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The role of *Period1* in non-photic resetting of the hamster circadian pacemaker in the suprachiasmatic nucleus

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Abstract

Non-photic stimuli, such as diurnal wheel running in rodents, phase shift the circadian clock and suppress the expression of *Per1* in the suprachiasmatic nucleus (SCN). The goal of the present study was to directly decrease *Per1* expression using antisense (AS) oligodeoxynucleotides to determine if such suppression produced non-photic phase shifts. Injections of *Per1*-AS suppressed expression of *Per1* within the SCN and produced phase shifts similar to those resulting from other non-photic manipulation, with large phase advances to injections during the subjective day. These results indicate that the decrease in expression of *Per1* is a cause rather than a consequence of non-photic phase shifts.

Keywords

Antisense oligonucleotides; In situ hybridization; Phase shift

Two classes of zeitgebers can reset the mammalian circadian system: photic and non-photic [17]. Photic resetting results from retinal illumination during the night, and is mediated by glutamate release from the retino-hypothalamic tract [14] at the suprachiasmatic nucleus (SCN), the site of the mammalian circadian pacemaker. This results in the rapid expression of the Period genes *Per1* and *Per2* in the SCN core [8]. Non-photic resetting of the circadian clock differs importantly from photic resetting. Non-photic shifts result from arousal [2] or activity [15] during periods of quiescence in nocturnal animals. Small phase delays are observed to late night non-photic pulses, while large phase advances are observed to daytime pulses [4]. The neurotransmitters neuropeptide Y (NPY) and serotonin (5-HT) have been implicated in non-photic resetting of the circadian system [15].

Expression of *Per1* and *Per2* in the SCN decreases following non-photic manipulations [6,9,12,13,20]. It has been suggested that light and activity “have convergent but opposite effects: light elevates *Per* and non-photic events decrease *Per*” (p. 29, Ref. [11]). It has yet to be determined if this non-photic effect on *Per* expression is the cause or a consequence of

the resulting non-photic phase shifts. In the current study, we demonstrate that directly decreasing *Per1* levels in the SCN using antisense oligodeoxynucleotides (AS-ODN) produces phase shifts consistent with the non-photic phase response curve.

Adult male hamsters (*Mesocricetus auratus*) obtained from Charles River (Kingston, NY) were given food and water ad libitum. The animal colony room was kept on a 12:12 h light/dark cycle (LD), with light intensity of 600 lux. The room was equipped with a white noise generator (91 dB spl) to mask environmental noise. For animals housed in constant darkness (DD), a dim red light (<1 lux; Delta 1, Dallas, TX) allowed for maintenance.

For studies performed under constant conditions, animals were housed in LD and then placed in DD for at least 1 week before being sacrificed. In this case, hamsters transferred to DD were placed in cages equipped with running wheels (diameter, 16 cm) and locomotor activity was monitored continuously using a computer-based data acquisition system (Dataquest, Data Sciences, St. Paul, MN).

All handling of animals conformed to the Institutional Animal Care and Use Committee guidelines of Columbia University.

Animals were anesthetized and a stainless steel guide cannula was stereotaxically implanted into the third ventricle using the following co-ordinates: 1.0 mm anterior and 0.0 mm lateral to bregma and 7.6 mm below the surface of the leveled skull. After surgery, hamsters were housed in DD and were allowed to establish stable free-running activity rhythms.

After at least 5 days of stable running, hamsters received a microinjection of either an ODN or saline through the guide cannula using a needle attached by polyethylene tubing to a 50 μ l Hamilton syringe. The ODN or control substance was injected slowly at one of four circadian times (CT, where activity onset is defined as CT12 by convention). The injection times CT0, 6, 12 or 18. After each injection, the needle was left in place for 5 min.

