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Masking Responses to Light in Period Mutant Mice

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Abstract

Masking is an acute effect of an external signal on an overt rhythm and is distinct from the process of entrainment. In the current study, we investigated the phase dependence and molecular mechanisms regulating masking effects of light pulses on spontaneous locomotor activity in mice. The circadian genes, *Period1* (*Per1*) and *Per2*, are necessary components of the timekeeping machinery and entrainment by light appears to involve the induction of the expression of *Per1* and *Per2* mRNAs in the suprachiasmatic nuclei (SCN). We assessed the roles of the *Per* genes in regulating masking by assessing the effects of light pulses on nocturnal locomotor activity in C57BL/6J *Per* mutant mice. We found that *Per1*^{-/-} and *Per2*^{-/-} mice had robust negative masking responses to light. In addition, the locomotor activity of *Per1*^{-/-}/*Per2*^{-/-} mice appeared to be rhythmic in the light-dark (LD) cycle, and the phase of activity onset was advanced (but varied among individual mice) relative to lights off. This rhythm persisted for 1 to 2 days in constant darkness in some *Per1*^{-/-}/*Per2*^{-/-} mice. Furthermore, *Per1*^{-/-}/*Per2*^{-/-} mice exhibited robust negative masking responses to light. Negative masking was phase dependent in wild-type mice such that maximal suppression was induced by light pulses at zeitgeber time 14 (ZT14) and gradually weaker suppression occurred during light pulses at ZT16 and ZT18. By measuring the phase shifts induced by the masking protocol (light pulses were administered to mice maintained in the LD cycle), we found that the phase responsiveness of *Per* mutant mice was altered compared to wild-types. Together, our data suggest that negative masking responses to light are robust in *Per* mutant mice and that the *Per1*^{-/-}/*Per2*^{-/-} SCN may be a light-driven, weak/damping oscillator.

Keywords

Aschoff's Type VI protocol; C57BL/6J; Circadian; Negative masking; *Period* double knockout; Suprachiasmatic nucleus (SCN)

INTRODUCTION

Light has salient effects on mammalian behavioral and physiological rhythms. Light can affect activity in two ways: (i) by entraining an endogenous pacemaker that controls activity or (ii) by masking, which is a direct and acute effect on activity (Aschoff, 1960). Both processes are likely critically important for the survival of animals in their natural environments, but relatively few studies have focused on understanding the molecular mechanisms regulating masking compared to entrainment.

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Previous studies examining the functions of the mammalian circadian genes, *Period1* (*Per1*) and *Per2*, have suggested that they are necessary components of the timekeeping machinery, since *Per1*^{-/-}/*Per2*^{-/-} mice are arrhythmic in constant darkness (Bae et al., 2001; Zheng et al., 2001). Furthermore, entrainment of the suprachiasmatic nucleus (SCN) oscillator by light appears to involve induction of the expression of *Per1* and *Per2* mRNAs in the SCN (Akiyama et al., 1999; Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Takumi et al., 1998; Tischkau et al., 2003; Wakamatsu et al., 2001; Zylka et al., 1998). Recent studies have demonstrated that the severity of the behavioral phenotypes of mice lacking functional PER1 or PER2 is dependent on genetic background. For example, *Per2*^{-/-} mice on a 129/sv or mixed C57BL/6J/129/S5 background become arrhythmic in constant darkness, whereas *Per2*^{-/-} mice congenic with the C57BL/6J strain have robust rhythmic locomotor activity in constant conditions (Bae et al., 2001; Pendergast et al., 2010a; Xu et al., 2007; Zheng et al., 1999). To gain a better understanding of the regulation of the masking responses to light, in the current study we examined negative masking of locomotor activity in C57BL/6J *Per* mutant mice.

MATERIALS AND METHODS

Animals

C57BL/6J *mPer1*^{ldc-/-}, *mPer2*^{ldc-/-}, and *mPer1*^{ldc-/-}/*m-Per2*^{ldc-/-} mice (Bae et al., 2001; Pendergast et al., 2009, 2010a) were bred and group-housed in 12:12 h light-dark cycle (12L:12D) and provided food and water ad libitum. Genotype was determined as previously described (Bae et al., 2001). Experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted in accordance with international standards (Portaluppi et al., 2010).

