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Research report

Non-photic phase resetting of *Dexras1* deficient mice: A more complicated story

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Abstract

Recently, it has been reported that mice deficient for *Dexras1* have a diminished phase-shifting response to photic stimuli but an enhanced response to non-photic stimuli; the latter is of additional interest in that mice generally show relatively weak and unreliable responses to non-photic events. Therefore, in situations in which both photic and non-photic stimuli are present, control of circadian rhythms, relative to wild-types, should tip toward non-photic stimuli in *Dexras1*$^{-/-}$ mice. However, we detected no differences in an experiment in which photic and non-photic entraining agents were presented 180° out of phase, i.e. were in conflict with each other. Furthermore, *Dexras1*$^{-/-}$ and wild-type mice did not differ in non-photic phase shifting to a pulse of confinement in a novel running wheel. Suppression of locomotion by light (masking effect) did not differ between the genotypes, indicating that the photoreceptor input to the non-image forming system is intact. The circadian phenotype of *Dexras1*$^{-/-}$ mice appears to be more complicated than previously thought.

Keywords: Conflict; Entrainment; Non-photic; Zeitgeber; Circadian rhythms; Novel wheel pulse; Masking

1. Introduction

There are two classes of stimuli that can entrain the circadian system, photic and non-photic. In mammals, light is detected by at least three types of photoreceptors [1], and information conveyed to the master clock, the suprachiasmatic nuclei (SCN) of the hypothalamus, by the retino-hypothalamic tract (RHT) [2]. A major pathway for non-photic stimuli runs via the geniculo-hypothalamic tract (GHT) from the intergeniculate leaflet of the hypothalamus (IGL) to the SCN [3]. Input pathways for non-photic stimuli, however, are not yet fully understood, and are likely to be more diverse, because non-photic entrainment agents vary from melatonin [4], and pharmacological compounds [5], to food [6], and locomotor activity [7–9].

Much of the work on non-photic clock resetting has been done with hamsters [10–12]. Because transgenic models have not been developed for hamsters, it would be useful to be able to obtain reliable non-photic clock resetting with single pulses of non-photic events in mice as well. Unfortunately, various attempts to achieve this have met with limited success [9,13].

In this context, recent findings of Cheng et al. [14] seem of particular importance for the understanding of locomotor activity as a zeitgeber in mice. In their paper, Cheng et al. [14] report diminished responses to light as a phase-shifting stimulus at ZT14 but greater responses to confinement of a novel wheel: *Dexras1*$^{-/-}$ mice showed consistent large phase shifts after non-photic pulses at ZT6 whereas wild-type controls did not. Furthermore, *Dexras1*$^{-/-}$ but not wild-type control mice lengthened their free-running period (τ) when access to the running wheel was blocked, again indicating that *Dexras1* plays some role in non-photic resetting of the clock. The proposed molecular explanation for the phenotype of *Dexras1*$^{-/-}$ mice attributes two functions to *Dexras1* [15]. (1) *Dexras1* mediates activation of the MAPK cascade after glutamate signaling via the RHT (photic input), and (2) *Dexras1* inhibits the $G_{i/o}$ mediated response to NPY signaling via the GHT (non-photic input). Impact of *Dexras1* on photic clock resetting was further elucidated in a more recent paper by the same authors that revealed

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two sizable advance portions, one peaking around midday and the other in the late part of the night but only a reduced delay portion in the first part of the night in *Dexras1*$^{-/-}$ mice [16].

To further explore the relationship between photic and non-photic clock resetting in *Dexras1*$^{-/-}$ mice, we used a conflict test in which a running wheel is made available only in the light (L) portion of a light–dark (LD) cycle [17]. For a nocturnal animal such as a mouse, this sets up photic and non-photic entraining schedules in opposition to each other, i.e. 180° out of phase. We predicted that if photic clock resetting was significantly altered with larger advances and smaller delays, and non-photic phase resetting stronger in *Dexras1*$^{-/-}$ mice, there should be a clear phenotypic distinction between *Dexras1*$^{-/-}$ and wild-type mice in the conflict test. Surprisingly, we did not detect such differences. We then conducted some additional tests designed to further characterize the response of *Dexras1*$^{-/-}$ mice to photic and non-photic stimuli.

2. Material and methods

2.1. Animals

We used 12 male B6.*Rasd1*^{tm1Pngr} (Ras-like gene, dexamethasone-induced; *Dexras1*$^{-/-}$) mice generated in the laboratory of J. Penninger [14] and made available by H.Y. Cheng (Columbus, OH, USA). Genotypes were confirmed by polymerase chain reaction (PCR) using AmpliTaq DNA polymerase (Promega, Madison, MI) with the following primer set: common forward primer (5′-ACGTGACGCACCTTAGCTGG-3′) together with a wild-type specific (5′-GTGTCCAGTATGTCCAACTGG-3′) and a knock-out specific reverse primer (5′-CATGCTCCAGACTGCCTTGGG-3') that was located within the neomycin cassette of the targeting construct. Common forward and wild-type specific reverse primer were designed based on the *Rasd1* nucleotide sequence from GeneBank (AL603710). The PCR protocol consisted of an initial 3 min denaturation followed by 30 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s), and extension (72 °C, 90 s), and a final extension (72 °C, 10 min). Product size was 429 and 459 bp for *Dexras1*$^{-/-}$ and wild-type, respectively, and products were distinguished by agarose gel electrophoresis.

