

CHRONOBIOLOGY INTERNATIONAL, 19(4), 671–682 (2002)

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8 **THREE DAYS OF NOVEL WHEEL ACCESS**  
9 **DIMINISHES LIGHT-INDUCED PHASE DELAYS IN**  
10 **VIVO WITH NO EFFECT ON *PER1* INDUCTION BY**  
11 **LIGHT**  
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20 **ABSTRACT**  
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22 The mammalian circadian clock, located in the hypothalamic suprachiasmatic  
23 nuclei, synchronizes endogenous behavioral and physiological rhythms to a  
24 24h period through responses to two types of stimuli: photic (light) and  
25 nonphotic (behaviorally induced arousal and/or increases in activity). Photic  
26 stimuli can block nonphotic effects and vice versa, although the mechanisms  
27 and levels of interactions between these two stimuli types are unknown. Here,  
28 we investigated whether 3 d of access to a novel running wheel alters the phase  
29 shift to light in vivo, and whether this effect could be seen on induction by  
30 light of the circadian gene *per1*. Through measurement of running wheel  
31 activity of golden hamsters, access to a new wheel for 3 d was shown to  
32 diminish photic phase delays with no effect on phase advances. As seen using  
33 in situ hybridization, however, there was no effect on levels of light-induced  
34 *per1* mRNA. This study indicates a possible role for this paradigm as a  
35 model of interactions between photic and nonphotic stimuli. (*Chronobiology*  
36 *International*, 19(4), 671–682, 2002)

37 **Key Words:** Circadian rhythm; Light; Locomotor activity; Novel wheel  
38 running; *Per*-induction; Phase response curve; Suprachiasmatic nucleus  
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## INTRODUCTION

The hypothalamic suprachiasmatic nuclei (SCN) generate and regulate mammalian circadian rhythms. This endogenous mechanism oscillates with a period near 24 h under constant, or free-running conditions, and entrains to the environmental 24h cycle through responses to two types of stimuli: photic (light) and nonphotic (behavioral manipulation). A nonphotic stimulus includes various behaviorally arousing stimuli such as exposure to novel environments, cage changes, and social interactions, although the most extensively characterized stimulus involves changes in locomotor activity (for review, see Ref. [1]). Either through forcing an animal to run in a wheel or by the presentation of a novel wheel, locomotor activity appears to have an influence that can be just as great as that of light on the circadian clock, though the phasing of photic and nonphotic effects differs. The photic phase response curve (PRC) for hamsters shows relatively small phase delays in the early subjective night and large phase advances later in the night.<sup>[2]</sup> Nonphotic stimuli, conversely, produce relatively large phase advances during the subjective day and smaller delays late in the night.<sup>[1]</sup>

Light input is thought to exert its effect on the clock by activating certain clock genes at times when the products of those genes are low. During the subjective night, light pulses of 5–30 min rapidly induce expression of *per1*, peaking within 30–60 min and returning to baseline levels after approximately 3 h.<sup>[3]</sup> Increases in activity, on the other hand, are thought to down-regulate acutely these genes at a time when the gene products are high (i.e., at midday).<sup>[4]</sup>

Circadian responses to light vary depending on the environmental situation surrounding the photic stimulus. For example, in the late subjective night, when light normally produces a phase advance, simultaneous confinement to a novel wheel with presentation of a light pulse greatly attenuates the photic phase shift.<sup>[5]</sup> In addition, phase shifts increase with extended time in constant conditions.<sup>[6]</sup>

In the course of another study, we noticed that changing a hamster from housing in a cage without a wheel to a cage with a wheel appeared to alter the phase shift to light. Here we describe this effect, show that it is separable from an effect of similar magnitude related to time under constant conditions, and begin to examine the underlying mechanism by measuring light-induction of *per1*. We hypothesized that if *per1* is a target gene in this process, a smaller degree of phase shifting seen in vivo would be correlated with lower amounts of photic induction of mRNA.

## MATERIALS AND METHODS

### Animals and Tissue Preparation

Male golden hamsters (LVG, Charles River,  $n = 62$ ) were supplied at 21 d of age. They were entrained to an LD 14h:10h cycle for 1–2 mon in our laboratory

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89 and housed without access to running wheels, then were transferred to constant  
90 dim red light (DRL) ( $1.8 \times 10^{-3}$  lux,  $6 \times 10^{-2}$   $\mu\text{W}/\text{cm}^2$ ) at *Zeitgeber* Time (ZT)  
91 12 and housed individually in cages equipped with a running wheel (17.5 cm  
92 diameter) and food and water available ad libitum. *Zeitgeber* time was defined as  
93 ZT 12 being the projected time of lights off in the animal room.

