Light-dependent behavioural phenotypes in PER3 deficient mice

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Abstract

A functional knockout of *Period3* in mice (*mPer3*$^{-/-}$) results in a mildly altered circadian phenotype, and *mPer3* shows a redundant role within the circadian clock. In this study, we re-evaluated the *Per3*$^{-/-}$ behavioural phenotype on a C57Bl/6J background and report altered responses to light. In constant light, free-running activity period was shorter than that of wild-type, whereas in constant darkness no difference was observed between genotypes. The effect of light was parametric and the difference in free-running period between genotypes increased under constant light with increasing light intensity. An attenuated response to light in *Per3*$^{-/-}$ mice was also demonstrated through reduced negative masking in activity in an ultradian protocol, and a slower re-entrainment to a shifted light-dark cycle when activity falls in the light period of the new light-dark cycle. Behavioural phase-shifts in response to a single delaying or advancing light pulse in the *Per3*$^{-/-}$ mouse were not compromised. This demonstrates that the *mPer3*$^{-/-}$ phenotype is characterised predominantly by altered sensitivity to light and not by the ability of the circadian system to respond to light. In addition to its redundant role within the molecular clock, these data suggest a new role for *Per3* outside of the circadian clock and contributing to light input pathways.

Keywords: Circadian, Clock gene, Light-pulse, Entrainment, Ultradian, Masking

Introduction

Daily rhythms in behavioural and physiological processes are driven by intrinsic circadian clocks that are entrained to the outside environment. One of the main entraining signals (Zeitgeber) is light-dark interaction, such as light-dark cycles and light or dark pulses. Besides being a strong Zeitgeber, light conditions can also exert a direct effect on behaviour, without containing timing information. These effects include direct suppression of activity in nocturnal animals (negative masking, (Mrosovsky, 1999)) and when light exposure is continuous (LL), free-running periods of activity (tau) extend (Aschoff's rule, (Aschoff, 1960)).

Circadian clocks are found in many tissues, a defining property of which is molecular transcriptional feedback loops. These loops include transcription factors such as CLOCK and BMAL1 that drive expression of proteins from the *Period* (*Per1, 2 & 3*) and *Cryptochrome* (*Cry1* & *2*) gene families. The protein products PERIOD and CRYPTOCHROME in turn suppress the expression of clock genes by inhibiting the action the transcription factors (for review see (Ko and Takahashi, 2006).

The mouse *Per3* (*mPer3*) gene is expressed in central and peripheral tissues, with high levels of central rhythmic expression in the suprachiasmatic nuclei (SCN) of the hypothalamus, and also peripheral tissues such as the retina and liver (Takumi *et al.*, 1998; Yamamoto *et al.*, 2004; Zylka *et al.*, 1998). *mPer3* is not critical to the SCN and when it is functionally knocked-out (*mPer3*$^{-/-}$), a 30-minute difference in free-running period in constant darkness (DD) was seen in the 129/sv background, but not in the 129/sv x C57BL/6 hybrid background (Shearman *et al.*, 2000). *mPer3* alone is not sufficient to maintain a functional clock and *mPer1*/*mPer2* double knockout mice are behaviourally arrhythmic in DD (Bae *et al.*, 2001; Bae and Weaver, 2007). Because the role of *Per3* in circadian rhythms seems uncertain, we investigated the phenotypes of *mPer3*$^{-/-}$ in more detail.

Material and Methods

Animals

Mice with a targeted disruption of the *Per3* gene (*Per3*$^{-/-}$) were generated as previously described (Shearman *et al.*, 2000). The animals used here are derived from these mice and have been backcrossed to a C57BL/6J background for at least 10 generations by Professor S. Yamazaki (Vanderbilt University, USA). *Per3*$^{-/-}$ and WT mice were bred in-house from pairs originating from heterozygous breeding. Genotyping was performed as previously described (Shearman *et al.*, 2000).

