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Phase shifts and *Per* gene expression in mouse suprachiasmatic nucleus

Lily Yan¹, Kelly J. Hochstetler², Rae Silver^{1,3,4}, and Abel Bult-Ito^{2,CA}

¹Department of Psychology, Columbia University, New York, NY 10027

²Behavioral and Evolutionary Neuroscience Laboratory, Alaskan Basic Neuroscience Program, Institute of Arctic Biology, University of Alaska Fairbanks, P.O. Box 757000, Fairbanks, AK 99775

³Department of Psychology, Barnard College, New York, NY 10027

⁴Department of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, NY 10027, USA

Abstract

In mammals, circadian rhythms controlled by the suprachiasmatic nuclei are entrained by photic stimuli. To investigate the molecular mechanism of photic entrainment, we examined light-induced behavioral phase delays and associated changes in *mPer1* and *mPer2* gene expression in the suprachiasmatic nuclei of two mouse lines artificially selected for nest-building behavior. Big nest-builders show larger phase delays than small nest-builders. Light-induced *mPer1* and *mPer2* expression was examined in individual mice previously tested for phase shifting at circadian time 16. Light-induced *mPer2* expression was significantly higher in big compared to small nest-builders. No difference was found between lines in light-induced *mPer1* expression. The results suggest a more important role for *mPer2* than for *mPer1* gene expression in behavioral phase delays.

Keywords

Circadian rhythm; Light pulse; Period gene; SCN

INTRODUCTION

Circadian rhythms in behavior and physiology are controlled by an internal clock located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus [1–3]. Light is the most prominent daily cue that synchronizes this clock to the environment. The discovery of clock genes (e.g. *Per1*, *Per2*, *clock*, *BMAL1*, *cry1*, *cry2*) has demonstrated that circadian oscillation is generated at the gene level [4]. The molecular mechanism of rhythmic oscillation in mammals is described as interlocked positive and negative transcription/translation feedback loops [5]. The molecular mechanism of photic entrainment, however, is less well understood. Clock genes *Per1* and *Per2* are rapidly induced by light in the SCN, and are believed to mediate photic resetting of the molecular clock [6,7]. However, the distinct function of these genes in producing behavioral phase delays is still unclear. Our previous study of regional distribution of light-induced responses suggests that *mPer2* but not *mPer1* expression is correlated with phase delays [8].

To further investigate molecular mechanisms underlying photic phase shifts, we studied light-induced *mPer* gene expression in two artificially selected mouse lines, termed big and small nest-builders, which differ in their thermo-regulatory nest building behavior. In both lines, the phase response curve to a 15 min light pulse, tested at 3 h intervals, are parallel at all circadian times, with significant phase delays between circadian time (CT) 12 and CT21, and no phase advances [9]. The big nest-builders, however, have larger light-induced phase delays than do the small nest-builders between CT15 and CT21 [9]. At CT16, there is a 2-fold difference in magnitude of phase delays and number of Fos-expressing cells [10]. In the present study we asked whether the magnitude of behavioral phase shifts is correlated with light-induced *mPer1* and/or *mPer2* gene expression in the SCN.

MATERIALS AND METHODS

Male mice, *Mus domesticus*, bidirectionally selected for thermoregulatory nest-building behavior for 57 generations were the subjects of this study [11]. Animals were housed individually, and food and water were available *ad lib*. All animal care and experimental procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (protocol 00-06).

Wheel-running behavior was recorded in 5 min bins with the VitalView data collection system (Minimitter Co., Inc., Bend, OR). Animals were kept in a 12:12 h light:dark cycle for 2–3 weeks and were then placed in constant darkness (DD), with no access to nesting material at any time. The experiment was performed in two series. In the first series, experimental animals were given a 15 -min light pulse (700 lux) at CT16, following housing in DD for 10, 28 and 46–53 days, whereupon they were sacrificed at CT17.5 after the third light pulse and brain tissue was processed for *in situ* hybridization (five big and eight small nest-builders). In the second series, animals (11 big and nine small nest-builders) were given a light pulse (as above) on day 21 of DD to test their behavioral phase shift. They were then randomly assigned to experimental and control groups. Experimental animals (seven big and four small nest-builders) were given a second light pulse on day 42–45 at CT16 then sacrificed at CT17.5. Control animals (four big and five small nest-builders) did not receive a light pulse and were sacrificed at CT17.5 on day 42–45.