94 For behavioral studies, a 5min light pulse (2000 lux,  $20 \times 10^3$   $\mu\text{W}/\text{cm}^2$ ,  
95 q1 Apollo Slide EXR Projector Lamp) was given 3 d later at various ZTs, covering  
96 the entire 24h period within the group of animals. After 2 wk following the first  
97 light pulses, a second set of light pulses were given, followed by two more weeks  
98 in DRL. The animals were then re-entrained to the LD 14h:10h cycle for 3 wk  
99 before returning to DRL for 3 d, followed by a third set of light pulses. Wheel  
100 revolutions were detected by a magnet attached to the wheel and a magnetic  
101 contact sensor (Sentrol, type 1085T, Tualatin, OR) attached to the roof of the cage,  
102 connected to a data collection and acquisition system (Clocklab, Actimetrics Inc.,  
103 Evanston, IL).

104 For in situ hybridization studies, animals were housed in cages either with  
105 ( $n = 11$ ) or without ( $n = 7$ ) a running wheel. All animals were housed with  
106 food and water available ad libitum. Of the animals housed with a running  
107 wheel, seven had access to the wheel for 3 d while four remained on the wheels  
108 for 18 d. At ZT 14, some animals received a 5min light pulse (2000 lux,  
109  $20 \times 10^3$   $\mu\text{W}/\text{cm}^2$ , Apollo Slide EXR Projector Lamp). At ZT 15, all animals  
110 were killed under DRL by overdose with halothane anesthesia and decapitation.  
111 Brains were removed and stored at  $-70^\circ\text{C}$  until cryostat sectioning of 20  $\mu\text{m}$ -  
112 sections.

### In Situ Hybridization

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117 [ $^{35}\text{S}$ ]-UTP-labeled cRNA probes were transcribed from an  $\sim 800$  bp  
118 (GenBank No. AF 249882, approx. nt320–1120) Syrian hamster *per1* cDNA  
119 fragment cloned into pBluescript II KS(–) (Stratagene) created by Drs. Lowrey  
120 and Takahashi (Northwestern University). The *haPer1* plasmid was linearized  
121 with *ApaI* or *BstXI* and transcribed with T7 or T3 RNA polymerase for antisense  
122 and sense probes, respectively.

123 All in situ procedures were adapted single-label versions of a dual-label  
124 protocol developed by Drs. Petersen, Lubbers, and Curran at the University of  
125 Massachusetts at Amherst. When all brains were sectioned, slides were thawed  
126 and fixed using 4% formalin in  $10 \times$  phosphate buffered saline (PBS), followed  
127 by rinsing with  $2 \times$  saline sodium citrate (SSC). The slides were then  
128 acetylated in 0.25% triethanolamine HCl/acetic anhydride and rinsed again in  
129  $2 \times$  SSC. The slides were dehydrated through a series of alcohol washes  
130 followed by a rinse in chloroform for delipidation and rehydrated through two  
131 alcohol rinses. Slides were air dried and stored in foil-covered slide racks until  
132 hybridization.

133 The antisense transcript was applied to slides at a rate of  $1 \times 10^6$  dpm/25  $\mu$ L  
134 of hybridization buffer (0.02 g Na pyrophosphate, 2 g dextran sulfate, 10 mL  
135 formamide, 2 mL  $20 \times$  SSC, 200  $\mu$ L  $100 \times$  Denhardt's solution, 10 mg tRNA,  
136 1760 USP units heparin sodium salt, 8 mg single-stranded sheared salmon sperm  
137 DNA, increased to 20 mL with nuclease-free H<sub>2</sub>O). The slides were coverslipped  
138 and slices were incubated overnight at 55°C.