Mice were housed in wheel running cages (ClockLab, Actimetrics, Wilmette, IL, USA) in light-tight, sound-attenuated cabinets. Activity was recorded in 1-min bins. Individual cage illumination was supplied by LEDs (NSPW500BS, Nichia Europe BV, Amsterdam, the Netherlands) through frosted glass. The LED spectrum showed a narrow peak at 455 nm and a broader peak at 562 nm. Relative spectral composition did not change with different light intensities. Light sources were calibrated with the sensor at the cage bottom directed towards the light. Temperature was kept at 19-22 ˚C and relative humidity at 55 ± 10%. Animals were provided with food (Transgenic mouse diet, B & K universal Ltd, Hull, UK) and water *ad libitum*. Experiments were approved by the University of Surrey Animal Ethics Committee, and carried out under UK Home Office Licence in accordance with the Declaration of Helsinki.

Constant light conditions

16 male mice for each genotype (8.9 weeks old, ± 4 days (AVG ± SEM)) were entrained to a 12h:12h light-dark (LD) cycle (L = 845 ± 14 mW m^{-2} (AVG ± SEM)) for 20 days and subsequently subjected to a 20-day episode of DD, and three episodes of LL of increasing intensity (10 ± 0.2, 188 ± 3 and 845 ± 14 mW m^{-2}, respectively). Thereafter, animals were re-entrained to a 12h:12h LD cycle.

Light pulses and re-entrainment

16 male mice for each genotype (9.8 weeks old, ± 4 days) were entrained to a 12h:12h LD cycle (L = 845 mW m^{-2}) for 14 days. In the last night of LD, animals received a single, 15-min light pulse (1236 ± 22 mW m^{-2}) and were then released in DD for 14 days. Phase shifts could not be interpreted reliably for 2 *Per3*$^{-/-}$ mice for ZT 14, and 1 WT and 3 *Per3*$^{-/-}$ mice for ZT 22.

The same mice were then re-entrained to a 12h:12h LD cycle for 14 days. Subsequently, the LD cycle was advanced or delayed by 4 hours by shortening or lengthening the dark period, respectively, then mice re-entrained to a 12h:12h LD cycle for at least 14 days. Both light pulse and re-entrainment experiments were performed in a cross-over design.

Ultradian light-dark cycles

6 male mice for each genotype (8.6 weeks old ± 1 day) were entrained to a 12h:12h LD cycle for 14 days and then subjected to a 3.5h:3.5h ultradian LD cycle. The initial light intensity was 3 ± 0.1 mW m^{-2} and increased every 10 ultradian cycles with the following intensities; 3 ± 0.1, 10 ± 0.2, 15 ± 0.3, 25 ± 0.4, 34 ± 0.5, 84 ± 1.2, 168 ± 2.3, 323 ± 4.6, 442 ± 6.7, 544 ± 8.6, 719 ± 11.9 mW m^{-2}. Mice were then released into DD.

Behavioural parameter measurements

Activity data rhythms were measured using periodogram analysis (Sokolove and Bushell, 1978) on the last 10 days of each condition, or the last 8.75 days for the ultradian protocol (last 3 ultradian light intensities). Onset was defined as the first instance when the smoothed, 2-h running average of activity exceeded the tau running average of activity, starting half a tau before the centre point of

gravity of activity. Offset of activity was determined inversely. Phase angles of entrainment were defined as the time between the activity onset and the onset of darkness, and alpha (daily active episode) was defined as the time between onset and offset.

Phase shifts were calculated by subtracting pre- and post-light-pulse phases on the day of the light pulse as predicted by linear regressions through the onsets of activity in the last 10 days pre-light pulse and from the 5th through the 14th day after the light pulse.

Statistics were performed using SigmaPlot (V11, Systat software Inc., San Jose, CA, USA) and SAS (V 9.1, SAS Institute Inc., Cary, NC, USA).

Results

There was no difference in entrainment in LD between genotypes, with phase angles between activity onset and light offset of 14.1 ± 0.6 min (AVG ± SEM) for WT and 15.2 ± 0.5 min for *mPer3*$^{-/-}$ (Mann-Whitney Rank Sum Test, $P > 0.05$).

