Per1 mutation enhances masking responses in mice

Nemanja Milićević, Arthur A. Bergen, and Marie-Paule Felder-Schmittbuhl

ABSTRACT
Light can restrict the activity of an animal to a diurnal or nocturnal niche by synchronizing its endogenous clock (entrainment) which controls the sleep wake cycle. Light can also directly change an animal’s activity level (masking). In mice, high illumination levels decrease activity, i.e. negative masking occurs. To investigate the role of core circadian clock genes Per1 and Per2 in masking, we used a 5-day behavioral masking protocol consisting of 3 h pulses of light given in the night at various illuminances (4–5 lux, 20 lux and 200 lux). Mice lacking the Per1 gene had decreased locomotion in the presence of a light pulse compared to wild-type, Per2 and Per1 Per2 double mutant mice. Per2 single mutant and Per1 Per2 double mutant mice did not show significantly different masking responses compared to wild-type controls. This suggests that Per1 suppresses negative masking responses in mice.

Introduction
Light profoundly affects the physiology and behavior of all living beings. Light can set the timing of behavior, i.e. synchronize the timing of the animal’s circadian activity pattern, a phenomenon called entrainment. Conversely, light can override the influence of the endogenous oscillator on behavior, a process called masking (Aschoff 1960; Mrosovsky 1999). The interrelationship between both of these processes is indispensable for the survival of nocturnal and diurnal animals in their natural conditions (reviewed in Smale et al. 2003)). However, substantially less is known about the molecular mechanisms that regulate masking compared to entrainment (Morin and Studholme 2014; Mrosovsky 1999; Pendergast and Yamazaki 2011).

Masking responses are mediated by classical photoreceptor input involving rods and cones (Thompson et al. 2008) and melanopsin (Opn4) positive, intrinsically photosensitive retinal ganglion cells (ipRGCs) (Mrosovsky and Hattar 2003). IpRGCs project to the central clock in suprachiasmatic nuclei (SCN) and to other brain areas via retinal projections, including the retinohypothalamic tract (RHT) (Berson et al. 2002). The RHT projections are necessary for masking responses (Li et al. 2005), but the role of the SCN is still debated (Li et al. 2005; Redlin and Mrosovsky 1999). Other brain regions modulate masking responses such as the dorsal lateral geniculate nucleus (Edelstein and Mrosovsky 2001), the visual cortex (Redlin et al. 2003) and the intergeniculate leaflet (Langel et al. 2014; Redlin et al. 1999) among others. The circadian system can, in turn, modulate masking responses in both diurnal and nocturnal species (Shuboni et al. 2012; Smale et al. 2003). The neural underpinnings of this link are not well understood, but extensive work on the Nile grass rat revealed that many brain regions play important roles, such as: the ventral subparaventricular zone (Gall et al. 2016), the olivary pretectal area (Gall et al. 2017; Langel et al. 2014), the superior colliculus (Gall et al. 2020), among others (reviewed in detail by Yan et al. 2020).

Circadian rhythms are driven on a cellular and molecular level by a complex network of interlocking transcriptional and translational feedback loops, involving core clock genes Bmal1 and Clock, with Per1-3 and Cry1-2 comprising the negative feedback loop (Cox and Takahashi 2019). The molecular outputs of this network coordinate the timing of a plethora of physiological...
processes by clock-controlled genes. Circadian oscillations were reported in wide variety of tissues and organs (Mure et al. 2018), including in the retina (Tosini and Menaker 1996). Numerous processes are under the control of the circadian clock in the retina including: melatonin release (Besharse and Iuvone 1983; Tosini and Menaker 1996), rod-cone coupling (Ribelayga et al. 2008), ion channel sensitivity (Ko et al. 2001) and light sensitivity (Barnard et al. 2006; Gegnaw et al. 2021) (reviewed by (Felder-Schmittbuhl et al. 2018; McMahon et al. 2014)).