Behavioral phase shifts were measured using actograms created by ClockLab (Actimetrics, Evanston, IL). Activity onsets were assigned by ClockLab, then checked and edited by eye to include activity bouts that exceeded 15% of daily maximum activity and were >15 min long [10]. Two animals were excluded from the study because of technical problems.

Ninety minutes after the start of the light pulse, mice were anesthetized (sodium pentobarbital: 200 mg/kg, i.p.) under a red safelight. At this time *Per1* and *Per2* mRNA levels both show a maximal response to light [7,8]. The animal's head was then wrapped in two layers of aluminum foil to prevent exposure to light prior to transcardial perfusion with 20 ml phosphate buffer (0.1 M; pH 7.4) and 100 ml 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed overnight at 4°C, then transferred to 20% sucrose in 0.1 M phosphate buffer for storage and overnight shipping to Columbia University for processing. All solutions were RNase free.

The *mPer1* and *mPer2* *in situ* probes used in this study were described previously [8]. Serial coronal sections (40 μ m) were made from the rostral to caudal end of the SCN using a cryostat (Reichert-Jung, Heidelberg, Germany). Alternate sections were collected for *mPer1* and *mPer2* probes. To minimize variation in the hybridization procedure, sections from both lines were processed simultaneously in the same well. Individual brains were identified with

unique markings. The *in situ* hybridization histochemistry was performed as described previously [8].

For quantification of optical density, images of serial sections were captured using a CCD video camera (Sony XC77) attached to a light microscope (BH-2; Olympus Optical, Tokyo, Japan). Expression of mRNA was quantified using the NIH Image program (version 1.61). Mean gray value per pixel was used to quantify the intensity of the signal in the SCN. For each animal, each SCN was evaluated separately in three sections taken from the mid-SCN region and the average was used for statistical evaluation. A hypothalamic area adjacent to the SCN was used to measure background. Relative optical density was calculated as $[\text{Optical Density}_{\text{SCN}} - \text{Optical Density}_{\text{background}}] / \text{Optical Density}_{\text{background}}$ [12].

The results of the two independent series did not significantly influence the overall statistical evaluation (not shown) and therefore the two series were pooled for analysis. Differences between selected lines were evaluated by two-way ANOVA. Effects of selected line (i.e. big and small nest-builder), light pulse (i.e. light pulse and no light pulse before processing for *in situ* hybridization), and selected line \times light pulse interaction were examined using type III sums of squares. Pairwise comparisons were evaluated with Student's *t*-test. All results are expressed as means \pm s.e.m.

RESULTS

A light pulse in early subjective night induced phase delays in both lines of mice, shown for representative individuals (Fig. 1a) and for the experimental group (Fig. 1b). Big nest-builders showed larger phase delays than did small nest-builders (Fig. 1b; $F(1,27) = 10.89$, $p < 0.0027$). Animals were screened for their behavioral response to a light pulse at CT16 once (series 2) or twice (series 1) in an initial part of the study. The first and second light pulses (see Materials and Methods) resulted in comparable ($t_{20} = 1.441$, NS) and highly correlated ($r = 0.808$, $p < 0.005$, $n = 11$) phase delays for each animal in series 1. Similarly, phase shift magnitude was not different between experimental and control animals tested in the behavioral part of the study (light pulse: $F(1,27) = 3.03$, NS; line \times pulse interaction: $F(1,27) = 0.03$, NS).