Gel purified phosphorothioate ODNs (Genelink, Hawthorne, NY) were injected with a volume of 7.5 μ l and a concentration of 1 nmol/ μ l. ODNs used were: *Per1*-antisense (AS) (5'-TAGGGGCCACTCATGTCT-3') (the initiation site is underlined), *Per1*-sense (S) (5'-AGACATGAGTGGCCCCCTA-3'; controlling for non-specific effects of ODN treatment), and CalB-AS (5'-AGGTGCGATTCTGCCATGG-3'; controlling for non-specific effects of gene silencing, as CalB is in the photic pathway [7]). Antisense technology permits reversible knockdown in the expression of a target gene with temporal and spatial specificity [5]. We have previously used this approach with CalB-AS to block phase shifts in response to light pulses [7].

Actograms were prepared for each animal covering about 7 days both preceding and following a manipulation. The phase of the locomotor activity rhythm was assessed visually by drawing a straight line through the onset of activity on successive days before the manipulation and again beginning about 3 days after the manipulation. A phase shift was assessed as the horizontal distance between the two lines the day following the manipulation. The differences in magnitude of phase shifts between treatments were analyzed by ANOVA followed by post-hoc Fisher's tests. All means are presented $\pm$ SEM.

To determine the effect of the experimental treatment on mRNA expression in the SCN, hamsters were given a third ventricle injection of *Per1*-AS at CT0. Control animals were given injections of *Per1*-S, CalB-AS or saline. They were sacrificed 4 h later by being given a lethal dose of pentobarbital (200 mg/kg), and were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were post-fixed overnight and then cryoprotected in 20% sucrose for 24 h. Frozen serial sections, 20–30 μ m thick, including the entire extent of the

SCN, were collected for in situ hybridization with digoxigenin (DIG) labeled cRNA probes using a free floating hybridization technique previously described [8]. Alternate sections were processed for *Per1* or vasopressin (VP) mRNA, and all groups were processed simultaneously.

Sections were photographed on Fuji 35 mm film and color prints were developed. For quantification of optical density, images of brain sections were captured using a CCD video camera (Sony XC77) attached to a light microscope (Olympus BH-2). mRNA expression was quantified by measuring stain density using the NIH Image program (NIH Image v1.61). Optical density measures, rather than cell counts, were used to assess changes in mRNA content as this approach is sensitive to changes in both the content of individual cells as well as the total number of cells expressing the mRNA.

Per1-AS injections produced non-photic-like phase shifts of locomotor activity rhythm (Fig. 1A,B). Injections during the subjective day produced significantly larger phase shifts than injections during the subjective night (Fig. 1A,B) ($F_{(3,10)} = 5.77$, $P < 0.05$). Significant phase shifts at CT6 were produced only by injections of *Per1*-AS (Fig. 1C) ($F_{(3,10)} = 24.638$, $P < 0.001$) and not by the various control substances injected (*Per1*-AS vs. saline, $P < 0.001$; vs. *Per1*-S, $P < 0.001$; vs. CalB-AS, $P < 0.001$).

Injections of *Per1*-AS at CT0 significantly decreased *Per1* mRNA expression in the VP region of the SCN (Fig. 2) ($F_{(2,8)} = 21.375$, $P < 0.0001$; *Per1*-AS vs. saline, $P < 0.001$; vs. *Per1*-S, $P < 0.001$). None of the treatments affected VP mRNA expression.

The present results demonstrate that the reduction in *Per1* following a non-photic treatment is the cause rather than merely a consequence of the phase shift. The results are consistent with previous studies that reported a decrease in *Period* gene expression following a variety of non-photic treatments [6,9,12,13,20]. While others have suggested that a decrease in *Per1* may be the mechanism underlying non-photic phase shifts [11], the present results are the first empirical demonstration of a causal link between such a decrease and the resulting phase shift. This phenomenon is specific to *Per1*, as silencing a gene involved in the photic pathway (i.e. calbindin [7]) was without effect.