Clock genes also play a role in masking. Clock mutant mice have impaired masking (Redlin et al. 2005). In contrast, deletion of the clock gene Rev-Erba leads to increased light sensitivity and negative masking to dim light pulses (Ait-Hmyed Hakkari et al. 2016). Per1 and Per2 mutant mice show robust masking responses to bright light pulses (Pendergast and Yamazaki 2011). However, it is unclear whether Per1-2 mutation(s) show such responses to dim light. To address this question, we subjected Per1−/−, Per2−/−, and double mutant Per1−/− Per2−/− mice to a 5-day negative masking protocol using 3 light intensities (4–5 lux, 20 lux and 200 lux). Data suggest that Per1 represses masking responses in mice.

Methods

Animals

Experiments were conducted using homozygote single and double mutant mice carrying the loss-of-function mutation of Per1 gene (Per1−/−; (Zheng et al. 2001)) and mutation of the Per2 gene (Per2−/−, (Zheng et al. 1999); hereafter defined as Per1−/−; Per2−/− and Per1−/− Per2−/−). Intercrosses between heterozygous (C57BL/6/J x 129 SvEvBrd) F1 offspring gave rise to F2 homozygous mutants. Mutant and wild-type (WT) animals on this mixed background were used in this study, maintained as described in (Albrecht, Albrecht et al. 2001). Mice were maintained in our animal facilities (Chronobiotron, UMS3415) on a light–dark cycle (12 L/12D, 300 lux during the light phase, <5 lux during the dark phase), with an ambient temperature of 22 ± 1°C. The animals were given free access to food and water. We used 2–5 month old mice (14 male and 2 female, see Supplementary Table S1 for details). Animals were acclimated to environmental conditions for at least 2 weeks before starting the experimental procedures. All experimental procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement on Use of Animals in Ophthalmic and Vision Research, as well as the European Communities Council Directive (2010/63/EU).

Behavioral masking

Negative masking is the light-dependent inhibition of locomotor activity in nocturnal animals such as rodents (Mrosovsky 1999). To assess the effect of genotype on light intensity-dependent masking responses, we subjected WT, single mutant Per1−/−, Per2−/−, and double mutant Per1−/− Per2−/− mice (N = 4/genotype) to a 5-day behavioral masking protocol. The mice were singly housed in cages and acclimated for 2 weeks prior to testing and received free access to food and water throughout the experiment. The cages were equipped with infrared detectors (CAMS, Circadian activity monitoring system, Lyon, France) and placed in a light–tight, ventilated compartment in 12 h L:12 h D. This custom-built chamber has a background illuminance of 0 lux and is equipped with automatically controlled lights. The mice received a 3 h light pulse 2 h after lights off (at ZT 14) on day 1 (4–5 lux), 3 (20 lux) and 5 (200 lux) as described by (Ait-Hmyed Hakkari et al. 2016). Locomotion was measured as infrared beam breaks/5 min (counts/5 min). Relative counts were calculated by dividing the mean value of beam breaks during the 3 h light pulse on test days (1, 3 or 5) with the activity during the dark phase of the baseline day (ZT 14–24, day 0). Data were collected using ClockLab software (Actimetrics).

Genotyping

Mice were genotyped by polymerase chain reaction (PCR) amplification of tail DNA with four sets of primers specific either for the genomic regions that were deleted in mutants but present in WT (5′-GTCTTG GTCTCATTCTAGGACACC and 5′-AACATGAGAGC TTCAGTCTTCTC for Per1 gene; 5′-AGTAGG TCTTCTT CTTATGCCCC and 5′-CTCTGCTT CAACTCTGTG TCTTG for Per2 gene), or for the recombinant alleles present in mutants only (5′-TCAAGAGGAGCAACCCATCTACC and 5′-ACT TCCATTTTGTCAGTCCCTGCAC for Per1−/−; 5′-TTTGG TTCTGGAGCTCTGAACGC and 5′-ACTTCCAT TTGTCAGTCTTGAC for Per2−/−).

