) mice that the absence of \( PER3 \) does not change the period of clock gene expression within the SCN but does affect circadian period in other central and peripheral tissues (29). This tissue-specific role for \( PER3 \) has been observed previously for \( Per1 \) and \( Per2 \), whose expression in the forebrain (18) and cortex (42) increases after sleep
deprivation and is restored to baseline levels after recovery sleep. In addition, _Per_ expression in the cortex is at a different phase to its expression in the SCN (30), as is _Per3_ expression in other brain areas and in the periphery (5, 29, 33). This implies that _Per_ expression in the forebrain tracks sleep loss in an SCN-independent way. Therefore, the overlapping roles of clock genes in circadian and sleep systems may have very distinct and non-overlapping localization (for review, see Ref. 14).

Perspectives and Significance

Although PER3 has been shown to interact with other components of the molecular clock, phenotypes revealed by its absence are non-circadian. In this study, we confirm a role for PER3 in waking activity and sleep homeostasis. While the effects of the absence of PER3 are relatively subtle, they nonetheless affect all of the established measures of sleep/wake regulation. Thus, while other core clock proteins may have overlapping roles in both the circadian and sleep systems, PER3 phenotypes from human and animal studies point towards a more prominent role for PER3 in the regulation of sleep and waking activity.

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GRANTS

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FIGURE LEGENDS

Fig. 1. Running wheel activity measures (means ± SEM) in WT (n = 54) and Per3−/− (n = 53) mice. (A) Total amount of active minutes per hour calculated for the last day of activity recordings. (B) Intensity of running wheel activity expressed as average activity per active minute. (C & D) Episode length of consecutive minutes with activity (C) and inactivity (D) was calculated over the last 10 days. Closed symbols & bars indicate WT and open ones indicate Per3−/− mice. Error bars show SEM. * P < 0.05, ‡ P < 0.005, # P < 0.001 (PROC MIXED – LSMEANS).

Fig. 2. Distribution of bouts of wakefulness (W) and total sleep (TS) throughout nine consecutive time bins (< 4, 8-12, 16-28, 32-60, 64-124, 128-252, 256-508, 512-1020, > 1020 seconds) for the 12-h light and 12-h dark periods of the baseline day. Only the lower interval for each bin is indicated. Histograms represent the mean number of W and TS episodes (± SEM; WT: n = 7, Per3−/−: n = 8) per bin expressed per hour of W and TS, respectively. * P < 0.05 and ‡ P < 0.005 (PROC FREQ followed by PROC TTEST).

Fig. 3. Time course of two-hourly binned values of Wakefulness (W), NREM sleep (NREMS) and REM sleep (REMS) for the baseline and recovery periods, expressed in percentage of recording time per 2 hours (± SEM; WT: n = 7, Per3−/−: n = 8). Black (WT) and white (Per3−/−) triangles represent the intervals with a significant increase (triangle up) or decrease (triangle down) effect of SD (triangle: P < 0.05; triangle inside square: P < 0.005; PROC
TTEST [paired]), compared to baseline (the three post-SD intervals were compared to the three first baseline intervals after light onset). The last post-SD light interval was also compared to the last baseline light interval (significant increase or decrease in WT are indicated by a black up or down triangle with a horizontal bar; \( P < 0.05 \); PROC TTEST [paired]). Significant interactions 'genotype x time of day' are found for baseline day in the three vigilance states (W: \( P < 0.05 \); NREMS: \( P < 0.0001 \); REMS: \( P < 0.05 \); PROC MIXED). ** \( P < 0.01 \), ‡ \( P < 0.005 \), # \( P < 0.001 \), ## \( P < 0.0005 \) (LSMEANS for 'genotype' effect).

Fig. 4. Time course of accumulated differences in sleep time (Total sleep [TS], NREMS & REMS) between the SD-Recovery and baseline conditions. Accumulated sleep values (mean ± SEM; WT: \( n = 7 \), Per3\(^{-/-}\): \( n = 8 \)) were expressed relative to baseline conditions (= black horizontal line). Significant interactions 'genotype x time of day' are found for all sleep states (TS: \( P < 0.005 \); NREMS: \( P < 0.001 \); REMS: \( P < 0.05 \); PROC MIXED). Dark-grey bars at the bottom of the panels indicate intervals in which 'genotype' effects were observed (\( P < 0.05 \); LSMEANS).