Masking Protocol

Male and female wild-type (mean age $\pm$ SEM: 96 $\pm$ 16 days), *Per1*^{-/-} (55 $\pm$ 4 days), *Per2*^{-/-} (96 $\pm$ 14 days), or *Per1*^{-/-}/*Per2*^{-/-} double knockout (117 $\pm$ 9 days) mice were singly housed in a cage (length $\times$ height $\times$ width: 29.5 $\times$ 11.5 $\times$ 12 cm) with unlimited access to a running wheel (diameter: 11 cm), food, and water. The cages were placed in light-tight, ventilated boxes (light intensity: 75–85 lux) in 12L:12D. Wheel-running activity was monitored using ClockLab (Actimetrics) and was collected by computer every minute. After at least 5 days of access to the wheel, mice received 1-h light pulses during the dark phase (75–85 lux, 20-W fluorescent lamp) starting at zeitgeber time (ZT) 14 (where ZT12 is the time of lights off), ZT16, or ZT18. Some mice were administered multiple pulses, but subsequent pulses were always separated by at least 1 wk to allow for entrainment to the LD cycle after phase shifts induced by the light pulses (i.e., an individual mouse was given a light pulse at ZT14, and then 1 wk later at ZT16, and then 1 wk later at ZT18). We could not reliably measure masking responses to light pulses applied after ZT18, because baseline activity typically decreased at the end of the dark phase. All experiments were performed from February 2010 to May 2010.

Analysis of Masking Effects

Baseline activity was calculated by averaging the number of revolutions in the hour of the light pulse in the 3 days prior to the light pulse. To evaluate masking, this baseline value was compared to the number of wheel revolutions during the 1 h (e.g., ZT14–15, ZT16–17, or ZT18–19) of the light pulse (only 1 day). Each replicate represents a single mouse administered a single light pulse at the specified ZT. In wild-type mice, the number of wheel revolutions during the light pulses was expressed as a percent of the number of wheel revolutions during baseline for each mouse, and then the mean ($\pm$ SEM) percent of baseline values was determined for each ZT.

Analysis of Phase Shifts

Phase shifts measured using Aschoff's Type VI protocol were defined as the time difference between the baseline onset of activity in the LD cycle and the onset of activity on the day after the light pulse. ClockLab was used to detect activity onset (by searching for a 6-h period of inactivity followed by a 6-h period of high activity). This was calculated by drawing a regression line to activity onset for the 3 days in the LD cycle preceding the light pulse and then extending the regression line to the day after the light pulse. A phase delay was obtained when activity started after the predicted onset and a phase advance was obtained when activity started before the predicted onset on the day after the light pulse.

Analysis of Wheel-Running Activity of *Per1*^{-/-}/*Per2*^{-/-} Mice

Per1^{-/-}/*Per2*^{-/-} mice were singly housed in 12L:12D (200–300 lux; in the same 12L:12D in which they were born and raised) with a running wheel and food and water provided ad libitum. After at least 5 days with access to the running wheel, the mice were released into constant darkness (DD). The phase angle of entrainment was defined as the time difference between lights off and the time of activity onset on the first day in DD.

Statistical Analysis

Statistical analysis was performed using SigmaStat (Systat Software, San Jose, CA). One-way analysis of variance (ANOVA) followed by post hoc Fisher's least significant difference (LSD) tests were used for comparison of more than two groups, and independent *t* tests (two-tailed) were used to compare two groups except when data were not normally distributed or variances were not homogeneous. The Kolmogorov-Smirnov test (with Lilliefors' correction) was used to test data for normality. For nonparametric analyses, the Mann-Whitney rank-sum test or Kruskal-Wallis one-way ANOVA on ranks followed by post hoc Dunn's method were used. Significance was ascribed at *p* < .05.

RESULTS

We first investigated the acute responses of *Per1*^{-/-} or *Per2*^{-/-} mice to light pulses at ZT14 (Figure 1). Similar to wild-type mice (*t* = 187.00, *p* < .001; Figure 1A), the wheel-running activity of *Per1*^{-/-} (*t* = 7.19, *p* < .001; Figure 1B) and *Per2*^{-/-} (*t* = 5.42, *p* < .001; Figure 1C) mice was acutely suppressed by light pulses at ZT14.