Constant light conditions

Figure 1A shows representative double-plotted behavioural actograms of WT and *Per3*$^{-/-}$ mice. Tau for the different light conditions for WT and *Per3*$^{-/-}$ mice are shown in Figure 1B and Supplemental Table T1. In DD, tau was approximately 23 hrs 40 mins. Tau in LL conditions was over 24 hours and lengthened with increasing light intensity. There were significant effects of both light condition ($P < 0.0001$) and genotype ($P < 0.0001$) and their interaction (gene x light, $P = 0.0009$) (SAS, proc mixed). Least square mean (LSM) pair-wise comparisons indicated that in DD, there was no difference between the tau of WT and *Per3*$^{-/-}$ mice, whereas in LL, WT tau was longer than for *Per3*$^{-/-}$ and this difference became greater with increasing light intensity (Ps < 0.0001).

Total activity decreased in higher light intensities for both genotypes equally (proc mixed; $P < 0.0001$), specifically between DD and LL 10 mW, m^{-2}, and between LL 10 mW m^{-2} and LL 188 mW m^{-2} (LSM; P's < 0.0001; Fig1C). Similarly, a shortening of alpha (proc mixed; light $P < 0.0001$) between the same light conditions was observed (LSM; $P < 0.001$ and $P < 0.01$, respectively; Fig1D).

Light-pulses and re-entrainment

A light-pulse at ZT 14 produced a phase delay of -54.3 ± 2.2 (AVG ± SEM) minutes for WT and -56.1 ± 3.6 minutes for *Per3*$^{-/-}$ mice. The phase advance after a light-pulse at ZT 22 was 50.1 ± 3.1 minutes for WT and 35.9 ± 2.5 for *Per3*$^{-/-}$ mice. While the phase shifts differed significantly between ZT 14 and ZT 22, WT and *Per3*$^{-/-}$ mice responded equally to the light pulses (two-way ANOVA, $P < 0.001$ for time of light pulse and $P > 0.05$ for genotype). Activity was reduced to 8% ± 4% (AVG ± SEM) and 6% ± 2% of the previous day at the equivalent time for WT and *Per3*$^{-/-}$ mice, respectively, during the light pulse at ZT 14, and 58% ± 32% and 56% ± 26%, respectively, for the light pulse at ZT 22. The response was different between the light pulses, but not between the genotypes (two-way ANOVA, $P < 0.01$ for time of light pulse and $P > 0.05$ for genotype).

Following the phase advance in LD entrainment, positive phase angles between onset of activity and lights-off were seen after 4.7 ± 0.4 (AVG ± SEM) days for WT and 4.2 ± 0.4 days for *Per3*$^{-/-}$ mice (see Supplemental Figure S1). Thus, 'anticipation' of lights-off was restored equally quickly for both phenotypes (t-test, $P > 0.05$). Similarly, post-shift activity onsets returned to pre-shift timing (defined as when the onset time is less than or equal to the average [± 2 SD] pre-shift onset) after 5.1 ± 0.4 (AVG ± SEM) days for WT and 4.7 ± 0.5 days for *Per3*$^{-/-}$ mice, which were not different between genotypes (t-test, $P > 0.05$). Both genotypes exhibited similar phase angles between activity and lights-on for any of the days during re-entrainment (proc mixed; day x genotype $P > 0.05$).

Re-entrainment to the phase delay was achieved in 2.1 ± 0.2 (AVG ± SEM) days in WT and 2.7 ± 0.3 days in *Per3*$^{-/-}$ mice (see Supplemental Figure S1), which was not different between genotypes (t-test, $P > 0.05$). A day-by-day comparison showed a difference in phase angles during re-entrainment between genotypes (proc mixed; day x genotype $P < 0.001$), where on the first day of re-entrainment *Per3*$^{-/-}$ mice shifted less than WT mice (LSM pair-wise comparison; $P < 0.0001$).

Ultradian light-dark cycles

Figure 2 shows representative examples of WT and *Per3*$^{-/-}$ activity during the ultradian paradigm. Both mice showed a circadian rhythm in activity, but WT mouse suppressed activity more in light. Periodogram analyses (Sokolove and Bushell, 1978) over the last three ultradian episodes revealed a more prevalent ultradian activity component for the WT mouse, and a stronger circadian rhythm in the *Per3*$^{-/-}$ mouse (Fig2).

