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# Sleep phenotype of *Period3* gene knockout mice under light-dark entrained condition

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## Abstract

Circadian rhythmicity contributes to sleep-wake phenotypes, and is regulated by clock genes. Although *Period3* gene (*Per3*) is not essential for circadian rhythm generation, it does modulate circadian clock outputs and polymorphisms of this gene related to some circadian sleep disorders. We studied the role of *Per3* gene on sleep-wake phenotypes by comparing *Per3* knockout mice (*Per3*$^{-/-}$) with wild-type (WT) mice under light-dark condition. Here we report that *Per3*$^{-/-}$ mice had more wakefulness and less non-rapid eye movement sleep (NREMS) at ZT 0 in the light period than WT mice. Likewise, in last hour of the light period on second day, wakefulness was higher and NREMS was lower in *Per3* mutants. *Per3*$^{-/-}$ mice began their main sleeping episodes later and waking episodes earlier than WT mice, whereas the average time spent in wakefulness, NREM and rapid eye movement (REM) sleep were not different between the genotypes. In addition, *Per3*$^{-/-}$ mice had significantly less sleep bouts than WT mice at early and late light periods. These results demonstrate the influence of *Per3* on sleep phenotypes related to light-dark transition and additionally show subtle effects on sleep times and sleep fragmentation.

***Keywords*****:** *Per3 gene, circadian rhythm, sleep phenotype, sleep-wake phenotypes, NREMS, REMS*

## 1. Introduction

Sleep is controlled by brain functions via numerous ways (Brown, Basheer, McKenna, Strecker, & McCarley, 2012). Environmental (Mistlberger, 2005) and genetic factors can significantly influence sleep and its architecture (Kimura & Winkelmann, 2007). Together with sleep-wake behavior, which is controlled by a circadian phase (Mistlberger, 2005), daily rhythms of behavioral states are intrinsically driven by the circadian clock (Kimura & Winkelmann, 2007; Mistlberger, 2005; Ukai & Ueda, 2010), which is further entrained by external cues such as light (Ukai & Ueda, 2010).

The circadian clock is cell-autonomous and self-sustained function, found not only in the suprachiasmatic nucleus (SCN) but also in the peripheral tissues. The clock mechanism is known to be similar at the molecular level, which consists of a network of transcriptional-translational feedback loops. Core clock components are defined as genes whose protein products such as *Circadian locomotor output cycles kaput* (*Clock*), *Brain and muscle aryl hydrocarbon receptor nuclear translocator like 1* (*Bmal1*), *Period* (in mice, *Per1*, *Per2*, and *Per3*), and *Cryptochrome* (*Cry1* and *Cry2*) are necessary for the generation and regulation of circadian rhythms (Ko & Takahashi, 2006; Lowrey & Takahashi, 2011; Ukai & Ueda, 2010). The molecular mechanism for circadian rhythm generation begins early in the circadian day by the formation of a CLOCK and BMAL1 heterodimer. These transcription factor proteins bind to E-boxes, which activate the transcription of several other clock genes and clock-related genes, including *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2* (Gekakis et al., 1998; Jin et al., 1999; King et al., 1997; Pace-Schott & Hobson, 2002; Reppert & Weaver, 2002; Richards & Gumz, 2013). The RNA products of *Per* and *Cry* genes are translocated from the nucleus to the cytoplasm. Following translation to PER and CRY

proteins, they dimerize to PER-CRY complex and reenter the nucleus to exert feedback effects by inhibiting the actions of the CLOCK-BMAL1, thus indirectly repressing their own transcription via a negative feedback loop (Kume et al., 1999; Lowrey & Takahashi, 2011; Pace-Schott & Hobson, 2002; Reppert & Weaver, 2002; Richards & Gumz, 2013; Shearman et al., 2000b; Vitaterna et al., 1999). Subsequently, the PER-CRY complex is degraded via the proteasomal degradation pathway, thus relieving CLOCK-BMAL1 inhibition at the end of cycle, such that CLOCK-BMAL1 can initiate a new cycle again (Lowrey & Takahashi, 2011).