Fig. 5. EEG spectral profiles of NREM and REM sleep during the baseline light period. The mean (± SEM; WT: \( n = 6 \), Per3\(^{-/-}\): \( n = 8 \)) of the EEG spectra was normalized to total EEG power for all frequencies within corresponding sleep states. Grey bars at the bottom indicate the frequency bins in which 'genotype' effects were observed. \( (P^{[\chi^2]} < 0.05 \); PROC NPAR1WAY).
Fig. 6. Time distribution per 3-h intervals of EEG delta power during NREM sleep. EEG delta power (mean ± SEM; WT: n = 6, Per3⁻/⁻: n = 8) was expressed relative to the 24-h baseline period. Significant interaction ‘genotype x time’ and ‘genotype’ effect occurred over the whole distribution ($P < 0.05$; PROC MIXED). Intervals with significant ‘genotype’ effect are indicated with * $P < 0.05$, ‡ $P < 0.005$, ## $P < 0.0005$ (LSMEANS). Per3⁻/⁻ mice had increased delta power at ZT 6 – 9 post-SD compared to baseline ZT 0 – 3 (upward triangle inside square, $P < 0.005$; PROC TTEST [paired]).

Fig. 7. Hourly time course of delta energy in NREM sleep. Baseline (bottom & left axis) and recovery (top & right axis) values of delta energy mean (± SEM; WT: n = 6, Per3⁻/⁻: n = 8) expressed as a percentage of the total delta energy for the last 18 hours (ZT 6 – 24) of baseline. Significant interaction ‘genotype x time of day’ is found during baseline day ($P < 0.0001$; PROC MIXED). Significant hourly ‘genotype’ effect $P$ values are represented with § ($P < 0.0001$; LSMEANS).
<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>Per3&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Wild-type</th>
<th>Per3&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>24h</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wakefulness (%)</td>
<td>63.57 ± 1.38</td>
<td>63.45 ± 1.65</td>
<td>83.4 ± 2.39</td>
<td>87.20 ± 3.21</td>
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<td>NREMS (%)</td>
<td>31.03 ± 1.14</td>
<td>31.13 ± 1.61</td>
<td>14.30 ± 1.92</td>
<td>11.61 ± 2.84</td>
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<tr>
<td>REMS (%)</td>
<td>5.40 ± 0.35</td>
<td>5.42 ± 0.24</td>
<td>2.29 ± 0.49</td>
<td>1.20 ± 0.37</td>
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<td>REMS/TST (%)</td>
<td>13.99 ± 1.00</td>
<td>12.34 ± 0.60</td>
<td>12.86 ± 1.54</td>
<td>8.69 ± 0.97</td>
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</table>

Percentage (mean ± SEM) of Wakefulness, NREMS and REMS (% of total recording time), and REMS/TST (% of Total Sleep Time). Statistics are represented by * = P < 0.05 (PROC MIXED). n = 7 for WT and 8 for Per3<sup>−/−</sup>.
Table 2. Vigilance states during recovery (R)

<table>
<thead>
<tr>
<th></th>
<th>Wakefulness (%)</th>
<th>NREMS (%)</th>
<th>REMS (%)</th>
<th>REMS/TST (%)</th>
</tr>
</thead>
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<tr>
<td><strong>First 6h light B</strong></td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>42.18 ± 2.49</td>
<td>48.94 ± 2.20</td>
<td>8.88 ± 0.39</td>
<td>15.40 ± 0.47</td>
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<td>*Per3−/−</td>
<td>33.89 ± 2.85</td>
<td>*55.36 ± 2.55</td>
<td>10.76 ± 0.80</td>
<td>16.28 ± 1.00</td>
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<td><strong>6h light SD</strong></td>
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<tr>
<td>Wild-type</td>
<td>99.93 ± 0.03</td>
<td>0.07 ± 0.03</td>
<td>0.00</td>
<td>n.a.</td>
</tr>
<tr>
<td>*Per3−/−</td>
<td>99.80 ± 0.03</td>
<td>*0.20 ± 0.03</td>
<td>0.00</td>
<td>n.a.</td>
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<tr>
<td><strong>6h light R</strong></td>
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<tr>
<td>Wild-type</td>
<td>38.50 ± 2.78</td>
<td>51.07 ± 2.43</td>
<td>10.43 ± 1.13</td>
<td>16.92 ± 1.48</td>
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<td>*Per3−/−</td>
<td>34.16 ± 1.89</td>
<td>55.82 ± 1.63</td>
<td>10.02 ± 0.43</td>
<td>15.21 ± 0.46</td>
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<td><strong>12h dark R</strong></td>
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<tr>
<td>Wild-type</td>
<td>83.90 ± 3.89</td>
<td>13.53 ± 3.25</td>
<td>2.57 ± 0.66</td>
<td>14.98 ± 1.33</td>
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<td>*Per3−/−</td>
<td>87.24 ± 2.43</td>
<td>11.02 ± 2.01</td>
<td>1.74 ± 0.43</td>
<td>12.90 ± 0.61</td>
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</table>

Percentage (mean ± SEM) of Wakefulness, NREMS and REMS (% of total recording time), and REMS/TST (% of Total Sleep Time). The 6 h light baseline (B) values are shown to contrast the values of 6 h light R. The values of Sleep Deprivation (SD) indicate the efficiency of SD. Statistics are represented by * = P < 0.05 (PROC MIXED). n = 7 for WT and 8 for Per3−/−.