139 Coverslips were removed using washes in  $1 \times$  SSC, and slices were  
140 incubated in 50% formamide/ $2 \times$  SSC at 52°C in a water bath. The slices were  
141 then washed twice in  $2 \times$  SSC followed by incubation in RNase buffer (0.5 M  
142 NaCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 50  $\mu$ g/mL RNaseA1) at 37°C in a  
143 shaking water bath. They were washed again in  $2 \times$  SSC and incubated for 5 min  
144 in 50% formamide/ $2 \times$  SSC at 52°C in a water bath. A series of 3min alcohol  
145 washes for dehydration were followed by a quick rinse in distilled water and a final  
146 rinse in 70% EtOH/dH<sub>2</sub>O. Air-dried slides were moved to autoradiography  
147 cassettes and exposed to Kodak Bio-Max Maximum Resolution film for at least  
148 2 d. The film was developed using Kodak GBX developer and Kodak fixer.

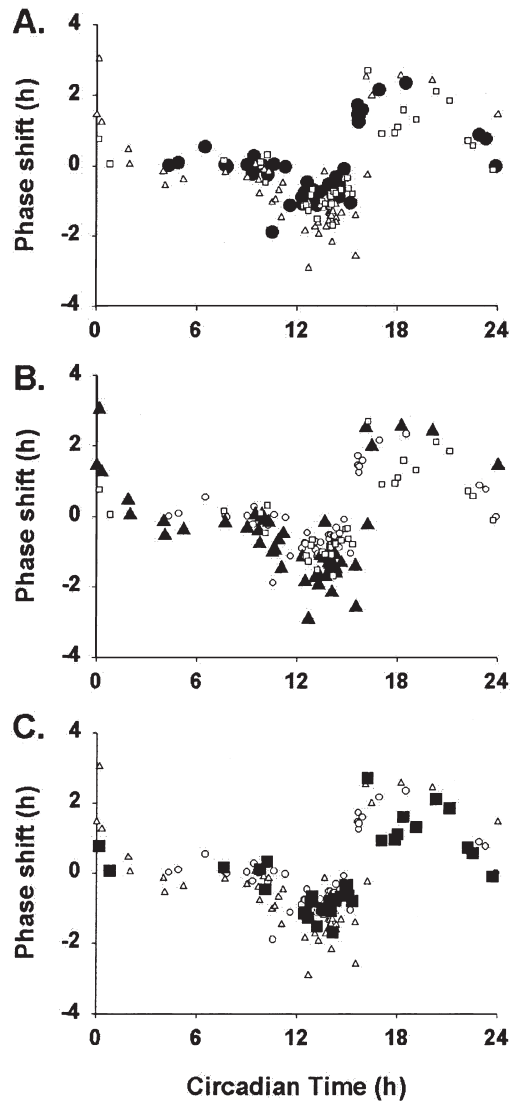
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151 **Q2**  
**Q1** **Data Analysis**

153 For behavioral studies, least-squares regression lines were fit to the activity  
154 onsets for the 3 d preceding light treatment and the 4 d following light treatment  
155 (for phase delays) or the 4–7 d following the light pulse to allow 3 d for transients  
156 (for phase advances). Phase shifts were determined by the difference in hours  
157 between the predicted onset from the first line and the predicted onset from the  
158 second line for the day following the light pulse. Onsets and offsets of activity are  
159 defined by the ClockLab program using a template matching algorithm to find a 6h  
160 period of inactivity followed by a 6h period of high-activity. The duration of  
161 activity was calculated as the time between onset and offset of activity on the day  
162 prior to light pulse administration. One-way analyses of variance (ANOVA) using  
163 SigmaStat software tested for differences among treatment conditions. Data are  
164 presented as means  $\pm$  SEM.

165 For in situ studies, slice images were scanned onto a Dell OptiPlex G110  
166 **Q1** using an Epson Expression 1600 scanner and Adobe Photoshop software. Images  
167 were quantified using Scion's PC version of NIH Image software, using a  
168 customized 2-Standard Deviation macro to determine the mean optical density  
169 above threshold in the SCN area. The threshold was selected from a standardized  
170 area to the right of the SCN and the perimeter of the SCN area quantified was  
171 individually determined in comparison to Toluidine blue-stained slices viewed  
172 under a microscope. The mean optical density for each subject was the average  
173 over all SCN-containing sections from one animal (average number of  
174 sections = 20), and that number was incorporated in the group mean. A one-  
175 way between groups ANOVA using SPSS software tested for differences among  
176 groups.