The study of some clock genes expressed in the SCN and circadian rhythms of locomotor behavior in *Per1* or *Per2* single mutant mice and *Per1*/*Per3* or *Per2*/*Per3* double-mutant mice indicated that *Per1* and *Per2* have different roles in central circadian clock functions, while *Per3* does not affect core clock mechanisms (Bae et al., 2001). In addition, *Per1* and *Per2* have distinct roles in the circadian system by displaying different patterns in sleep rhythm parameters of mutants, but are not necessary for sleep homeostatic regulation (Kopp, Albrecht, Zheng, & Tobler, 2002; Shiromani et al., 2004). *Per3* in mice has mild effects on circadian period (Shearman, Jin, Lee, Reppert, & Weaver, 2000a). By contrast, other studies in *Per3*$^{-/-}$ mice have reported a role for *Per3* in sleep-wake timing and sleep homeostasis regulations (Hasan, van der Veen, Winsky-Sommerer, Dijk & Archer, 2011). *Per3* may also be important for timekeeping and period determination in specific peripheral oscillators such as the pituitary gland, lungs, and liver (Pendergast, Friday, & Yamazaki, 2010; Pendergast, Niswender, & Yamazaki, 2012).

Recent evidence suggests that the *Per3* gene has a functionally distinct role separate from the proposed role in negative feedback. Although the *Per3* gene is not essential for maintaining circadian rhythmicity (Von Schantz, 2008), variable-number tandem-repeat (VNTR) polymorphisms in this gene are significantly associated with delayed sleep phase disorder (DSPD) (Dijk & Archer, 2010; Von Schantz, 2008). However, the role of *Per3* gene in circadian rhythm seems unclear. We investigated the phenotypes of *Per3*$^{-/-}$ mice on C57BL/6J genetic background in more detail focusing on the sleep-wake phenotype over 1-day period.

## 2. Objectives

This research paper aims to monitor the sleep-wake cycle and sleep phenotype in mice with deletion of the *Per3* gene under entrained condition.

## 3. Materials and methods

### 3.1 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*$^{-/-}$) (Shearman et al., 2000a) and their wild-type (WT) controls were bred in-house from heterozygous breeding pairs. Heterozygous mice (strain number J010493) were purchased from Nanjing Biomedical Research Institute of Nanjing University, China. We confirmed the different genotypes of the F4 offspring, then homozygous F4 breeding pairs were used to replace the F1 generation when available. Mice were housed in light-tight, ventilated environmental compartments in a temperature- and humidity-controlled facility. Storage cages held up to 6 animals. Food and water were available ad libitum. Mice were maintained on a 12:12-h light-dark cycle. Mice were genotyped at weaning by polymerase chain reaction amplification of genomic DNA extracted from tail biopsies.

Adult male mice 10 week old were individually housed in acrylic cages in an experimental room with an ambient temperature 24±1°C under a 12:12-h light-dark cycle (lights on at 07:00 AM; 80-100 lx, fluorescent tube 40 W). Food and water were available ad libitum. Animals were kept under these conditions for at least 7 days before the experiment.

### 3.2 Genotyping

Genotypes were determined by PCR and/or Southern blot analysis of tail biopsy DNA (Shearman et al., 2000a) (Figure 1). Tail samples were cut 3 mm by aseptic technique, centrifuged at 600 rpm in tail digestion buffer (Sangon Biotech Co. Ltd., Shanghai, China) at 65°C for 1-h until sample dissolved. Next PA buffer was added and the sample was mixed by vortex, stored at -20°C for 5 min then centrifuged at 10000 rpm for 5 min. The supernatant was transferred to a new tube, mixed with isopropanol and centrifuged at 10000 rpm for 5 min. The supernatant was decanted, and the DNA precipitate was rinsed two times with 75% ethanol and centrifuged at 10000 rpm 2 min,

dried for 30 min and re-dissolved with TE buffer (10 mM Tris-HCl， 1 mM EDTA， pH 8.0, Sangon Biotech Co. Ltd., Shanghai, China). DNA extracts were immediately transfered to PCR experiment or kept at -20°C until the next process. The PCR was done with three primers, a forward primer for wild-type (WT) TCTGTGAGTTCTTCCGTGTCT, mutant (*Per3*$^{-/-}$) TGCCCCAAAGGCCTACCCGC, and a common primer TCTTTGGGTCCAGTTGTTCC. The PCR protocol consisted of 5 min at 95°C, 35 cycles of amplification (each consisting of 30 sec at 94°C, 35 sec at 65°C, and 45 sec at 72°C), and a final extension phase (3 min at 72°C). Products were separated on 1.0% agarose gels and viewed by UV transillumination with ethidium bromide.