For the analysis of *Per* gene expression, animals were next randomly assigned to the experimental or control groups. Light-induced *mPer1* was detected in the SCN in both lines (Fig. 2a, upper panel) and was concentrated in the ventral or core region of the SCN (see inserts in Fig. 2a, upper panel). In non-light pulse controls of both lines, little *mPer1* expression was found in the SCN (Fig. 2a, lower panel). As shown in Fig. 2b, results indicate light-induced *mPer1* expression in the SCN in both lines ($F(1,27) = 58.76$, $p < 0.0001$), and no difference in light-induced *mPer1* expression between lines (selected line: $F(1,27) = 0.86$, NS; line \times pulse interaction: $F(1,27) = 0.96$, NS).

A strong *mPer2* signal was detected in the SCN of big nest-builders that received a light pulse, while a moderate *mPer2* signal was detected in small nest-builders (Fig. 3a, upper panel). High magnification images (see inserts in Fig. 3a, upper panel) show that *mPer2* mRNA-expressing cells were located throughout the SCN. Little *mPer2* signal was found in the SCN (Fig. 3a, lower panel) of non-light pulse controls from both lines. As shown in Fig. 3b, results indicate light-induced *mPer2* expression in the SCN in both lines ($F(1,27) = 209.63$, $p < 0.0001$), with significantly higher expression in big nest-builders ($F(1,27) = 11.91$, $p < 0.0019$). The interaction effect was significant ($F(1,27) = 8.81$, $p < 0.0062$) because the difference between big and small nest-builders in *mPer2* expression in the SCN in controls was much smaller than in experimental animals (Fig. 3b).

DISCUSSION

Complex behaviors, such as circadian rhythmicity [4], have a polygenic basis [13]. Selected house mouse lines likely represent normal phenotypic and allelic variation that are products of natural selection [13], thereby presenting an opportunity to study the mechanistic basis of circadian physiology and behavior. Allelic variations in the *Period* gene are associated with alterations in free-running period and temperature compensation in *Drosophila* [14]. Our lines of mice have been selected for their thermoregulatory nest-building behavior, and it is of interest to note that selection for this trait is associated with altered circadian behaviors including changes in free-running period [9].

The present study investigates *Per* gene expression in two lines of mice which differ in their behavioral phase shifts to a light pulse at CT16 [9,10]. The results indicate greater light-induced *mPer2* expression in big nest-builders than in small nest-builders, with no difference in light-induced *mPer1*. *Per* expression within the SCN is highly localized, as reported previously [8], with *mPer1* restricted to the core SCN region, while *mPer2* is expressed throughout the SCN. Using anterograde tracer cholera toxin- β , retinal efferents have been shown to be concentrated in the core SCN, and avoid the shell region in mice [15] (and confirmed in our unpublished findings). Thus, present results suggest that *mPer1* expression is induced directly by photic input, while *mPer2* is regulated by retinal input and some unknown mechanism(s).

Distribution of light-induced *mPer1* expression is the same in both lines, and similar to other mouse strains, including BALB/C [6] and C57BL/6 [8]. The absence of differences between big and small nest-builders in *mPer1* induction in the core SCN suggests that line differences in behavioral phase shifts cannot be accounted for by *mPer1* induction or by photic input. This is consistent with studies of *mPer1* mutant animals, which show normal phase delays to a light pulse in early subjective night [16,17]. However, in mice an antisense phosphotioate oligonucleotide to *mPer1* mRNA significantly reduced light-induced phase delays [18].

More interesting is the greater *mPer2* expression in the SCN of big nest-builders, associated with bigger phase delays, and lesser *mPer2* expression in small nest-builders, associated with smaller phase delays. This suggests that *mPer2* is important in regulating phase delays, consistent with work on *mPer1* and *mPer2* mutant animals [16]. Alternatively, second-order neurons receiving indirect photic input may be more sensitive to the consequences of photic information potentially leading to the activation of several genes. This activation might result in increased communication among SCN neurons leading to increased phase delays without direct involvement of *mPer2*.