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**Figure 1.** Phase response curves (PRCs) obtained from animals housed under different environmental conditions. Individual points represent phase shifts measured from animal exposed to a 5min light pulse at a particular circadian time. The data shown on all three panels are the same, with different groups highlighted to allow comparison. (A) Hamsters given 3 d of DRL and their first exposure to a running wheel (circles) show reduced delays relative to other groups. (B) Hamsters housed under DRL for 2 wk following the first light pulse show increased delays (triangles). (C) Hamsters re-entrained to LD 14h:10h and then exposed to light pulses after 3 d of DRL show intermediate delays (squares). Negative values for phase shifts indicate phase delays.

## RESULTS

## In Vivo

Figure 1 shows the PRCs under the various experimental conditions. The data shown on all three panels of Fig. 1 are identical, with different groups highlighted to allow comparison. As is apparent from inspection of Fig. 1, the general shape of the PRC seemed similar across the groups; in this study we focus largely on the delay zone. Figure 2A and B represent the phase delays obtained in each condition from light pulses given between circadian time (CT) 13.5 and 14.5 (CT 12 is defined as the start time of the animal's daily activity period). After 3 d of wheel access in DRL, the average phase delay to light pulses given between CT 13.5 and 14.5 was  $0.57 \pm 0.06$  h ( $n = 7$ ) while phase advances in the late subjective night, from CT 16 to 18.5, were  $2.03 \pm 0.23$  h ( $n = 3$ ). After two more weeks in DRL, hamsters did not show larger phase advances to light in the late subjective night ( $1.71 \pm 0.66$  h,  $n = 4$ ; n.s.), but did show larger phase delays in the early subjective night ( $1.33 \pm 0.11$  h,  $n = 15$ ;  $p < 0.01$ ). After 3 d in DRL following re-entrainment to an LD 14h:10h cycle, the average phase delay induced was  $0.98 \pm 0.09$  h ( $n = 9$ ), which was significantly different from both the first treatment ( $p < 0.01$ ) and the second ( $p < 0.05$ ) condition. The average phase advance following the third light pulse was  $1.46 \pm 0.34$  h ( $n = 5$ ). One-way ANOVAs showed there was no difference among treatment conditions for phase advances ( $F = 0.351$ , n.s.) but there was for the delays ( $F = 11.1$ ,  $p < 0.05$ ). The mean number of revolutions on the day prior to the light pulses between ZT 13.5 and 14.5 were not different ( $13,302 \pm 2455$ ,  $n = 7$ ;  $12,121 \pm 1409$ ,  $n = 15$ ;  $9590 \pm 1488$ ,  $n = 9$ ;  $F = 1.02$ , n.s.) and the duration of activity on the day prior

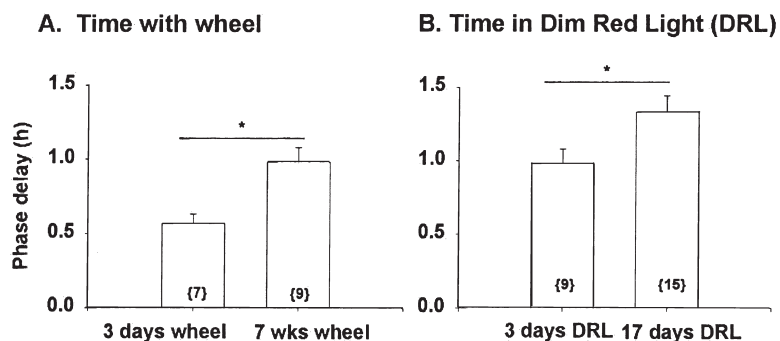
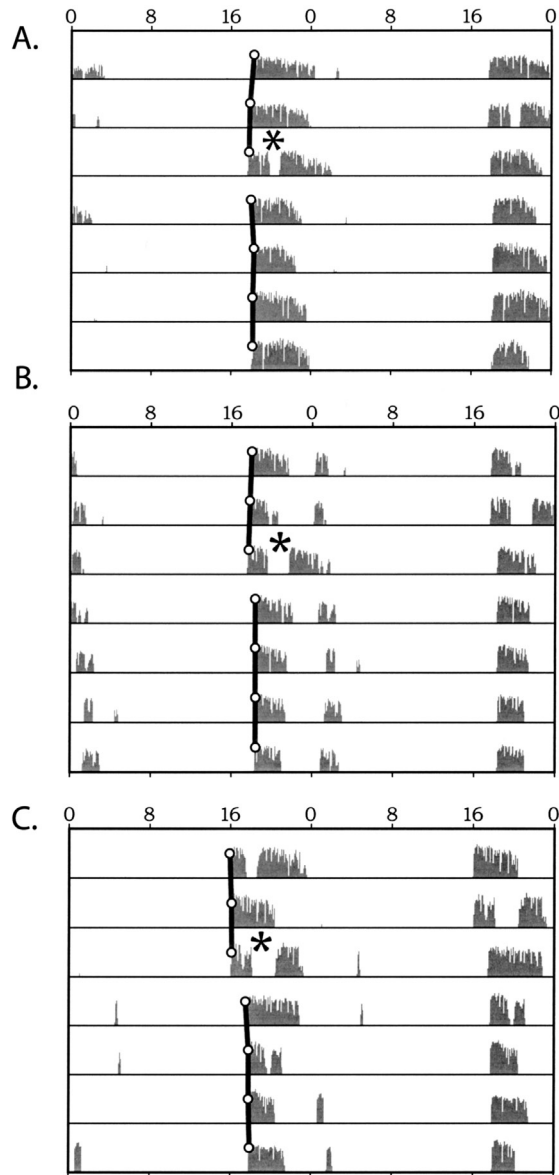