3.3 Surgery

EEG and electromyogram (EMG) electrodes were implanted under deep chloral hydrate anesthesia (400 mg/kg ip). The implantation was performed as previously reported (Franken, Malafosse, & Tafti, 1998), two stainless steel screws (diameter 1 mm) served as EEG electrodes and were screwed through the skull over the right cerebral hemisphere (frontal: 1.7 mm lateral to midline, 1.5 mm anterior to bregma; parietal: 1.7 mm lateral to midline, 1.0 mm anterior to lambda). Two flexible multi-strand insulated stainless steel wires served as EMG electrodes and were inserted into the neck muscles ~ 5 mm apart. The electrodes, which were soldered to recording leads before implantation, were cemented to the skull with dental acrylic. The animals were allowed 7–10 days of recovery from surgery and habituation before the experiment, and data collection started when the mice were 12-13 weeks of age (24.5-26 g).

3.4 Experimental protocol

One week after recovery from surgery, the animals were connected to lightweight recording cables and adapted for 1 week. The cables permitted complete mobility and normal behavior including rearing, turning, and assuming a curled sleep posture. Each animal was adapted and measured for EEG and EMG in its own acrylic cage in an observation room which was a sound-proof and electrically shielded, under 12-h light-dark condition (light-on at 7:00AM; as zeitgeber time 0 (ZT 0), light-off at 7:00PM; as ZT 12). The EEG and EMG signals were recorded consecutively for 48-h on electroencephalograph (MP 150, Biopac systems, Inc.), starting at lights on, and by using AcqKnowledge 4.2 (Biopac systems, Inc.). The analog EEG and EMG signals were sampled at 500 Hz, amplified (20,000x for EEG, 2,000x for EMG) and filtered. EMG filtered with a high-pass (10 Hz) and a low-pass (500 Hz), EEG filtered with high-pass (1.0 Hz).

3.5 Analysis of sleep data

The EEG/EMG data were determined using SleepSign 2.0 (Kissei Comtec, Nagano, Japan). Vigilance states for consecutive 10 sec epochs were classified by visual inspection of the EEG and EMG signals, according to standard criteria (Lee-Chiong, 2006) as follows: 1) wakefulness (high and variable EMG activity and a low-amplitude EEG signal), 2) NREM sleep (high EEG amplitude, dominated by delta wave (0.5-4 Hz), low EMG), and 3) REM sleep (low EEG amplitude, theta wave (4-8 Hz) and loss of muscle tone) (Figure 2).

The time spent in each vigilance state was expressed as a percentage per hour and average percentage in 12-h light-dark period. The occurrence of bouts of total sleep (TS; i.e., NREM sleep + REM sleep) was assessed per hour of total sleep, over 1-h bins (by counting the number of NREMS and REMS for each 10 sec epochs) and number of sleep bouts over 12-h light-dark period. A two-tailed *t*-test (Microsoft Excel 2010) was used to compare differences in sleep parameters between genotype.

## 4. Results

4.1 Daily rhythm of vigilance states

The percentage of time spent in waking, NREM sleep, and REM sleep in each hour is depicted in Figure 3. Sleep-wake circadian in entrained light-dark (LD) conditions were unaffected in *Per3*-deficient mice (Figure 3). Differences between WT mice and *Per3*$^{-/-}$ mice were observed at some time-points, particularly after light onset. At the first hour after light-on (ZT 0) data show significant differences in time spent of wakefulness and NREMS, *Per3*$^{-/-}$ mice had more wakefulness than WT mice (85.64 ± 5.64% vs. 36.64 ± 8.05% on first day, $P = 0.0001$; 82.82 ± 7.77% vs. 56.22 ± 6.49% on second day, $P = 0.017$), in contrast, *Per3*$^{-/-}$ mice had less NREMS than WT (13.22 ± 4.97% vs. 56.53 ± 6.53% on first day, $P = 0.0001$; 14.94 ± 6.20% vs. 40.64 ±

5.79% on second day, $P = 0.0072$). In addition, in the last hour of the light period on the second day, wakefulness was higher and NREMS was lower in *Per3* mutants than in WT mice. REM sleep of *Per3* mutants was lower than WT mice at ZT 0 on first day, but higher at ZT 4 on the second day (Figure 3).