Per1 could compensate for the loss of *Per2* in *Per2*^{-/-} mice in regulating the masking response and vice versa. Therefore, we next investigated whether masking of activity in response to light pulses at ZT14 were intact in *Per1*^{-/-}/*Per2*^{-/-} mice (Figure 2). The activity of most *Per1*^{-/-}/*Per2*^{-/-} mice (4 of 5 mice) was strongly suppressed in response to light pulses administered at ZT14 (Figure 2).

Since our initial masking study in *Per1*^{-/-}/*Per2*^{-/-} mice suggested that their phase of activity relative to the LD cycle differed from wild-type mice, we further characterized the activity of *Per1*^{-/-}/*Per2*^{-/-} mice by maintaining them in 12L:12D (200–300 lux) and then releasing them into DD (Figure 3). Some mice displayed rhythmic wheel-running activity for 1–2 days, the phase of their activity onset was advanced relative to lights off (~2.4 h; Figure 3A, B), and they had short free-running periods (~19–21 h). Other *Per1*^{-/-}/*Per2*^{-/-} mice did not clearly maintain circadian rhythmicity in DD (Figure 3C, D).

We next investigated whether masking in wild-type mice was phase dependent. We administered 1-h light pulses at ZT14, ZT16, and ZT18 to wild-type mice maintained in 12L:12D (Figure 4). Light pulses at ZT14 induced strong suppression of activity (Figure 4A). In contrast, the effect of light pulses at ZT16 (Figure 4B) and ZT18 (Figure 4C) was

more variable. Though baseline levels of wheel-running activity did not differ at ZT14, ZT16, and ZT18 (Figure 4E), the number of wheel revolutions during the light pulse was significantly smaller at ZT14 compared to ZT18 ($H = 8.50$, $p = .01$, Dunn's $p < .05$; Figure 4F). Quantification of the masking response during the light pulse as the percent of baseline also showed that the inhibition of activity was greater during light pulses administered at ZT14 compared to ZT18 ($H = 8.34$, $p = .02$, Dunn's $p < .05$; Figure 4F).

Since the masking protocol used for this study is equivalent to Aschoff's Type VI protocol for assessing phase shifts (Aschoff, 1965), we quantified the phase shifts induced by 1-h light pulses administered at ZT14, ZT16, and ZT18 (Figure 5). Wild-type mice had larger phase delays to light pulses at ZT14 than *Per1*^{-/-} and *Per2*^{-/-} mice (Figure 5D). Phase shifts induced by light pulses at ZT14 were small (<10 min) or were not detected in *Per1*^{-/-}/*Per2*^{-/-} mice. Light pulses at ZT16 induced phase delays in wild-type and *Per1*^{-/-} mice, but very small (<5-min) phase shifts in *Per2*^{-/-} mice. When light pulses were administered at ZT18, wild-type, *Per1*^{-/-}, and *Per2*^{-/-} mice did not display robust phase shifts.

DISCUSSION

Since Aschoff first coined the term "masking" in 1960 to describe the direct effect of an external signal on a rhythm (separate from entrainment of the pacemaker), few studies have investigated the molecular mechanisms and anatomical substrates of this phenomenon. We found that masking responses to light pulses administered at ZT14 are intact in *Per1*^{-/-} and *Per2*^{-/-} mutants. Furthermore, *Per1*^{-/-}/*Per2*^{-/-} mice also exhibit robust masking.

Previous studies of *Per1*^{-/-}/*Per2*^{-/-} mice (on a 129/sv or C57BL/6J/129 mixed genetic background) showed that their wheel-running activity immediately became arrhythmic upon release from 12L:12D to DD (Bae et al., 2001; Zheng et al., 2001). In contrast, we identified several lines of evidence suggesting that the SCN is rhythmic in C57BL/6J *Per1*^{-/-}/*Per2*^{-/-} mice in the LD cycle. C57BL/6J *Per1*^{-/-}/*Per2*^{-/-} mice become active before lights off in 12L:12D, which differs from wild-type mice. Some *Per1*^{-/-}/*Per2*^{-/-} mice display a free-running activity rhythm with a short period (~19–21 h) for 1–2 days after release from 12L:12D to DD. In addition, their advanced onset of activity on the first and second days in DD is consistent with their advanced phase of activity onset in LD. That being said, we observed variability in the activity of *Per1*^{-/-}/*Per2*^{-/-} mice, and not all mice clearly exhibited a free-running rhythm in DD. Furthermore, all *Per1*^{-/-}/*Per2*^{-/-} mice lost their circadian activity rhythm by the third day in DD. Taken together, these data lead us to postulate that the *Per1*^{-/-}/*Per2*^{-/-} SCN is a light-driven, weak, and/or damping oscillator that is severely compromised, even if it can maintain rhythmicity in an LD cycle. Further experiments are required to examine the light-entrainability and oscillatory phenotype of the *Per1*^{-/-}/*Per2*^{-/-} SCN. Analyses of the limits of entrainment and of systematic changes in the phase of activity in different T cycles are required to determine if *Per1*^{-/-}/*Per2*^{-/-} mice entrain to the LD cycle. Additionally, single-cell imaging of *Per1*^{-/-}/*Per2*^{-/-} SCN explants will reveal if individual cells are capable of maintaining self-sustained oscillations.

