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THE RESPONSE OF *PER1* TO LIGHT IN THE SUPRACHIASMATIC NUCLEUS OF THE DIURNAL DEGU (*OCTODON DEGUS*)

Jessica M. Koch¹, Megan H. Hagenauer², and Theresa M. Lee^{1,2}

¹Department of Psychology, University of Michigan, Ann Arbor, Michigan, USA

²Neuroscience Program, University of Michigan, Ann Arbor, Michigan, USA

Abstract

Several studies suggest that the circadian systems of diurnal mammals respond differently to daytime light than those of nocturnal mammals. We hypothesized that the photosensitive “clock” gene *Per1* would respond to light exposure during subjective day in the suprachiasmatic nucleus of the diurnal rodent, *Octodon degus*. Tissue was collected 1.5–2 h after a 30 min light pulse presented at five timepoints across the 24 h day and compared to controls maintained under conditions of constant darkness. *Per1* mRNA was quantified using in situ hybridization. Results showed that the rhythmicity and photic responsiveness of *Per1* in the degu resembles that of nocturnal animals.

Keywords

Diurnality; Chronotype; *Per1*; Photic; Day-active; Clock gene

Species evolved alternate chronotypes to compensate for time-dependent stressors, such as food availability, environmental temperature, and predation (DeCoursey, 2004). These chronotypes by definition are likely to experience different durations and intensities of daily light exposure. Diurnal, or day-active, animals are exposed regularly to bright daylight, and nocturnal, or night-active, animals frequently spend the daylight hours hidden or asleep. Therefore, we hypothesized that the photosensitive timekeeping system in the suprachiasmatic nucleus (SCN) of the hypothalamus would show adaptations related to chronotype (Hagenauer & Lee, 2008).

Behaviorally, there is support for this argument. Several authors have commented that diurnal species appear to have a greater duration of sensitivity to the circadian phase-shifting properties of light during the daytime (Challet, 2007; Hagenauer & Lee, 2008; Honma & Honma, 1999; Pohl, 1982; Smale et al., 2003). During the night, the circadian system responds to light by producing large phase shifts in behavioral rhythms. During the daytime, light produces little phase response in nocturnal animals for several hours. This midday segment of the phase response curve is referred to as the *dead zone* (Johnson, 1992). Diurnal species, including our model species, the *Octodon degus* (degu), arguably have a condensed dead zone (e.g., Hagenauer & Lee, 2008; Hut et al., 1999; Mahoney et al., 2001; Slotten et al., 2005; although also see Honma & Honma, 1999).

Physiological comparisons of the circadian photic pathway of diurnal and nocturnal species are relatively rare, but suggest dramatic differences between chronotypes (e.g., Jiao & Rusak, 2003; Jiao et al., 1999; Krajnak et al., 1997; Meijer et al., 1989). For example, unlike nocturnal mammals, both the diurnal degu and 13-lined ground squirrel show prevalent suppression of SCN firing rate in response to light (Jiao & Rusak, 2003; Jiao et al., 1999; Meijer et al., 1989). Some diurnal species, including the degu, also show an unusual responsiveness to light during the subjective day (the dead zone), as evidenced by measurements of FOS protein in the SCN (Abe et al., 1995; Krajnak et al., 1997; Schumann et al., 2006). FOS is the product of the immediate early gene *cFos*, and its photic induction closely correlates with behavioral phase shift in nocturnal species (as reviewed in Morin & Allen, 2006). Therefore, we tested the response to light in the SCN of another highly photosensitive gene, the “clock gene” *Per1*, in the diurnal degu.

Per1 expression also correlates with the behavioral phase response to light (Shigeyoshi et al., 1997). The *Per1* gene is rhythmically expressed in the SCN, and the phase of its rhythmic expression can be rapidly reset by light. The photic phase shift of the *Per1* gene expression is thought to underlie the photic phase shift of behavioral rhythms (Shigeyoshi et al., 1997). Light pulses shift both behavioral and *Per1* rhythms in an advancing manner in the morning and a delaying manner in the evening. Based on this observation, it was proposed that the behavioral dead zone could be attributed to *Per1* expression reaching peak levels during the midday and further photic induction being blocked (Hastings & Herzog, 2004; Maywood & Mrosovsky, 2001; Shigeyoshi et al., 1997).

In a recent study, we observed that the nocturnal rat exhibited a broader midday peak in the daily rhythm of *Per1* transcript in the SCN, while the diurnal *Octodon degus* (degu) showed a peak in *Per1* that quickly returned to lower levels of expression (Vosko et al., 2009). Therefore, we hypothesized that these differences in peak duration left the diurnal degu with a broader window of time during which *Per1* expression could be induced by light. To test this hypothesis, we compared *Per1* expression throughout the subjective day and night in degus that were kept in constant conditions or treated with a 30 min pulse of light.