4.2 Time spent of vigilance states

In the 12-h light and dark period on each day, the only differences shown between the genotypes were average time spent in light period NREM on first day ($P = 0.049$). Averages of time spent in wakefulness, NREM and REM sleep were not different between the genotypes (Table 1). During the 12-h light and dark period over total 48-h recording time, averages of percent time spent in all vigilance states were also not different between the genotypes (Table 2). In addition, the average times spent in NREM sleep as a percentage of TST (%NREMS/TST) were not different between *Per3*$^{-/-}$ mice and WT mice (Table 1 and Table 2).

4.3 Number of sleep bouts

The number of sleep bouts per hour in entrained light-dark condition during 48-h is depicted in Figure 4 (mean ± SEM). The number of total sleep bouts showed minor differences between the genotypes. *Per3*$^{-/-}$ mice had less bouts of sleep than WT mice at ZT 0 on first day (2.80 ± 1.08 vs. 9.50 ± 2.10, $P = 0.002$), on the second day, at ZT 1 (8.60 ± 1.20 vs. 12.60 ± 0.92, $P = 0.011$), ZT 2 (7.30 ± 1.67 vs. 12.80 ± 1.95, $P = 0.046$) and ZT 11 (6.30 ± 1.46 vs. 12.60 ± 0.83, $P = 0.001$). Averages sleep bouts per hour over 24-h period showed a slight difference between genotypes (Figure 5). Sleep bouts of *Per3* mutants were less than WT mice at early and late light periods (at ZT 0; 3.80 ± 1.37 vs. 8.45 ± 1.61, $P = 0.041$, ZT 10; 10.50 ± 1.05 vs. 13.70 ± 1.01, $P = 0.042$, and at ZT 11; 7.75 ± 1.30 vs. 11.30 ± 0.37, $P = 0.017$). Total episodes of sleep bouts during 12-h LD periods over 48-h and average to 24-h period showed that the number of sleep bout were significantly reduced during light period while not significantly elevated during dark period in mutant mice (Table 3). These results suggested less fragmented sleep in *Per3*$^{-/-}$ mice at the early and late light periods, also less fragmented sleep over 12-h light period when compared with WT mice.

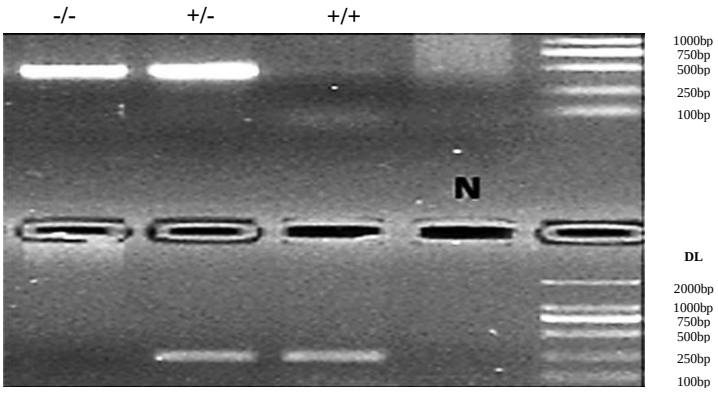


**Figure 1** PCR genotyping of DNA extracted from mouse tails biopsy for *Per3* gene, show DL-2000 DNA marker product. (mutant ~ 460 bp, wild-type ~242 bp, N as blank control)

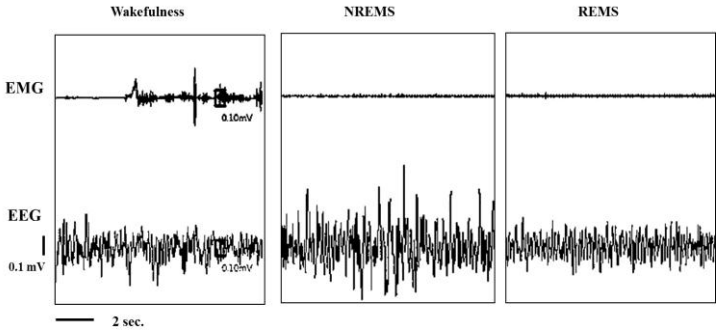


**Figure 2** Representative data depicting 10 seconds EEG/EMG samples for wakefulness, non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS).