We found that negative masking of spontaneous activity is phase dependent in wild-type mice such that maximal suppression is induced by light pulses at ZT14, and gradually weaker suppression occurs during light pulses at ZT16 and ZT18. These findings are consistent with other studies that have found positive masking (increase in activity) in canaries and hamsters, and negative masking of novel wheel-induced activity in hamsters were phase dependent (Aschoff & von Goetz, 1988, 1989; Redlin & Mrosovsky, 1999). The phase dependence of the masking response could be the result of circadian changes in the responsiveness of the sympathetic nervous system, which has been shown to mediate masking responses in the pineal and liver, or extra-SCN brain regions, which receive direct

or indirect projections from the retina, and could be candidates for mediating light masking (Brown et al., 2010; Ecker et al., 2010; Redlin, 2001). It is also possible that phase-dependent masking responses are the result of circadian changes in the sensitivity of the retina, since the electroretinogram (ERG) has a circadian rhythm.

The masking protocol we used in this study is equivalent to Aschoff's Type VI protocol for measuring phase shifts, i.e., measure shifts after administering light pulses to animals maintained in an LD cycle (Aschoff, 1965), and provides insight into the characteristics of the rhythms of the mutant SCN in the LD cycle. We found that the phase responses of *Per1*^{-/-} mice entrained to the LD cycle (Type VI) are strikingly different from our previous studies assessing the responses of *Per1*^{-/-} mice administered light pulses in DD (Type I) (Pendergast et al., 2010b). The high-amplitude phase shifts to light pulses administered in DD likely reflect the endogenous low-amplitude oscillations of the *Per1*^{-/-} SCN (Pendergast et al., 2009). In contrast, using the Type VI protocol, the phase shifts of *Per1*^{-/-} mice are similar to the phase responses of wild-type mice. These data suggest that the rhythm of the *Per1*^{-/-} SCN is robust in the LD cycle and that light input to the SCN may drive high-amplitude gene expression. The phase responses of *Per2*^{-/-} mice assessed using the Type VI protocol are similar to those measured with the Type I protocol: the phase response curve (PRC) of mice is phase-advanced compared to wild-types. The phase shifts that we measured using the Type VI protocol were similar to ones found in previous studies that assessed light responsiveness in different lines of *Per1*^{-/-} and *Per2*^{-/-} mice (*Brdm1* mice) using Aschoff's Type II protocol, in which the light pulse is administered on the first day in DD following entrainment to the LD cycle (Albrecht et al., 2001; Spoelstra et al., 2004).

Per1^{-/-}/*Per2*^{-/-} mice (at least some) appear to have rhythmic SCN that maintain a stable phase relationship between activity onset and lights off in the LD cycle, but their phase shifts to light pulses at ZT14 are negligible. It is possible that the sensitivity of *Per1*^{-/-}/*Per2*^{-/-} mice may be compromised, so that the relatively dim light pulse (75–85 lux) that we used in this experiment may not induce a large phase shift.

Together, our data suggest that negative masking responses to light are robust in *Per* mutant mice, including *Per1*^{-/-}/*Per2*^{-/-} mice, whose SCN may be a light-driven, weak/damping oscillator.

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FIGURE 1.