It is well established that light-induced behavioral phase shifts are correlated with Fos expression in the SCN [19]. Our results point to a similarity between fos and *mPer2* expression, but different expression patterns for fos and *mPer1*. Big nest-builders, which show bigger phase delays, also show higher *mPer2* mRNA and c-FOS protein expression than do small nest-builders [10], while *mPer1* expression does not differ between lines. This can be explained by taking the regional localization of expression of these genes into account. Light-induced c-Fos mRNA is initially observed in the ventral or core region of the SCN and subsequently spreads throughout the SCN [20]. Light-induced expression of c-FOS protein was observed in both ventral (core) and dorsal (shell) regions of the SCN [10]. In the present study, *mPer1* is only observed in the core, and *mPer2* is observed throughout the SCN. These data suggest that c-fos expression in the SCN is the product of both direct retinal input and light-induced changes in SCN cells that are not themselves directly

retinorecipient. We speculate that similar processes may underlie *mPer2* induction in the SCN.

Photic entrainment is a unique process that involves three components of the circadian system: input, pacemaker, and output. In mammals, the major input pathway to convey light information to the SCN is the retinohypothalamic tract originating from a subset of retinal ganglion cells [21,22]. Light resets the phase of pacemaker cells in the SCN, thereby resetting the phase of all circadian rhythms [23]. SCN output pathways are not fully understood, but both synaptic and diffusible signals may be involved [3,24–26]. The present study suggests that differences between lines in phase shifting behavior are not due to differences in the input pathway, but rather in pacemaker mechanisms and/or output pathways. While strain differences in protein expression remain to be determined, the results demonstrate that behavioral phase delays are associated with line differences in *mPer2*, but not *mPer1* mRNA.

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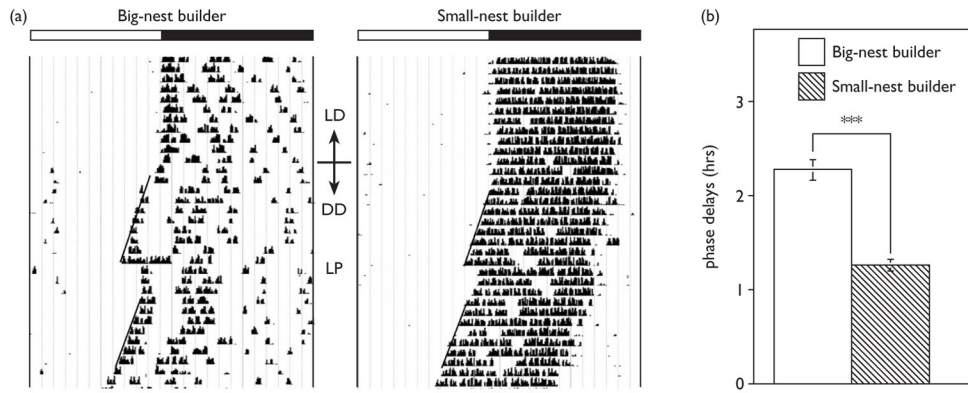


Fig. 1. Light-induced phase shifts in the two mouse lines. (a) Representative actograms show locomotor activity of a big and small nest-builder. Mice maintained in a 12:12 h light:dark cycle (indicated by the white-black bar on the top) were placed in DD and then were given a light pulse at circadian time 16 as indicated by the label to the right of each figure. (b) In the light-pulsed experimental group, big nest-builders showed a larger phase delay than did small nest-builders. *** $p < 0.005$ (t -test), $n = 11$ for each group.