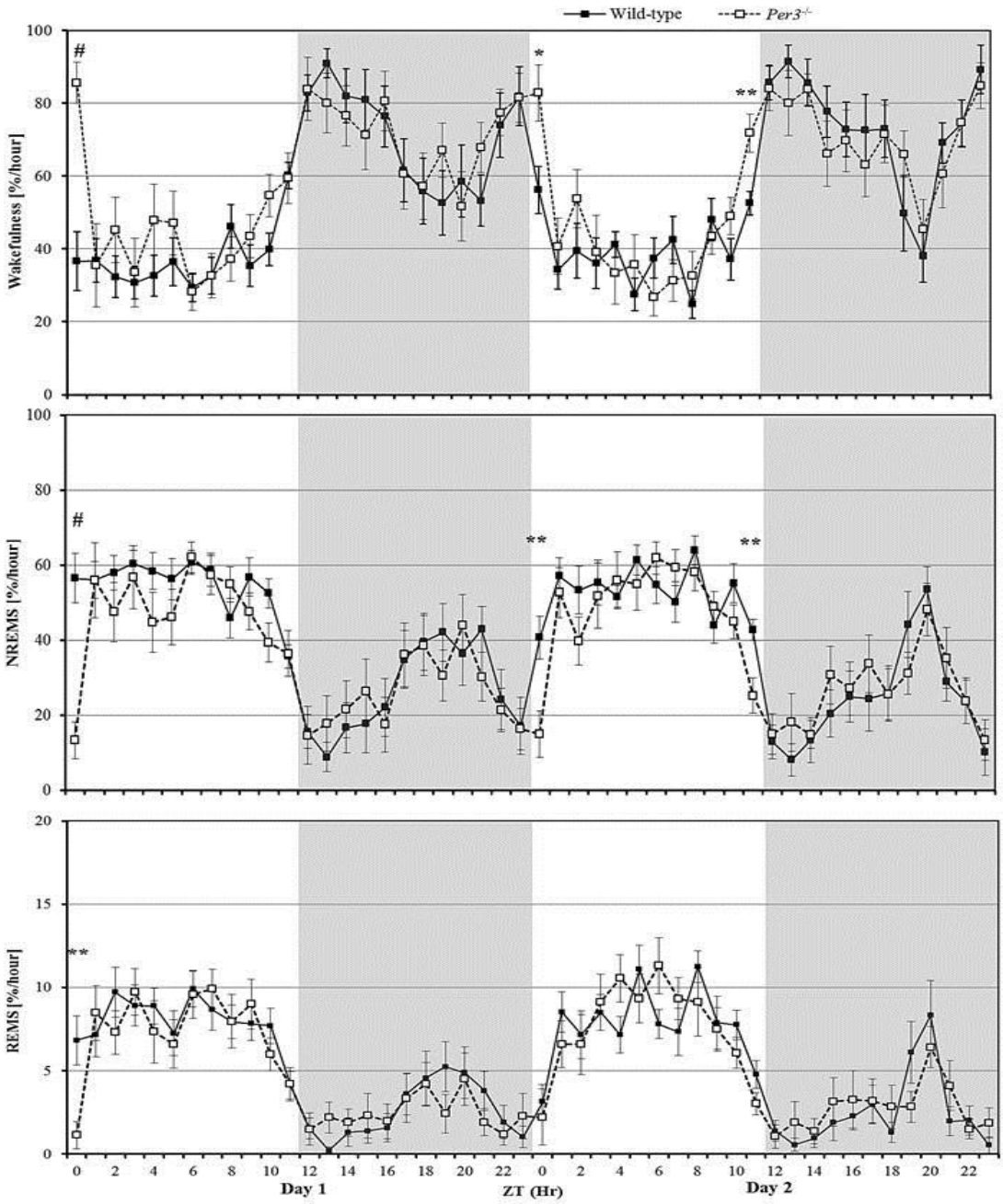


**Figure 3** Diurnal rhythm during 48-h of wakefulness, NREMS and REMS in mutant mice (*Per3*$^{-/-}$) ($n$ = 10) compared to WT mice ($n$ = 10). All vigilance states are expressed as percentage of recording time per hour (mean ± SE). * $P$ < 0.05, ** $P$ < 0.01, # $P$ < 0.001 (two-tailed unpaired t-test). The white and gray backgrounds of each graph indicate the 12-h light : 12-h dark cycle respectively.