Masking responses to light are intact in *Per1*^{-/-} and *Per2*^{-/-} mice. Effect of 1-h light pulses (75–85 lux) administered at ZT14 on the wheel-running activity of wild-type (A; n = 11), *Per1*^{-/-} (B; n = 5), and *Per2*^{-/-} (C; n = 5) mice maintained in 12L:12D. Each symbol represents an individual mouse. Baseline was calculated as the total number of wheel revolutions from ZT14–15 (wheel revs/h) averaged over the 3 days prior to the light pulse. The light-pulse value represents the total number of wheel revolutions during the light pulse. Single-plotted actograms of wheel-running activity (scaled, 1-min bins) of wild-type (D), *Per1*^{-/-} (E), and *Per2*^{-/-} (F) mice. Gray shading indicates darkness and arrows indicate the day the light pulse was administered. The top panel of actograms (D, E, F) shows typical examples of the masking response of each genotype. The bottom panel of actograms (D, E, F) shows the atypical masking response of each genotype and correspond to the mice represented by the star symbol (*) in A, B, and C, respectively.

FIGURE 2.

Intact masking responses to light in *Per1*^{-/-}/*Per2*^{-/-} mice. (A–E) Single-plotted actograms of wheel-running activity (scaled, 1-min bins) of *Per1*^{-/-}/*Per2*^{-/-} mice. Gray shading indicates darkness. *Per1*^{-/-}/*Per2*^{-/-} mice (n = 5) were maintained in 12L:12D and administered 1-h light pulses at ZT14 (75–85 lux). Arrows indicate the day the light pulse was administered. (F) Each symbol represents an individual mouse. Baseline was calculated as the total number of wheel revolutions from ZT14–15 (wheel revs/h) averaged over the 3 days prior to the light pulse. The light-pulse value represents the total number of wheel revolutions during the light pulse.

FIGURE 3.

The phase of activity onset in some *Per1*^{-/-}/*Per2*^{-/-} mice is advanced relative to lights off. Double-plotted actograms of wheel-running activity (normalized, 6-min bins) of *Per1*^{-/-}/*Per2*^{-/-} mice maintained in 12L:12D (200–300 lux) for 5 days (in the same 12L:12D in which they were born and raised) and then released into DD. Gray shading indicates darkness.

FIGURE 4.

Phase-dependent masking in wild-type mice. Effect of 1-h light pulses (75–85 lux) administered at ZT14 (A; $n = 11$, data from Figure 1 are replotted), ZT16 ($n = 7$), and ZT18 ($n = 10$) on wheel-running activity. Each symbol represents an individual mouse administered a single light pulse. Baseline was calculated as the total number of wheel revolutions from ZT14–15 (A), ZT16–17 (B), or ZT18–19 (C) (wheel revs/h) averaged over the 3 days prior to the light pulse. The light-pulse value represents the total number of wheel revolutions during the light pulse. The mean ($\pm$ SEM) number of wheel revolutions/h during baseline (E) and light pulses (F) are shown. (G) The number of wheel revolutions during the light pulses was expressed as a percent of the number of wheel revolutions during baseline for each mouse, and then the mean ($\pm$ SEM) percent of baseline values was plotted at each ZT (G). * $p < .05$.

FIGURE 5.

Phase responses to light pulses in wild-type and *Per* mutant mice measured using the Type VI protocol. Mice were maintained in 12L:12D, and 1-h light pulses (75–85 lux) were administered at ZT14, ZT16, or ZT18 to wild-type, *Per1*^{-/-}, and *Per2*^{-/-} mice. *Per1*^{-/-}/*Per2*^{-/-} mice received light pulses only at ZT14. Representative single-plotted actograms of wheel-running activity (normalized, 6-min bins) of wild-type (A), *Per1*^{-/-} (B), and *Per2*^{-/-} (C) mice given light pulses at ZT14. Actograms of *Per1*^{-/-}/*Per2*^{-/-} mice administered light pulses at ZT14 are shown in Figure 2. Gray shading indicates darkness. (D) Mean phase shifts ($\pm$ SEM) are shown (number of replicates reported in previous figures for wild-types; *Per1*^{-/-}: ZT14, n = 7; ZT16, n = 1; ZT18, n = 6; *Per2*^{-/-}: ZT14, n = 4; ZT16, n = 3; ZT18, n = 5; *Per1*^{-/-}/*Per2*^{-/-}: ZT14, n = 2). No error bar is shown for the ZT16 pulse of *Per1*^{-/-} mice because only one pulse was administered, or for *Per1*^{-/-}/*Per2*^{-/-} mice because the onset of activity could only be determined in two of five mice. Light pulses were not administered to *Per1*^{-/-}/*Per2*^{-/-} mice at ZT16 and ZT18 (indicated by ND; not determined).