Figure 3 shows the total amount of activity during the light-phase, corrected for total activity, which was less for WT (proc mixed; genotype $P < 0.05$). Both genotypes reduced activity in the light with increasing light intensity. However, *Per3*$^{-/-}$ mice showed consistently more activity during the light (proc mixed, light $P < 0.0001$, genotype $P < 0.0001$).

Discussion

WT and *Per3*$^{-/-}$ mice exhibited lengthening tau under increasing light intensities, but *Per3*$^{-/-}$ mice showed shorter tau in LL, a difference that became larger under higher light intensities. Similar free-running periods have been reported previously in mice on a sv129 x C57BL/6J hybrid background, although a 30-minute shortening in *Per3*$^{-/-}$ tau was observed on a 129/sv background (Shearman *et al.*, 2000).

WT and *Per3*$^{-/-}$ mice showed equal phase-shifting responses to light pulses, and re-entrained similarly to a phase advance in LD. Only during re-entrainment to a phase delay in LD did the *Per3*$^{-/-}$ mouse show a smaller shift on the first day of re-entrainment. Only in the phase delay condition is the circadian system motivating activity in the light while adjusting to the new LD cycle, suggesting that the observed genotypic difference is linked to light. Also, the tau difference in LL, but not DD, indicates a decreased light sensitivity for the *Per3*$^{-/-}$ mouse. Indeed, in the ultradian LD cycle, *Per3*$^{-/-}$ mice showed less negative masking during light.

Circadian or light-related phenotype?

The *Per3*$^{-/-}$ mouse is different from other *Per* mutants. Tau in DD for *Per1* mutant mice is shorter than for WT (Cermakian *et al.*, 2001; Zheng *et al.*, 2001), whereas *Per2* mutants become behaviourally arrhythmic (Zheng *et al.*, 1999). Tau in LL for *Per1* mutant mice is longer than 24-hours (Steinlechner *et al.*, 2002) and rhythms in *Per2* mutants are restored in LL, but with tau's shorter than 24 hours and *shortening* with increasing light. *Per3*$^{-/-}$ and WT mice did not respond differently

to a light-pulse, while *Per1* and *Per2* mutant mice do not show phase advances and delays in response to a light-pulse, respectively (Albrecht *et al.*, 2001; Spoelstra *et al.*, 2004).

The absence of a *Per3*$^{-/-}$-specific phenotype in DD and in responses to light pulses is different from *mPer1* and *mPer2*, and does not agree with a strong role for *Per3* in the circadian system. Therefore, even though *Per1* and *Per2* are well-established 'clock-genes', no strong case for *mPer3* as a clock gene can be made.

Our data suggest that the *Per3*$^{-/-}$ mouse has a lower sensitivity to light. Similarly, the melanopsin knockout mouse (*Opn4*$^{-/-}$) has a reduced light sensitivity and shows smaller increases in tau in LL (Ruby *et al.*, 2002), but unlike *Per3*$^{-/-}$, shows altered phase shifting responses to light (Panda *et al.*, 2002). When given a three-hour light pulse, *Opn4*$^{-/-}$ mice show an initial negative masking response that is similar to WT, but normal activity resumes after 100 minutes (Mrosovsky and Hattar, 2003). Our data suggest that *mPer3* could play an important role in the light input of the clock, but to a lesser extent within the SCN, where it is not light-induced, unlike *Per1* and *Per2* (Takumi *et al.*, 1998; Zylka *et al.*, 1998). Taken together, these data also emphasise the value of performing additional investigations on the role of *Per3* within the retina.