**Table 1** Percentage of time spent in vigilance states during 12-h LD period over 48-h

| | Wakefulness (%) | NREMS (%) | REMS (%) | NREMS/TST (%) |
|---|---|---|---|---|
| 1st day 12-h light period | | | | |
| *Wild-type* | 37.44 ± 1.44 | 54.65 ± 1.45 | 7.91 ± 0.26 | 87.29 ± 0.52 |
| *Per3* $^{-/-}$ | 45.91 ± 4.08 | 46.82 ± 3.42 * | 7.27 ± 0.74 | 86.84 ± 0.64 |
| 1st day 12-h dark period | | | | |
| *Wild-type* | 70.93 ± 2.70 | 26.48 ± 2.38 | 2.59 ± 0.38 | 91.32 ± 0.76 |
| *Per3* $^{-/-}$ | 71.32 ± 4.02 | 26.21 ± 3.40 | 2.48 ± 0.69 | 91.91 ± 1.11 |
| 2nd day 12-h light period | | | | |
| *Wild-type* | 39.80 ± 1.61 | 52.5 ± 1.67 | 7.70 ± 0.49 | 87.12 ± 0.77 |
| *Per3* $^{-/-}$ | 45.07 ± 3.71 | 47.36 ± 3.02 | 7.57 ± 0.80 | 86.65 ± 0.92 |
| 2nd day 12-h dark period | | | | |
| *Wild-type* | 73.31 ± 2.42 | 24.16 ± 2.01 | 2.53 ± 0.49 | 91.27 ± 1.26 |
| *Per3* $^{-/-}$ | 70.85 ± 3.62 | 26.36 ± 3.14 | 2.79 ± 0.62 | 90.62 ± 1.17 |

Percentage (means ± SE) of time spent in wakefulness, NREM sleep and REM sleep (% of total recording time), and NREMS/TST (% of total sleep time) in 12-h LD period on each day, compared between genotype. * $P < 0.05$, two-tailed unpaired t-test.

**Table 2** Percentage of time spent in vigilance states during LD period

| | Wakefulness (%) | NREMS (%) | REMS (%) | NREMS/TST (%) |
|---|---|---|---|---|
| 12-h light period | | | | |
| *Wild-type* | 38.62 ± 1.41 | 53.57 ± 1.48 | 7.81 ± 0.28 | 87.21 ± 0.60 |
| *Per3* $^{-/-}$ | 45.49 ± 3.81 | 47.09 ± 3.15 | 7.42 ± 0.74 | 86.74 ± 0.75 |
| 12-h dark period | | | | |
| *Wild-type* | 72.12 ± 2.33 | 25.32 ± 1.99 | 2.56 ± 0.41 | 91.30 ± 0.96 |
| *Per3* $^{-/-}$ | 71.08 ± 3.74 | 26.28 ± 3.20 | 2.63 ± 0.63 | 91.27 ± 0.99 |

Vigilance states in 12-h light and dark period were averaged from 2 days recording times. Expressed as percentage (means ± SE) of time spent in wakefulness, NREM sleep, REM sleep (% of total recording time), and NREMS/TST (% of total sleep time).