METHODS

Animals and Housing

Adult male degus ($n = 53$) were obtained from the University of Michigan breeding colony. The animals were housed at a room temperature ($18^{\circ}\pm 1^{\circ}\text{C}$) in $48\times 26.8\times 20.3$ cm cages under a 12 h: 12 h light-dark (LD) cycle (250 lux, fluorescent room light) with food and water available ad libitum. All animal procedures were approved by the University Committee for the Care and Use of Animals at the University of Michigan and are in compliance with international ethical standards (Portaluppi et al., 2008).

Before use in the study, degus were screened for diurnality using activity monitored via infrared devices and evaluated using Vitalview and Actiview software (Minimitter Company, Inc.; Bend, Oregon, USA). Animals were considered to be diurnal if they had a LD activity ratio >1 , intermediate chronotype if they had an LD activity ratio >0.8 and <1 (due to prominent crepuscular activity), and nocturnal if the LD activity ratio was <0.8 .

Degus were randomly separated into two groups, a light-pulsed group (LP) and a constant darkness control group (DD). Both LP and DD degus were placed into constant darkness for 24–48 h (Aschoff II methodology). Degus in the LP group received a 30 min light pulse (250 lux fluorescent room light) beginning at one of the following times (ZT, extended from the previous LD cycle): 4, 8, 12, 16, or 22 (lights-on corresponding to ZT = 0). Following the light pulse, degus were returned to darkness until sacrifice, which occurred between 1.5–

2 h after the beginning of the light pulse. The DD degus remained in constant darkness until the designated time of sacrifice. Therefore, both the LP and DD degus were sacrificed via decapitation within 30 min of the following timepoints: ZT (extended) 6, 10, 14, 18, and 24. At the time of sacrifice, animals were rapidly anesthetized with isoflurane prior to decapitation. Brains were collected and flash frozen in acetone cooled with dry ice (20–30 s). Tissue was sliced into four series of 16 μ m coronal sections via cryostat. One of these series was utilized for this study, and three of the series were partially utilized for another study (Vosko et al., 2009). Tissue was stored at -80°C until processed for in situ hybridization.

In situ Hybridization

Similar to the methodology used by Vosko et al. (2008), an anti-sense degu *Per1* mRNA probe was used to label the SCN of one series of sliced tissue per animal. Plasmids containing the degu *Per1* fragment (pGEMT) (892 bp, GenBank accession no. EU715821, courtesy of Drs. Jeremiah Shepard and Steven McKnight, Southwest Medical School, Texas, USA) were purified with a QIAprep Spin Miniprep Kit. Plasmids were digested with restriction enzymes, purified, and evaluated for complete linearization with gel electrophoresis.

Radioactive riboprobes were generated from a solution of linearized DNA, DNA polymerase, appropriate transcription buffer, nucleotides, and radioactively labeled nucleotides ^{35}S -UTP and ^{35}S -CTP (GE Health-care, Piscataway, New Jersey, USA; MPBiomedicals, Solon, Ohio, USA). This solution was incubated for 1.5 h in a 37°C water bath. Probes were treated with DNase and purified with Micro Bio-Spin Chromatography Columns (Bio-Rad Laboratories, Hercules, California, USA).

Slides containing four 16 μ m coronal brain sections were fixed in 4% paraformaldehyde for 1 h, washed three times in $2\times$ SSC solution, and placed in 0.1M TEA before being washed in dH_2O . Slides were dehydrated in graded alcohols and left to dry. Hybridization solution containing enough radioactively labeled probe to ensure 3×10^6 cpm per μL was applied to each slide and coverslipped. Labeled slides were incubated in a 55°C oven overnight in boxes containing 50 ml of 50% formamide. Following incubation, cover slips were removed in a $2\times$ SSC solution, rinsed three times in $2\times$ SSC, and incubated at 37°C in RNase solution for 1 h. Slides were washed and incubated at 65°C in $0.1\times$ SSC for 1 h before being dehydrated through graded alcohols and left to dry.

Biomax film (Kodak, Rochester, New York, USA) was exposed to the radioactive slides overnight and digitized with Microtek ScanMaker 1000XL (Microtek, Cerritos, California, USA) and SilverFast Ai software (Lasersoft Imaging Inc., Sarasota, Florida, USA). As a control, a probe containing sense degu *Per1* mRNA was used to label the SCN. The results of this control are published as part of another study (Vosko et al., 2009).