Despite the homology between *mPer3* and *mPer1* and *mPer2*, it is not critical for the circadian clock, nor is it redundant with *mPer1* or *mPer2*. Differences in tau in *Per3*$^{-/-}$ mice could be interpreted as an intrinsic property of the clock, and the *mPer3*$^{-/-}$ phenotype as a circadian one. We propose that the *mPer3*$^{-/-}$ phenotype is predominantly light-dependent. An alteration in light sensitivity may affect behavioural and physiological timing mechanisms in numerous ways, through clock function, or via pathways not involved in rhythmic processes. These data may also help to understand other non-circadian *PER3* phenotypes that have been observed in humans (Viola *et al.*, 2007), and these mechanisms now need to be investigated at a lower organisational level.

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Figure legends

Figure 1

A. Double-plotted actograms of running wheel activity (black bars) for a WT and a *Per3*$^{-/-}$ mouse. **B**. Differential lengthening of tau with increasing light. **C**. Percentage of total activity in LD decreased similarly for WT (black circles) and *Per3*$^{-/-}$ (grey circles) mice with increasing intensity. **D**. Length of alpha in DD and three LL conditions. For the highest intensity, tau could not be calculated for 1 WT and 3 *Per3*$^{-/-}$ mice. Error bars indicate SEM. Grey areas indicate darkness and varying light intensities. Between conditions ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$; between genotypes # $P < 0.0001$.

Figure 2

Running wheel activity for representative WT (**A**) and *Per3*$^{-/-}$ (**B**) mice. On the left, activity (black lines) is plotted in each row for a 70-h episode under a particular light condition, starting with LD, then 11 episodes of ultradian LD cycles (3.5 h light – 3.5 h dark) of increasing light, and in DD. On the right, periodograms (solid line) over the last three ultradian light conditions for activity of both mice are shown. The dotted line represents the cut-off for <chi>-square significance ($P < 0.05$). Grey areas indicate darkness, while white areas indicate light of the designated intensity. See Supplemental Figure S2 for the same activity data double-plotted on a 24-h timescale.

Figure 3

Bars indicate the amount of activity in the light (as a percentage of total activity) during LD and 11 subsequent ultradian cycles of increasing light. With increasing light intensity, WT (black bars) and *Per3*$^{-/-}$ (grey bars) mice allocate activity to the dark period ($P < 0.0001$ for the effect of light). *Per3*$^{-/-}$ mice show consistently more activity in the light than WT mice ($P < 0.0001$). Error bars indicate SEM.