Statistics

GraphPad Prism software was used for generating the graph and performing statistics (version 8.3.0, La Jolla, CA, USA). Normality of distribution was tested by the Kolmogorov–Smirnov test. The effect of light intensity
(in lux) and genotype on relative beam-break counts was assessed by a two-way ANOVA with repeated measures. Differences between groups were assessed by the two-stage step-up method of Benjamini, Krieger and Yekutieli (Benjamini et al. 2006).

Results

Behavioral masking

A dim light pulse (0.1 lux) significantly suppressed the activity of Rev-Erbα−/− mice compared to WT mice (Ait-Hmyed Hakkari et al. 2016). Considering that negative masking responses are robust in mice carrying mutations in Per genes (Pendergast and Yamazaki 2011), we hypothesized that Per mutant mice might have enhanced negative masking responses to dim light. To test this hypothesis, we subjected WT, single mutant Per1−/−, Per2Brdm1, and double mutant Per1−/− Per2Brdm1 mice to a 5-day behavioral masking protocol in which mice received a 3 h light pulse at ZT14 (2 h after lights off) (Figure 1a) as described by (Ait-Hmyed Hakkari et al. 2016). The activity of mice was plotted as actograms (Figure 1b–e). We compared relative beam breaks by repeated measures two-way ANOVA (Figure 1f). We found that light intensity (F (1.86, 22.36) = 8.47; p = .0022) and genotype (F (3, 12) = 4.97; p = .018) significantly affected the activity of mice. The interaction of genotype x light intensity was not significant (F (6, 24) = 0.15; p = .99). Post-hoc testing for the effect of genotype revealed that the activity of Per1−/− mice was significantly lower compared to WT (q = 0.043; p = .12), Per2Brdm1 (q = 0.002; p = .0039) and Per1−/− Per2Brdm1 (q = 0.0013; p = .0012) (Table S2). Post-hoc tests performed for the effect of genotype within each light intensity suggest that a 20-lux pulse might suppress the activity of Per1−/− compared to Per2Brdm1 (q = 0.053; p = .019) and Per1−/− Per2Brdm1 (q = 0.053; p = .0033) mice (Table S2). We provide raw uncorrected beam breaks and statistics in the supplementary material (Figure S1 and Table S3). These results suggest that Per1 might be involved in suppressing masking responses in mice.

Discussion

The present study describes a distinct phenotype of Per1−/− compared to WT, Per2Brdm1 and Per1−/− Per2Brdm1 mice in response to light, with Per1−/− mice exhibiting enhanced negative masking behavior.

In our study, we used Per1−/−, Per2Brdm1 and double mutant Per1−/− Per2Brdm1 mice, all of which retain the ability to entrain to a 12:12 LD cycle (Zheng et al. 2001, 1999). Among them, Per1−/− mice show rhythmic behavior in constant darkness (DD) with shorter periods, whereas Per2Brdm1 mice lose their rhythmicity in such conditions (Zheng et al. 2001, 1999). By contrast, Per1−/− Per2Brdm1 mice are arrhythmic under DD (Zheng et al. 2001). To the best of our knowledge, no masking studies were performed using the same mixed background mice as used in the present study. However, it is known that mPer1Idc−/−, mPer2Idc−/− and mPer1Idc−/− mPer2Idc−/− mice on a C57BL/6 J background show robust masking responses (Pendergast and Yamazaki 2011).

The interrelationship between masking and the circadian system is complex (Shuboni et al. 2012). In nocturnal animals (e.g. mice), light is most effective in suppressing activity in the early dark phase of the light-dark cycle (ZT14) (Shuboni et al. 2012). This suppressing effect is also observed in the early subjective night (CT14, i.e. 14 h after the onset of constant darkness) in mice (Shuboni et al. 2012). Moreover, light pulses administered in the early dark phase can reduce wakefulness in mice (‘photosomnolence’) (Lupi et al. 2008; Morin and Studholme 2009; Tsai et al. 2009). In the present study, the light pulses were also administered in the early dark phase (ZT14–17). As expected, we observed that light suppressed the activity in all mice. This suppressive effect is similar to the one elicited by a 75–85 lux (Pendergast and Yamazaki 2011). Our results suggest that light suppressed the activity of Per1−/− compared to WT, Per2Brdm1 and Per1−/− Per2Brdm1 mice.