Figure 2. Phase delays induced by a 5min light pulse between CT 13.5 and 14.5. Data are means  $\pm$  SEM. (A) The reduction in phase shift magnitude associated with first introduction of a running wheel, with both conditions involving 3 d of pre-treatment with DRL following entrainment to LD 14h:10h. (B) The increase in phase delay associated with extended time in DR, comparing both conditions with extended experience with the running wheel (7 wk with the wheel for the "3d DRL" group, 17 d with the wheel for the "17d DRL" group). All treatments are significantly different from each other ( $p < 0.05$ ).

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**Figure 3.** Actograms for animals in the three conditions shown in Fig. 2. Phase delays induced by a 5min light pulse between CT 13.5 and 14.5 administered on the day marked by an asterisk. (a) The phase shift associated with a light pulse given 3 d following the first introduction of a running wheel. Hamsters were given 3 d of pre-treatment with DRL following prior entrainment to LD 14h:10h. (b) The phase shift associated with a light pulse administered after 3 d in DRL following entrainment to LD 14h:10h, in an animal housed with a wheel for 7 wk. (c) The phase delay associated with a light pulse administered after 17 d of DRL in a hamster housed with a wheel for 17 d.

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to the light pulse was not different ( $6.44 \pm 0.9$ ,  $n = 7$ ;  $5.04 \pm 0.5$ ,  $n = 15$ ;  $5.88 \pm 0.9$ ,  $n = 9$ ;  $F = 0.96$ , n.s.). Representative actograms are shown in Fig. 3.

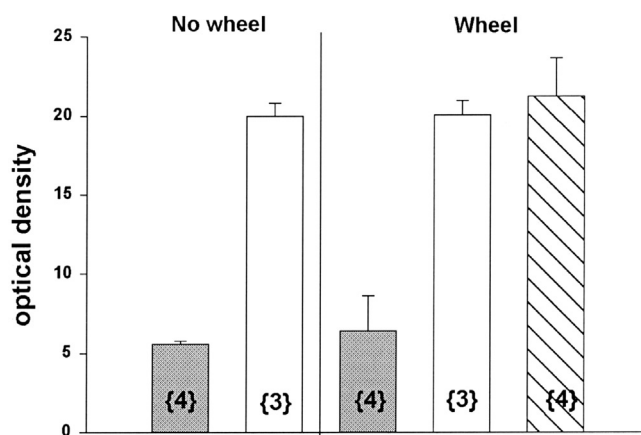
### F3 In Situ

Comparison between light-treated groups and nonlight-treated groups with a one-way ANOVA showed a significant effect ( $F = 22.7$ ,  $p < 0.01$ ). The light-treated groups, however, were not significantly different from each other. Mean optical density values among groups are shown in Fig. 4, and representative slices (film radiography and Toluidine blue stain images) are shown in Fig. 5.

### F5 DISCUSSION

The first presentation of a running wheel to a naive hamster has a potent effect on the magnitude of phase delay to a light pulse in early subjective night. As we suspected from previous research, the phase delay to light increases with the length of time under DRL, an effect shown here to be additive to the effect of the new wheel. Our experiments were inconclusive as to the underlying mechanism of these effects, suggesting only that light induction of *per1* mRNA is not obviously different across these conditions.