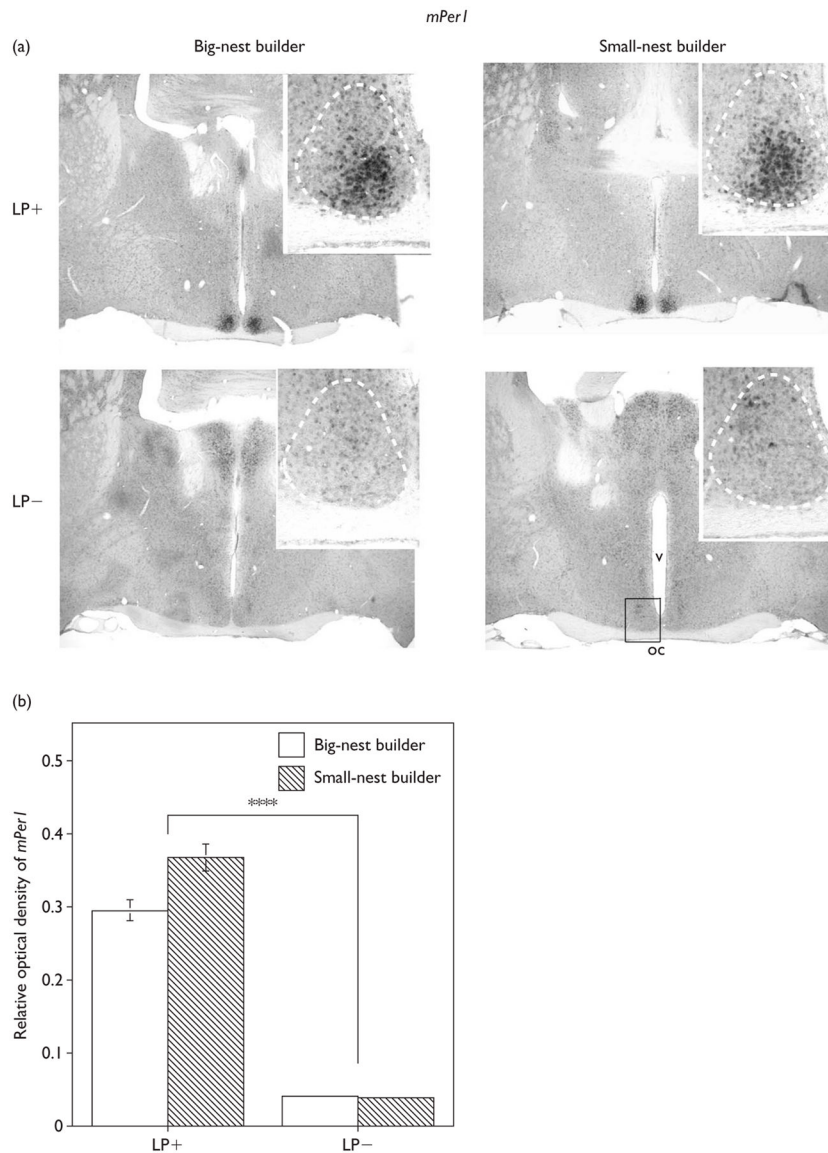


Fig. 2. Light-induced *mPer1* in the suprachiasmatic nucleus (SCN) of the two mouse lines. Experimental animals (LP+) were given a light pulse (700 lux, 15 min) at circadian time 16, then killed at circadian time 17.5. Control animals (LP-) that were not exposed to light were killed at the same circadian time. (a) *mPer1* staining in the hypothalamus containing the SCN in brain sections from both mouse lines. Inserts show a high-magnification micrograph of the SCN. (b) in the LP+ group, light-induced *mPer1* expression in the SCN (relative optical density) did not differ between the big and small nest-builders. White dashed line shows the border of the SCN. OC, optic chiasm; V, third ventricle; **** $p < 0.0001$ (ANOVA): LP+: $n = 11$ for each group, LP-: $n = 4$ (big), $n = 5$ (small).