It is not clear why the Per1 gene represses behavioral masking. However, enhanced negative masking responses were observed in mice carrying a mutation in the circadian clock gene Rev-Erbα, which suggest that a common converging pathway underlies the masking phenomenon. A hypothetical model was proposed in which masking responses are driven by ipRGC output (Ait-Hmyed Hakkari et al. 2016; Felder-Schmittbuhl et al. 2018). This output results from a summation of intrinsic light stimulation (Opn4-dependent) and synaptic input from rod and cone-specific bipolar cells. At lower light intensities, this input is insufficient to depolarize ipRGCs of WT mice. By contrast, Rev-Erbα−/− mice have increased Opn4-dependent intrinsic sensitivity and input from the rod pathway, thus eliciting enhanced masking at lower light intensities (Ait-Hmyed Hakkari et al. 2016; Felder-Schmittbuhl et al. 2018). It is plausible that Per1−/− mice may have higher expression of Opn4 and/or enhanced rod sensitivity.

An alternative explanation is that Per1 inhibits masking responses by a pathway in the brain. This
possibility is supported by studies on the effects of Per genes in entrainment. For example, Per1−/− mice show a greater phase response curve (PRC) amplitude compared to WT mice, whereas Per2−/− mice were not significantly different compared to WT mice (Pendegast et al. 2010). Others have found that Per1 mutant mice cannot advance the phase of the clock in response to a nocturnal light pulse at ZT22, whereas Per2 mutant mice cannot delay the phase of the clock in response to a light pulse at ZT14 (Albrecht, Albrecht et al. 2001). Yan and Silver reported differential localization of Per1 and Per2 mRNA expression in the SCN upon light pulses that entrain the clock (Yan and Silver 2002). In the SCN shell, they found that a phase advancing light pulse increased Per1, but not Per2 mRNA expression. In contrast, they found that Per2, but not Per1 mRNA, was increased in the SCN shell after a phase delaying light pulse (Yan and Silver 2002). Because there is an intertwined relationship between masking and entrainment (Shuboni et al. 2012), it is...
tempting to speculate that there is a link in the neuronal circuitry underlying these processes. In our study, pulses were given at ZT14 (i.e. phase delaying). Thus, it is plausible that enhanced masking responses of Per1<sup>−/−</sup> mice is mediated by neural processing of light by the SCN.

There are limitations in our study. Although the suppression of locomotor activity in Per1<sup>−/−</sup> is observed at dim light, the light pulse is higher than the one required for Rev-Erbα<sup>−/−</sup> mice (0.1 lux) (Ait-Hmyed Hakkari et al. 2016). Therefore, the contribution of Per1 for masking is of less significance compared to other clock genes such as Rev-Erbα<sup>−/−</sup> (Ait-Hmyed Hakkari et al. 2016) or Clock (Redlin et al. 2005).

Another limitation is that we used only one day between the light pulses. Previous masking studies used study designs in which the periods between pulses were at least 3 days (Shuboni et al. 2012), and even 5–6 days (Morin and Studholme 2014). Thus, there may be a confounding effect of the previous pulse(s) on masking responses in the present study. However, our short protocol is less than a week and minimizes the need for animal handling (e.g. we do not need to replenish food, water, to clean cages). Thus, our protocol might have removed the potential confounding effects of handling on masking responses (Mrosovsky et al. 1989). Also, prior work showed that this protocol was sufficient to detect masking responses in the Rev-Erbα<sup>−/−</sup> mice (Ait-Hmyed Hakkari et al. 2016).

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Nemanja Miličević http://orcid.org/0000-0002-8062-7270
Arthur A. Bergen http://orcid.org/0000-0002-6333-9576
Marie-Paule Felder-Schmittbuhl http://orcid.org/0000-0003-3539-1243

Data availability statement

Raw data that support the findings of this study are available from the corresponding authors, upon reasonable request.

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