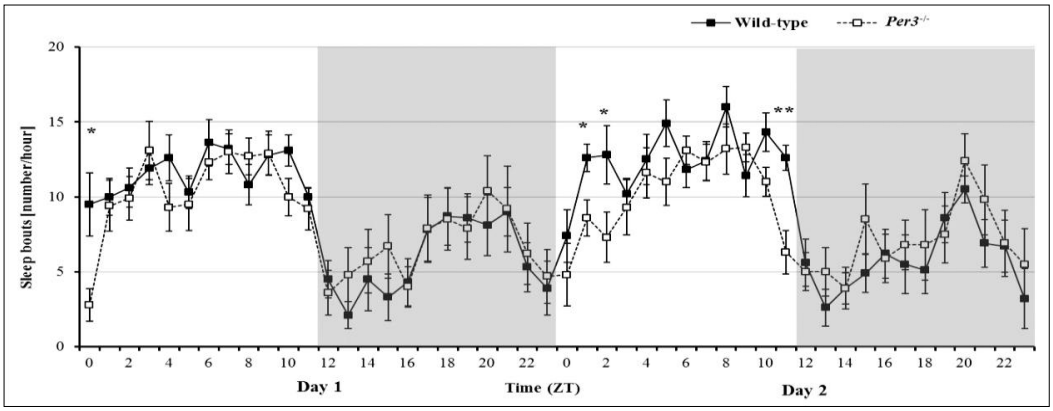


**Figure 4** Number of sleep bouts (mean ± SEM; WT: $n = 10$, *Per3*$^{-/-}$: $n = 10$) for 1-h interval. Stars above the curves indicate significant differences between genotype for the corresponding interval (* $P < 0.05$; ** $P < 0.01$, two-tailed unpaired t-test). The white and gray backgrounds indicate the 12-h light:12-h dark cycle.

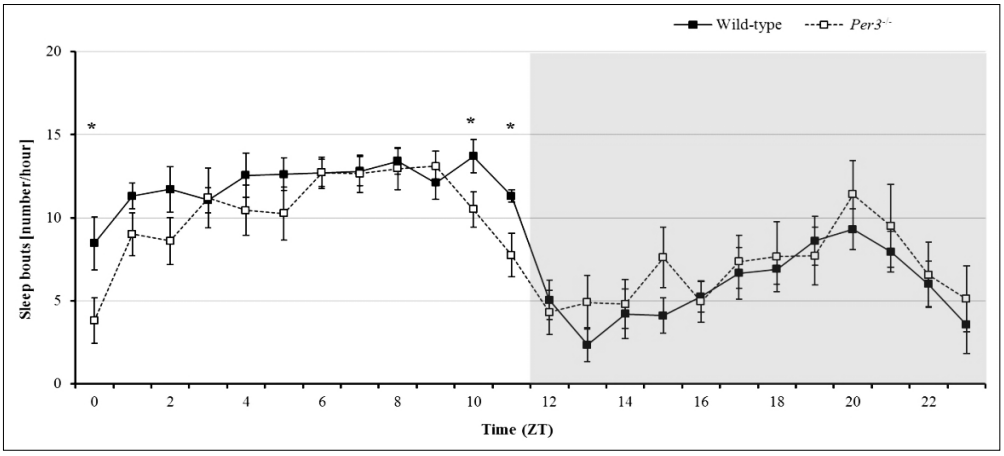


**Figure 5** Average of sleep bouts during 2 days represented in mean ± SEM (WT: $n$ = 10, mutants: $n$ = 10) for 1-h interval. The white and gray backgrounds of panel indicate the 12-h light: 12-h dark cycle. * $P < 0.05$, two-tailed unpaired t-test.

**Table 3** Total number of sleep bouts during 12-h LD period

| | 1st day light period | 1st day dark period | 2nd day light period | 2nd day dark period | Average light period | Average dark period |
|---|---|---|---|---|---|---|
| **Wild-type** | 138.40 ± 7.02 | 70.10 ± 8.04 | 148.90 ± 8.26 | 69.70 ± 7.18 | 143.65 ± 7.51 | 69.90 ± 6.19 |
| ***Per3*** $^{-/-}$ | 124.10 ± 9.75 | 79.60 ± 15.11 | 121.80 ± 9.34 | 84.00 ± 15.21 | 122.95 ± 9.24 | 81.80 ± 15.02 |
| ***P*** | 0.0532 | 0.3176 | 0.0005 | 0.1098 | 0.0014 | 0.1389 |

Total number of sleep bouts during LD period of each day and average from 2 days represent in mean ± SEM (WT: $n$ = 10, mutants: $n$ = 10), two-tailed unpaired t-test.