The images were subsequently analyzed using Scion Image to quantify the intensity (optical density [OD] of grayscale units) and area of labeling in the SCN. Using the corpus callosum as background threshold, the area of the SCN containing labeling with an intensity surpassing 2.0 standard deviations above the threshold was measured as labeled area. This labeled area was then multiplied by its OD and divided by the total area of the SCN to get a measurement of OD/unit area or integrated optical density (IOD). Measurements were taken bilaterally from two mid-SCN slices representative of each degu by an experimenter blind to group identity. The location of the SCN was identified using ventral landmarks (e.g., optic chiasm, third ventricle) in reference to previous anatomical characterization performed in our lab (Goel et al., 1999). Any tissue with damage to the ventral hypothalamus was discarded.

Statistical Analyses

A one-way multivariate ANOVA was used to examine SCN tissue from degus kept in DD for evidence of rhythmicity in *Per1* IOD, labeled area, and OD in the SCN across the five timepoints. A 2×5 multivariate ANOVA was used to examine the effect of treatment (LP vs. DD) on *Per1* IOD, labeled area, and OD across the five timepoints in the degu SCN. *Post-hoc* independent *t*-tests (Sidak corrected for multiple comparisons) compared LP and DD tissue at each timepoint. Planned contrasts (Student's *t*-test) were used to determine whether there was generally photic induction of *Per1* IOD, labeled area, and OD in the SCN (LP vs. DD tissue) during the subjective day (extended ZT6 and ZT10) or subjective night (extended ZT14, ZT18, and ZT24), the term *extended* referring to the previous LD cycle.

RESULTS

Pre-screening of locomotor rhythms indicated an average diurnality score (L/D ratio) of 1.48 (± 0.11 SE). Of these animals, 80% could be strongly classified as a diurnal chronotype (L/D activity ratio >1), 12% fit an intermediate chronotype (L/D activity ratio <1 but >0.8), and 8% were nocturnal (L/D activity ratio <0.8). Only degus with a diurnal or intermediate chronotype were included in the rest of the study (see Figure 1A).

Tissue from the SCN of degus kept under constant conditions (DD, $n = 17$, 3–4 degus per timepoint) showed daily rhythms in *Per1* IOD ($F(4,12) = 7.167$, $p = 0.003$) and OD ($F(4,12) = 6.368$, $p = 0.005$), with a peak occurring at extended ZT6 and trough at ZT18. The labeled area was also significantly rhythmic ($F(4,12) = 6.270$, $p = 0.005$), but showed a peak at extended ZT10 and trough at ZT18. All three measures indicated that *Per1* transcript levels were elevated above 50% of peak for 8 h or longer during the day.

When compared to the DD tissue, LP tissue at most timepoints showed elevated levels of *Per1* transcript in the SCN (LP $n = 19$, 3–4 degus per timepoint; see Figure 1B–1E). Therefore, a significant main effect of treatment was found for OD ($F(1, 26) = 8.730$, $p = 0.007$), IOD ($F(1, 26) = 4.886$, $p = 0.036$), and labeled area ($F(1,26) = 11.531$, $p = 0.002$).

Post-hoc independent *t*-tests, which compared the treatment groups at each timepoint, did not reveal significant differences after correcting for multiple comparisons ($p > 0.05$), but planned contrasts indicated that an induction of *Per1* occurred in response to light pulses presented during the subjective night (IOD: $t = 4.212$, $p < 0.001$; OD: $t = 4.372$, $p < 0.001$; labeled area: $t = 3.224$, $p = 0.004$) but not during the subjective day (IOD: $t = 0.944$, $p = 0.364$; OD: $t = 0.464$, $p = 0.651$; labeled area: $t = 0.899$, $p = 0.387$). Therefore, there was also a significant interaction between the effects of treatment and time on *Per1* OD ($F(4, 26) = 2.947$, $p = 0.039$) and IOD ($F(4, 26) = 4.499$, $p = 0.007$), although not labeled area ($F(4, 26) = 1.682$, $p = 0.184$).

DISCUSSION

This is the first study to analyze the effects of a light pulse on the transcription of *Per1* mRNA in the SCN of the useful diurnal laboratory species, the degu. In accordance with our previous study, we found that degus kept in DD displayed rhythmic expression of *Per1* with increased transcription during the subjective day and decreased transcription throughout the subjective night (Vosko et al., 2009). This expression pattern resembles that of other nocturnal and diurnal species (e.g., Caldelas et al., 2003; Mrosovsky et al., 2001; Novak et al., 2003; Shigeyoshi et al., 1997). In contrast to our previous study (Vosko et al., 2009), we did not find that the peak in *Per1* expression was of shorter duration than that typically found in nocturnal species. This discrepancy is most likely due to the fewer timepoints sampled in the current study (5 vs. 12).