The *Dexras1*$^{-/-}$ mice were backcrossed to C57BL/6J for at least 10 generations. Hence, we purchased 12 male C57BL/6J mice (wild-type) from Jackson Laboratories (Bar Habour, ME, USA) as control animals. All animals arrived in the lab at 11 ± 1 weeks of age. Experiments started after a habituation period of 2 weeks and ended for all animals at 33 weeks of age. All animals were held individually in polypropylene cages (44 cm × 23 cm × 20 cm) equipped with a running wheel (17.5 cm diameter) on standard bedding (Beta Chip, USA) in a sound attenuated and temperature controlled room at 22 ± 1.5 °C with food (LabDiet #5001, PMI, USA) and tap water available *ad libitum*.

Upon arrival the animals were assigned to one of two experimental groups. Both groups were used in all experiments in a counterbalanced design. While group 1 was used in the conflict paradigm, group 2 was tested for masking and non-photic shifting, and then the groups were interchanged so that group 1 was tested for masking and non-photic shifting whereas group 2 was studied in the conflict paradigm.

2.2. Conflict paradigm

In this test, wheel running is restricted to the light phase of the LD cycle promoting activity at an unusual time of day for a nocturnal animal. For details of the apparatus see elsewhere [17]. In addition to the wheel revolutions that were recorded in all three experiments, general activity was monitored via a passive infrared motion detector (PID) and continuously recorded by a computerized data acquisition system. A hook-shaped brass rod hanging into the cage could block the wheel automatically, thereby restricting the time the animals could turn the wheel but not the access to the wheel. Prior to the onset of this experiment, mice (n = 12 of each genotype) were held in a dim LD cycle with 12 h of light per day (LD 12:12) for at least 10 days. The illumination at cage level was about 0.5 lx (EX2 lux meter, B. Hagner AB, Solna, Sweden) from an incandescent light bulb (60 W).

At the start of the experiment (day 0), the running wheels were blocked for 26 h starting at the onset of darkness. Simultaneously, the LD cycle was inverted by having 1 day in constant light with the new dark phase beginning at the time of the former lights on. Thus, the dark phase was delayed by 12 h. Starting on the first inverted day, the wheels were unblocked only throughout the 12 h of the new light phase, which was identical with former dark phase. After 21 days of exposure to the new conditions, the illumination level was raised to approximately 6 lx for another 18–21 days; the animals were then transferred to constant darkness (DD) and *ad libitum* wheel access in order to assess clock phase under free-running conditions.

In contrast to the phase shift experiments (see below), the onset of activity for the conflict data was determined using ClockLab's (Actimetrics Software, Wilmette, IL, USA) built-in function with a 12-h window [17]. Days with an activity onset that differed from the proceeding as well as from the subsequent one by more than 2 h were excluded from further analysis. In total, less than 2.1% of all activity onsets had to be excluded. To compare the two genotypes, we pooled activity onsets of seven consecutive days for each animal (pooled periods: week before inversion, first week, . . ., sixth week of inversion). Thus, we calculated a repeated measures ANOVA with *genotype* as between variables and *week of experiment* as repeated measure.

2.3. Masking

In order to investigate possible differences in the non-image forming system, the masking capabilities of the animals were tested as described elsewhere [18]. In short, animals were entrained to LD 12:12 and then exposed to a 3 h light pulses from ZT13-16 ranging from 0.002 to 283 lx in brightness (all values are given in Fig. 2B). The percentage of masking was defined as the number of wheel revolutions during the light pulse × 100 divided by the number of wheel revolutions during the same interval in the night before the light pulse (baseline). Fluorescent light sources (Sylvania Octron 4100 K, 32 W) were dimmed to appropriate illumination levels by addition of neutral density filters (Cinegel #3404, Rosco).

2.4. Novel wheel pulse

In this experiment, we aimed to confirm in our laboratory the reported non-photic phenotype for *Dexras1*$^{-/-}$ mice. At ZT6 animals were transferred to novel wheels within the same room, and lights were turned off at the same time. The novel wheels were equipped with a plastic mesh surrounding the running wheel, and were practically identical to the ones used by Cheng et al. [14]. At ZT9, animals were placed back in their home cages and DD was established for at least 5 days following the pulse day. After the animals had re-entrained to LD 12:12, we carried out an Aschoff type II control experiment [19]. Here, lights were turned off at ZT6 and the animals remained in their home cages without getting the novel wheels. This control is a crucial element of the Aschoff type II protocol since it distinguishes between the specific effect of the novel wheel and the effect of darkness in the middle of the day [19,20]. Phase shifts were calculated as difference between onset of activity on the last day in LD and on the first day after the non-photic pulse or in the case of the control experiment the first day after transfer to DD. Again, ClockLab's built-in function was used to determine the onset but here with a 6-h sliding window. A one-sample t-test determined whether the control shift minus the novel wheel shift differed significantly from 0 min (no shift).

In order to control for possible changes in τ that might be responsible for differences in phase shifting, animals were held in DD for longer either after the control or the novel wheel pulse; starting on the first day in DD, the free-running period was determined for no less than seven consecutive days using the built-in chi-square algorithm of ClockLab.

2.5. Statistical analysis

All data are given as mean ± standard error of the mean (S.E.M.). All data were analyzed using repeated measurements two-way analyses of vari-