## 5. Discussion

Our data have shown that the most prominent difference between the *Per3* mutants and WT mice on C57BL/6J genetic background was the distribution of sleeping and waking during the light period. *Per3* mutants began their main sleeping episodes later and waking episodes earlier than WT mice. When vigilance states were analyzed with a 1-h resolution, their temporal distributions under entrained conditions were different between the genotypes, with *Per3*$^{-/-}$ mice showing more wakefulness and less NREM and REM sleep at the beginning of the light period. The significant differences that we observed in wakefulness and NREMS at ZT 0 in the light period between *Per3*$^{-/-}$ and WT mice (Figure 3). In contrast with previous studies, *Per3*$^{-/-}$ mice on 129/sv genetic background have no significant differences in sleep rhythmicity compared to WT (Shiromani et al., 2004). While *Per3*$^{-/-}$ mice on C57BL/6J genetic background have only subtle behavioral changes in circadian properties, with these mutant mice showing less wakefulness, and more NREM and REM sleep at the beginning of the light period (Hasan et al., 2011). However, our results are similar to previous studies, suggesting that the timing of the genotypic differences in relation to the dark-light transition in other previously reported cases are light-dependent behavioral phenotypes in *Per3*$^{-/-}$ mice (Hasan et al., 2011; van der Veen & Archer, 2010).

On the other hand, consistent with previous reports (Hasan et al., 2011; Shiromani et al., 2004), we observed no genotype difference in the percentage of total time spent in wakefulness, NREM sleep, REM sleep, and amount of NREM sleep as a percentage of total sleep time (Table 1 and Table 2). Although most of the time spent in wakefulness and NREM sleep during light period was not significantly different between genotypes, we did notice a tendency to more wakefulness and less NREM sleep in *Per3*$^{-/-}$ mice (Table 1 and Table 2). These results suggested that absence of or disruption of *Per3* is not strong enough to affect

time spent during sleep and does not affect sleep quality.

Under the entrained condition, *Per3*$^{-/-}$ mice had significantly less sleep bouts than WT mice at the early and late light periods (Figure 5). This resulted in less fragmented sleep-wake transitions in *Per3*$^{-/-}$ mice. However, most of the 1-h bins sleep bouts had no significant difference between genotypes, but *Per3*$^{-/-}$ mice had a tendency for less sleep bouts during the day time and more sleep bouts during night time, especially in the early and late time points of each period. These results suggested that *Per3* has subtle effects on the fragment of sleep-wake behavior consistent with sleep profiles at the same time.

Although our finding contrasted with previous studies (Hasan et al., 2011; Shiromani et al., 2004; van der Veen & Archer, 2010), a recent study reported that *Per3* is not involved in the processing of acute responses to light, but the animals were changed in behavior under chronic light conditions (Pereira et al., 2014). From our published accounts combined with previous reports, we propose that behavioral phenotypes of *Per3*$^{-/-}$ mice are light-dependent. Our conclusion is bolstered by a previous study that suggested that *mPer3* could play an important role in the light input of the clock (van der Veen & Archer, 2010) and affect behavioral activities of mice in direct response to light (Pereira et al., 2014). The mutant *Per3* gene has a lesser effect within the SCN, where it is not light-induced, unlike *Per1* and *Per2* (Takumi et al., 1998). In addition, although the *Per3* functions are not necessary for core clock loop, but are responsible for peripheral clock and clock output from the SCN (Bae et al., 2001; Pendergast et al., 2010; Pendergast et al., 2012), we can hypothesize that *Per3* may alter sleep-wake timing by regulating clock components outside of the SCN. These data, could contribute to sleep phenotypes in human who have variable-number tandem-repeat (VNTR) polymorphism in the coding region of the circadian clock gene *Per3* that relate to diurnal preference in humans, including delayed sleep phase syndrome (DSPS) (Archer et al., 2003). Besides, polymorphisms in the clock gene *Per3* have influences on subsequent sleep episodes which respond to evening light exposure (Chellappa et al., 2014). Moreover, our data reveal differences to previous studies in regards to the role of *Per3* on sleep-wake phenotypes (Hasan et al., 2011; Shiromani et al., 2004; van der Veen & Archer, 2010). Finally, while not addressed in our experiments, it is possible that latitude could also have an influence on clock genes. (Pereira et al., 2005).

## 6. Conclusion

In summarize, *Per3* gene have influence on sleep-wake phenotypes by a light-dependent manner, via numerous pathways. Thus, both genetic and environmental factors influence the complex behavior of sleeping. These data may be helpful in furthering the understanding and treatment of sleep and circadian rhythm disturbances, which require in depth studies to understand their mechanisms.

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