With respect to the light pulse data, we originally hypothesized that the behavioral dead zone of the nocturnal rat is due to the broad peak of *Per1* expression occurring throughout the midday, thereby preventing any further induction of *Per1* transcription (Shigeyoshi et al., 1997). Based on this assumption, we predicted that the relative lack of a dead zone in the diurnal degu would be reflected by increased responsiveness of *Per1* expression during the late subjective day (Hagenauer & Lee, 2008). In contrast to this hypothesis, we saw no elevation of *Per1* mRNA levels following a light pulse in tissue collected at extended ZT6 or ZT10, whereas during the subjective night, degus responded to light pulses in a manner similar to nocturnal species, showing reliable *Per1* induction. These results resemble those from the diurnal *Arvicanthis ansorgei* (Caldelas et al., 2003) and *Arvicanthis niloticus* (Novak et al., 2006; Ramanathan et al., 2009) and suggest that diurnal and nocturnal mammals are relatively equivalent at this level of the circadian photic pathway.

These results are provocative when one takes into account the findings of a recent study using the diurnal *Arvicanthis niloticus* (Ramanathan et al., 2009), which similarly indicated a lack of *Per1* or *Per2* induction in response to light exposure during the subjective day at a timepoint (ZT4) when this species would normally be sensitive to behavioral photic phase shift (Mahoney et al., 2001). Ramanathan et al. (2009) confirmed that the observed lack of *Per1* and *Per2* induction at ZT4 was not due to a different time course of induction by sampling at three intervals (30, 90, and 140 min) following the light pulse. They also characterized *Per1* and *Per2* induction by subregion (core vs. shell) within the SCN, because previous studies indicated that photic information in mammals propagates from the retinally innervated ventrolateral SCN (“core”) to the endogenously rhythmic dorsomedial SCN (“shell”) (Antle & Silver, 2005; Hastings & Herzog, 2004). It is believed that a “gating effect” allows this photic input to spread to the shell only during the night and not the day (Antle & Silver, 2005; Hastings & Herzog, 2004). However, in the *Arvicanthis niloticus*, a region-specific, time-lapsed evaluation indicated that a gating effect is not responsible for the lack of *Per1* induction following a light pulse at ZT4, as neither the core nor shell was responsive to light at this timepoint (Ramanathan et al., 2009).

Our data similarly show a lack of photic induction of *Per1* transcript following light exposure during the subjective day in the diurnal degu. We have not confirmed whether this result is due to a gating effect using a time-lapsed analysis with stronger anatomical resolution (e.g., digoxigenin-labeled mRNA and microscopy), but taken in tandem with the results of the *Arvicanthis* study (Ramanathan et al., 2009), our data indicate that future research on chronotypical differences in the circadian photic pathway should focus downstream of the *Per1* transcript. For example, recent research suggests that post-translational mechanisms may play a role in mammalian circadian entrainment (Jakubcakova et al., 2007). Therefore, a logical next step may be to examine the stability, intracellular location, and phosphorylation of PER protein in response to light exposure across the day in diurnal species.

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FIGURE 1.

The expression of *Per1* within the SCN of degus kept under conditions of constant darkness (DD) or 1.5–2 h following the beginning of a 30 min light pulse. (A) Sample actogram produced during the pre-screening of degus for diurnal chronotype. Three days of locomotor activity are illustrated, as quantified by the number of times the degu passed under an infrared beam during each 10 min bin. For each day, the y-axis shows activity level (0–50 beam crossings) and the x-axis shows clock time, with a light-dark bar denoting the 12 h: 12 h LD cycle. This degu was classified as diurnal because the majority of its activity occurs during the lighted period of the LD cycle (L:06:00 to 18:00 h). (B) Representative autoradiographs showing *Per1* labeling in coronal slices of the degu brain. Both brains come from degus sacrificed during the night at ZT18. The upper autoradiograph shows a lack of *Per1* labeling in the SCN of a degu kept in DD, whereas the lower autoradiograph shows the induction of *Per1* following a 30 min light pulse presented at ZT16 (arrow). (C–E) *Per1* is expressed rhythmically in the SCN across the 24 h day under DD. Light exposure induces *Per1* transcription during the subjective night (ZT12–ZT24). This pattern is observed when quantifying the area of the SCN labeled using antisense *Per1* probe (labeled area, C), the intensity of *Per1* labeling (optical density, D), and a measurement of *Per1* labeling that integrates labeling area and intensity (integrated optical density or IOD, E). In each graph, the x-axis indicates the timepoint when the animal was sacrificed (extended from the previous LD cycle). Error bars represent standard error from the mean.