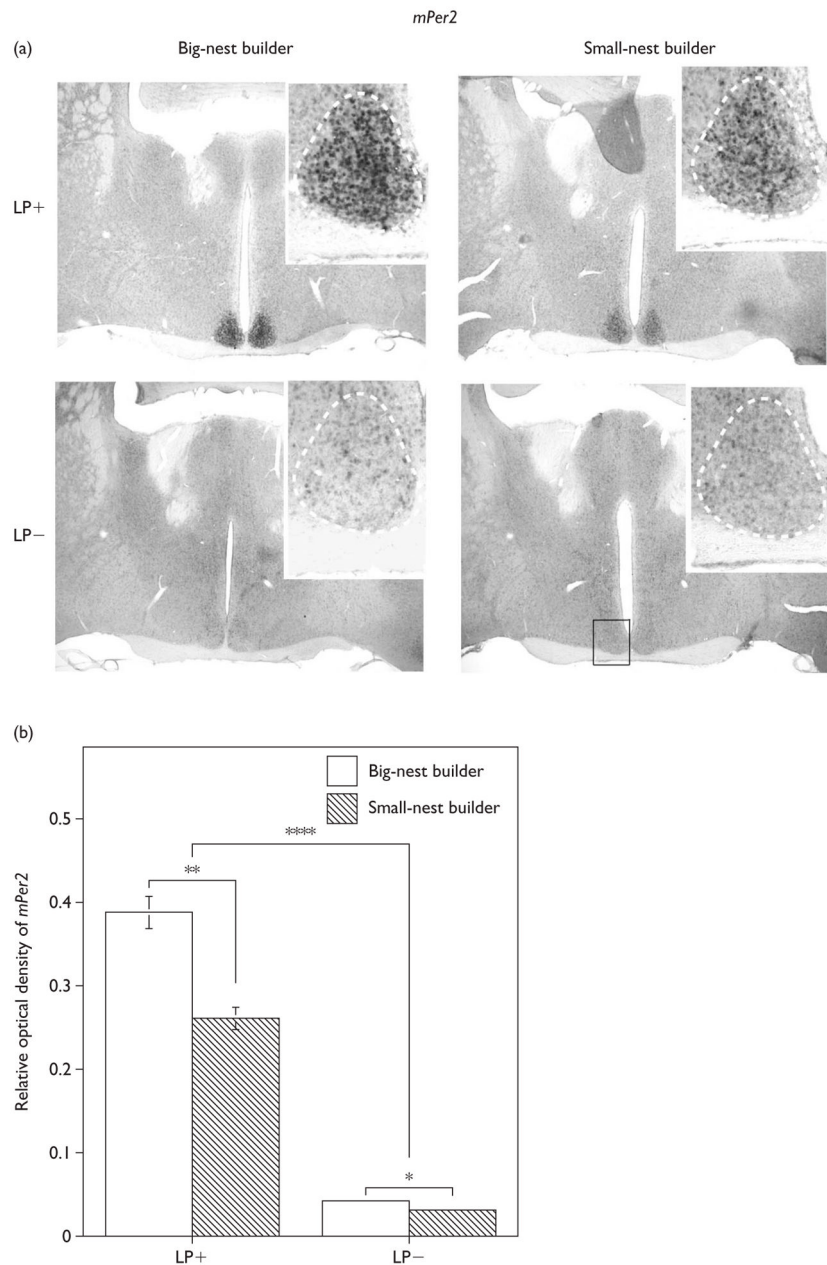


Fig. 3. Light-induced *mPer2* in the suprachiasmatic nucleus (SCN) of the two mouse lines. Experimental animals (LP+) were given a light pulse (700 lux, 15 min) at circadian time 16, then killed at circadian time 17.5. Control animals (LP-) that were not exposed to light were killed at the same circadian time. (a) *mPer2* staining in the hypothalamus containing the SCN in brain sections from both mouse lines. Inserts show a high-magnification micrograph of the SCN. (b) in the LP+ group, light-induced *mPer2* expression in the SCN (relative optical density) was significantly higher in big nest-builders compared to small nest-builders. White dashed line shows the border of the SCN. OC, optic chiasma; V, third ventricle; * $p < 0.05$, ** $p < 0.01$ (t -test) **** $p < 0.0001$ (ANOVA); LP+: $n = 11$ for each group, LP-: $n = 4$ (big), $n = 5$ (small).