Figure 1

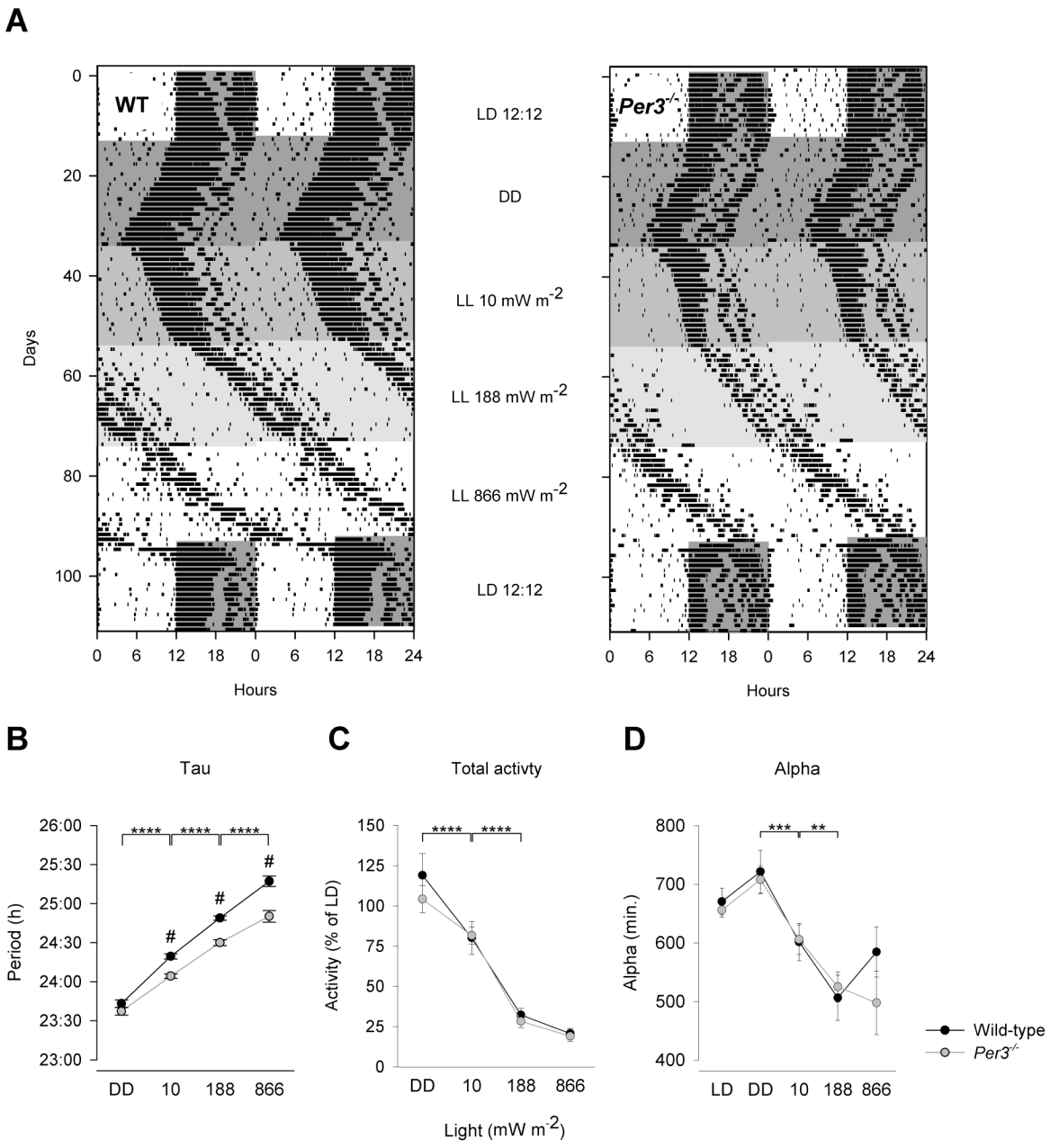
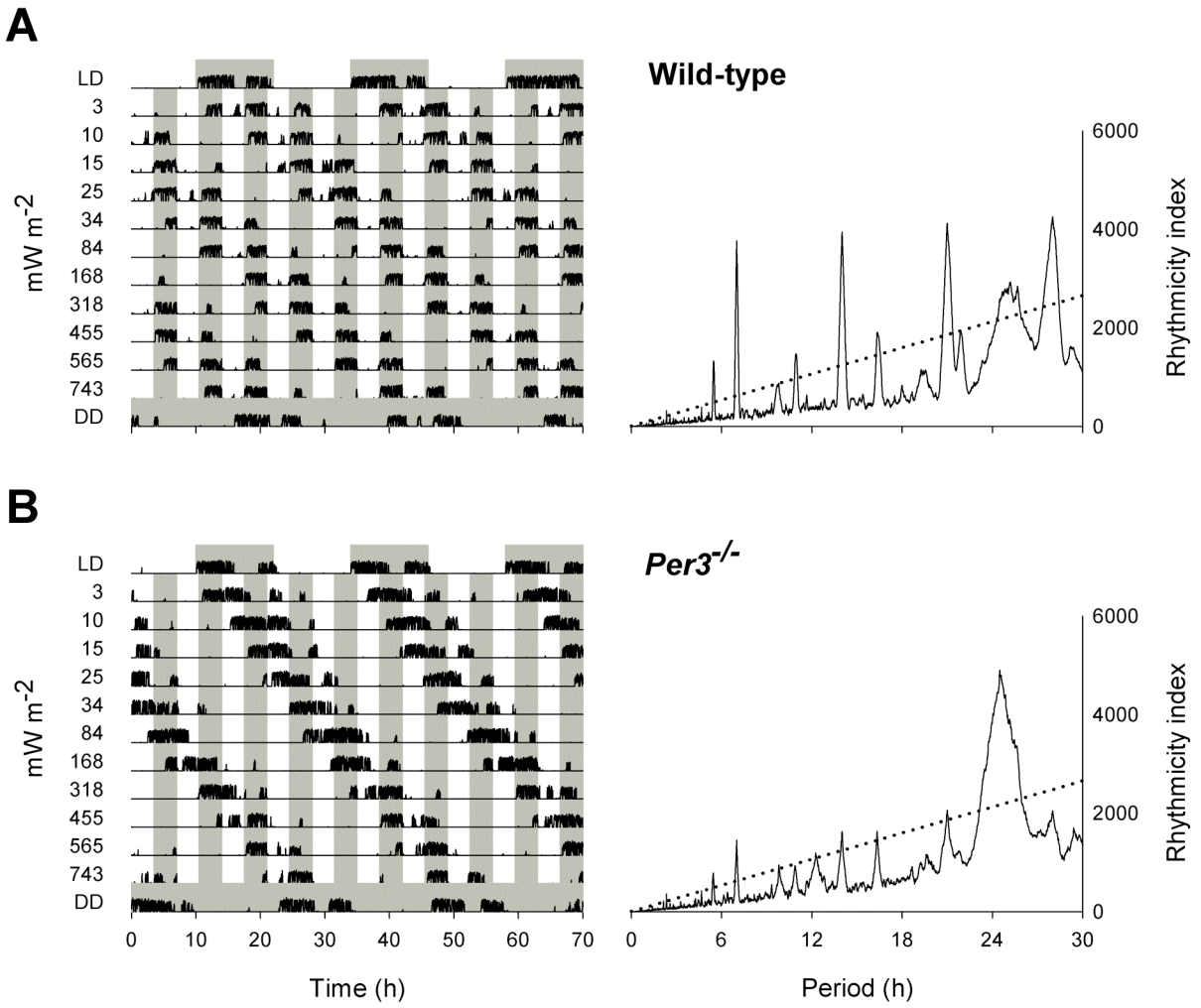