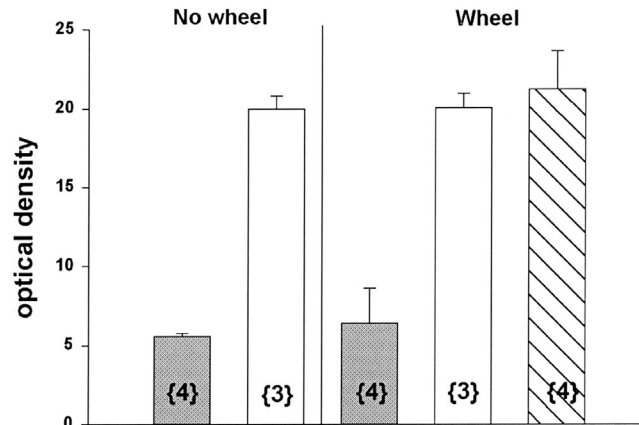
The increased phase delay seen here following two more weeks in DRL, comparable to previously reported shifts,<sup>[2]</sup> confirms previous results showing that



**Figure 4.** Means  $\pm$  SEM optical density of *per1* mRNA levels observed in five treatment groups. Light-treated groups are statistically significant from nonlight-treated groups ( $F = 22.7$ ,  $p < 0.01$ ), but there is no significant difference in optical density of label in SCN among light-treated groups in different conditions. The number of subjects in each group is designated on the bars. Gray bars indicate groups that did not receive a light pulse while white bars indicate those that did. Hatched white bar indicates group that had 18 d of wheel access; all other groups had either no running wheel ("No Wheel") or 3 d of wheel access.

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**Figure 5.** Representative in situ hybridization film images from slices labeled with antisense transcript for *haPer1* mRNA. There is a high expression of *haPer1* in the SCN in light-treated subjects. Toluidine blue-stain images are provided for comparison.

the amplitude of phase delays to light increases with time in DD, both in mice<sup>[7]</sup> and hamsters.<sup>[6,8]</sup> The much larger phase delays after extended time in DD seen in *tau* mutant hamsters has been shown to closely parallel an increase in the duration of activity or *alpha*.<sup>[6]</sup> Oscillator amplitude may become damped in constant conditions, subsequently increasing phase shift amplitude.<sup>[7]</sup>

The finding that the magnitude of phase delay induced after 3 d with a novel wheel is significantly different from that induced after 3 d in DRL indicates that 3 d of novel wheel access constitutes a nonphotic stimulus strong enough to diminish a photic response. Phase advances induced by light in the late subjective night can be attenuated by activity simultaneous to light presentation, although phase delays are not affected.<sup>[5]</sup> Phase delays to light in mice depend on the size of the running wheel.<sup>[9]</sup> Here, we see diminished delays but not advances, a result similar to that seen in comparisons of time in DD. This could indicate that this effect is not mediated by the same pathway but a different one instead, perhaps that involved in larger phase shift magnitude following extended time in constant conditions. Continuous access to a running wheel has been shown to affect circadian rhythms in a way that indicates a form of feedback on the circadian clock. For example, exposure to a running wheel has been shown to shorten free-running periods in hamsters<sup>[10]</sup> and rats.<sup>[11]</sup> Theoretically, in order to entrain to a 24h cycle, a shortening of the free-running period would necessitate a greater phase delay shift to the entraining stimulus. In fact, it is therefore puzzling to see a smaller phase delay in this paradigm of continuous wheel access. We do not know, however, whether the free-running period was affected in this particular experimental design, as introduction of a running wheel and transfer to constant conditions is simultaneous. Perhaps measuring activity using infrared motion detectors in constant conditions for several days followed by subsequent re-entrainment to an

397 LD cycle prior to introducing a running wheel would allow for measurement of a  
398 baseline free-running period from which to measure variation.

399 The number of wheel revolutions on the day prior to the light pulse was not  
400 significantly different across treatment conditions, suggesting that the overall  
401 amount of activity did not vary. In addition, there was no difference in duration of  
402 activity (*alpha*). Activity was not measured, however, while the animals were still  
403 housed in the colony room without running wheels. It would therefore be  
404 beneficial to do so in order to quantify any increase in amount or duration of  
405 activity associated with introduction of a running wheel.