Non-photic treatments produce small behavioral phase delays late in the subjective night [4], when *Per1* mRNA expression starts to increase [16]. Decreasing *Per1* at this stage of the cycle would delay its increase in expression and would result in the observed behavioral delay. By comparison, non-photic treatments produce large advances during the subjective day [4], when *Per1* mRNA expression is reaching its peak in the SCN [16]. Decreasing *Per1* at this stage of the cycle would result in an earlier trough in *Per1* expression, thereby allowing an advanced rise in expression during the next cycle. This would result in the observed behavioral advance. In fact, the amplitude of the non-photic phase response curve correlates with the amplitude of *Per1* expression as follows: larger phase shifts are observed at phases when *Per1* expression is at its highest, suggesting that the magnitude of the phase shift may be directly related to the magnitude of the suppression in *Per1* expression.

In the current study, *Per1*-AS produced large phase advances when administered at CT0 and CT6 and had little phase shifting effect when administered at CT12 and CT18. Behavioral treatments are usually without effect at CT0 [4], suggesting that the kinetics of AS treatment may be slower than occurs with direct activation of non-photic inputs to the SCN. In mice, lateral ventricular injections of *Per1*-AS produced large phase delays at CT1, no phase change at CT8, and small phase delays at both CT15 and CT21 [1]. The differences between our findings and those of Akiyama et al. [1] may reflect differences in methods. Antisense delivered to the lateral ventricle could affect more areas than just the SCN. Such widespread activity could have led to the result reported by Akiyama et al. [1]. Although the absolute

amount of antisense delivered in this study was similar to that delivered by Akiyama et al. [1] (7.5 and 6 nmol, respectively), mice are five to six times smaller than hamsters, making the dose/kg bodyweight proportionally much higher in mice, which could have contributed to the different effects observed. Alternatively, the differences observed between the current results and those of Akiyama et al. [1] may reflect differences in the properties of the circadian systems between mice and hamsters. Many studies have reported robust phase shifts of up to 180 min to non-photic stimuli in hamsters (reviewed in Ref. [15]). In mice there is only one report of non-photic phase shifts, and these shifts were small, averaging only about 40 min [10]. Furthermore, in vivo, 8-OH-DPAT produces non-photic phase shifts in hamsters [18] but not mice [3].

Numerous studies have reported that non-photic treatments decrease *Per2* expression as well as *Per1* [6,9,12,13,20]. The present study focused on *Per1* mRNA because non-photic manipulations decrease levels of this mRNA before they decrease *Per2* mRNA [6,20]. It is not known if, and entirely possible that, suppression of *Per2* with antisense would also produce non-photic phase shifts. Furthermore, it is possible that suppression of both *Per1* and *Per2* would result in a synergistic reaction leading to larger phase shifts. Such an approach has been used in investigating the role of *Per1* and *Per2* in photic phase shifts [19].

The present study demonstrates that a non-photic treatment has regionally specific effects on the expression of canonical clock gene. Furthermore, the change in the expression of this gene causes the resulting non-photic phase shift. Finally, antisense technology, when used with the proper control treatments, can be a powerful tool for behavioral neuroscience research.

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Fig. 1.

Per1-AS induces non-photic-like phase shifts of locomotor activity rhythms. (A) Actograms of locomotor activity in animals given third ventricle injections of *Per1*-AS (7.5 nmol) at CT0, 6, 12, 18, respectively. (B) Phase response curve for *Per1*-AS. (C) The effect of *Per1*-AS and control treatments on the locomotor activity rhythm administered at CT6. Values shown are mean $\pm$ SEM. *** $P < 0.001$, post-hoc Fisher's test. $n = 3$ –4 hamsters/group.

Fig. 2. *Per1*-AS treatment decreases *Per1* mRNA expression in the SCN. (A,B) Photomicrographs and quantification of *Per1* mRNA expression following third ventricular administration at CT0 of *Per1*-AS, *Per1*-S or saline. Animals were sacrificed at CT4 for DIG in situ hybridization 4 h after injections. Values shown are mean ± SEM. ****P* < 0.001, post-hoc Fisher's test. *n* = 3–5 hamsters/group.