A
WT
Per3-/-
LD 12:12
DD
LL 10 mW m-2
LL 188 mW m-2
LL 866 mW m-2
LD 12:12
Days
Hours
B
Tau
Period (h)
C
Total activty
Activity (% of LD)
Light (mW m-2)
D
Alpha
Alpha (min.)
Wild-type
Per3-/-

Figure 2


A
B
LD
3
10
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25
34
84
168
318
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565
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DD
mW m-2
Wild-type
Per3-/-
6000
4000
2000
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Rhythmicity index
0
10
20
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Time (h)
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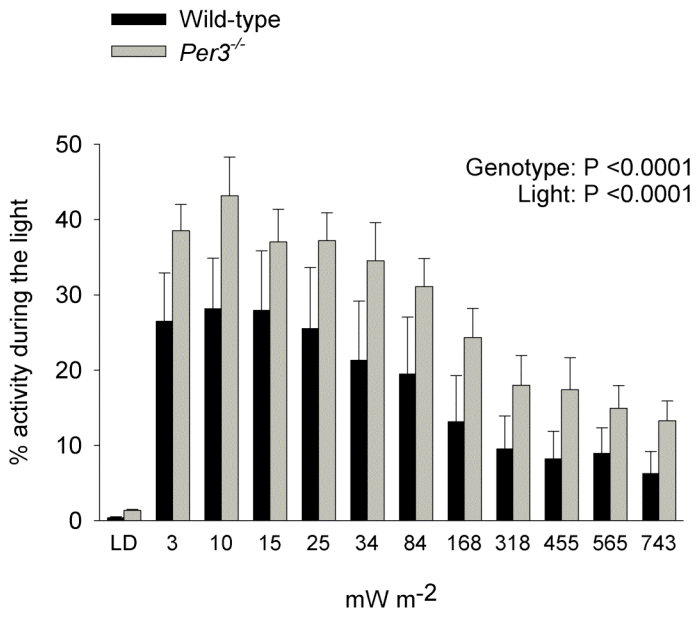
Period (h)


Wild-type
Per3-/-
Genotype: P <0.0001
Light: P <0.0001
% activity during the light
50
40
30
20
10
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LD
3
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318
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565
743
mW m-2

Figure 3