406 Three days of access to a new wheel and 3 d in DRL negatively influence the  
407 phase shift response to light in an additive manner; the difference between the  
408 phase shift induced after 3 d in DRL following re-entrainment and the phase shift  
409 after 3 d with a new wheel is approximately equal to the difference between 2 wk  
410 in DRL and 3 d in DRL. Novelty-induced running in the mid-subjective day has a  
411 nonadditive effect on phase shifts to a 1h light pulse in the following subjective  
412 night.<sup>[12]</sup> Perhaps, a difference in paradigms (an acute stimulus of activity in the  
413 mid-subjective day vs. 3 d of continuous wheel access) influences whether  
414 interactions with photic stimuli occur in an additive or nonadditive manner. Here,  
415 we do not see that the photic PRC has been shifted, but rather that its amplitude has  
416 been dampened and/or enhanced, depending on the housing condition.

417 Absence of a statistical difference among the light-treated groups in the in  
418 situ hybridization studies indicates that neither 3 d with a novel wheel nor 3 d in  
419 DRL constitute a behavioral stimulus that acts on the molecular pacemaker by  
420 inhibiting the induction of *per1* by light, at least as measured 1 h after the light  
421 pulse. It is possible that the stimulus of 3 d of novel wheel access works through  
422 another gene in the circadian oscillator. In fact, previous experiments have  
423 indicated that *per2* may be more involved in nonphotic influences on photic phase  
424 shifting.<sup>[13]</sup> On the other hand, it is possible that the mechanisms by which a  
425 nonphotic stimulus down-regulates a photic response depend on the properties of  
426 the nonphotic stimulus itself.

427 In mice, behavioral resetting by light has been shown to be strongly  
428 correlated with *mPer1* induction. Both light-induced clock resetting and *mPer1*  
429 induction are directly proportional and have similar thresholds of light intensity  
430 for response.<sup>[14]</sup> Here, the change in magnitude of phase shift to light after 3 d and  
431 light 2 wk later was not reflected by changes in induction of *per1* by light. There  
432 may be species differences in the regulatory pathways, leading to differences in  
433 correlations between *per1* induction by light and photic phase shifting. On the  
434 other hand, our methods of using a rather bright light stimulus may have precluded  
435 our ability to determine differences in induction levels. Further experiments using  
436 a lower light stimulus may therefore be useful. It is noteworthy that a  
437 benzodiazepine, brotizolam, reduces phase advances but not delays to light;<sup>[15]</sup>  
438 this effect is accompanied by a decrease in both light-induced *per1* and *per2*.  
439 Studies of *per1* and *per2* mutant mice indicate *per2* but not *per1* is critical for  
440 phase delays to light.<sup>[16,17]</sup>

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441 These results indicate that for the purposes of molecular experiments  
442 looking at *per1*, 3 d in DRL, housed with or without a wheel is sufficient to  
443 produce the same light-induction of *per1* achieved after much longer access to  
444 the wheels and much longer time in DRL. This is important for the design of  
445 future protocols of in situ hybridization experiments where wheel access and  
446 associated behavioral stimuli may be of concern. It suggests that housing  
447 hamsters in DRL conditions for only 3 d rather than 2 wk could save time  
448 without affecting the outcome of the in situ hybridization experiment.  
449 Additionally, it allows for more freedom in deciding whether or not to house  
450 hamsters with access to running wheels.

451 The magnitude of light-induced resetting of circadian rhythms depends  
452 dramatically upon environmental conditions. Both novelty of the running wheel  
453 and length of time in constant conditions alter phase shift magnitude. This study  
454 indicates that 3 d of novel wheel access could serve as a new paradigm for  
455 investigating interactions between photic and nonphotic stimuli.

## ACKNOWLEDGMENTS

456  
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458  
459 This work was supported by the Arnold and Mabel Beckman Foundation (CAC) and  
460 NIH R01 NS26496 (MEH). Equipment was provided in part by a grant from the Arthur  
461 Vining Davis Foundation. The assistance of Dr. Paola Yannielli, Dr. Judy McKinley  
462 Brewer, Dr. Dick Briggs, and Meagan Ward is gratefully acknowledged. This work was  
463 submitted as a senior honors thesis (CAC), Smith College, 2001.

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511 Received September 4, 2001

512 Returned for revision October 10, 2001

513 Accepted March 14, 2002

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