

Research report

Morphine-induced activity attenuates phase shifts to light in C57BL/6J mice

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Abstract

Circadian rhythms can be phase shifted and entrained by daily schedules of light and by non-photic stimuli such as locomotor activity. Relatively little is known of how photic and non-photic stimuli interact to regulate circadian phase. Morphine injections were used to examine the effects of locomotor activity on phase shifts to light pulses in mice free-running in constant dark. Morphine injections scheduled early or late in the active period (subjective night) induced hyperactivity, but did not induce phase shifts. Light pulses late in the subjective night induced phase advance shifts that were significantly attenuated (63% smaller, $p < 0.01$) by pretreatment with morphine. This inhibitory effect of morphine on light-induced phase advances was blocked by preventing mice from running for 6 h after the injections. Light pulses early in the subjective night induced phase delay shifts that were only weakly attenuated (15% smaller, $p = 0.06$) by morphine. These results demonstrate behavioral inhibition of light-induced phase resetting of circadian rhythms in mice, and suggest that the strength of this effect may be phase dependent, although other interpretations are possible. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Circadian rhythms in mammals are entrained by daily schedules of light exposure, and can be shifted by single ‘pulses’ of light. In animals ‘free-running’ in constant darkness, brief exposure to light early in the ‘subjective night’ (the active phase in nocturnal rodents) induces phase delay shifts, while exposure late in the subjective night induces phase advance shifts [14]. Several non-photic stimuli are also capable of inducing entrainment or phase shifts [14]. In nocturnal rodents, a daily scheduled bout of locomotor activity can entrain free-running rhythms, and a single 1–3-h bout of activity, stimulated during the middle of the subjective day (the rest phase in nocturnal rodents), can induce a phase advance shift [e.g., Refs. [9,12,22,25]].

Animals in natural environments are likely to be exposed to photic and non-photic stimuli in combination, therefore it will be important to examine how these stimuli interact to set the phase of steady state entrainment, and to effect adjustments of circadian phase when entraining stimuli (i.e., zeitgebers) are shifted. Several types of inter-

actions have been documented. In some instances, shifts induced by photic and non-photic zeitgebers may be additive [e.g., Refs. [7,16,17,23]]. In other cases, interactions may be non-linear. For example, light pulses that do not induce shifts in the subjective day may block phase shifts induced by a behavioral stimulus [8,18]. Conversely, two studies have demonstrated that a behavioral stimulus (running stimulated by confinement to a novel wheel) can attenuate phase advances in response to light pulses late in the subjective night without inducing significant shifts at those phases [13,20]. Evidence for these interactive effects of photic and behavioral stimuli has so far been derived exclusively from studies of the Syrian hamster.

In the present study, we sought to extend the analysis of photic and non-photic zeitgebers in combination by examining the effects of morphine injections on phase shifts to light pulses in the C57BL/6j mouse. We have previously shown that morphine injections during the subjective day can induce phase advance shifts in free-running C57BL/6j mice [10]. The drug also induces a prominent bout of hyperactivity in this strain, and preventing activity for 6 h after drug treatment prevented phase shifts. Although endogenous opiate containing fibers or cell bodies have been localized within the suprachiasmatic nucleus (SCN) circadian pacemaker or its afferents [3,15], these results indicate

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that the effects of morphine on circadian phase are mediated indirectly by its effects on behavioral state. Morphine can thus be employed as a tool for examining interactions between photic and behavioral zeitgebers in the mouse.

2. Materials and methods

Twenty-one male C57BL/6j mice (Charles River, Quebec) were housed in constant dark in separate plastic cages ($45 \times 25 \times 20$ cm) equipped with running wheels (17 cm diameter) and wire mesh floors over litter trays. Wheel running activity was monitored continuously by computer (Activity Counting System, Simon Fraser University), and wheel revolutions were summed and stored to disc in 10-min bins.

At intervals of approximately 3 weeks, mice received single intraperitoneal injections of morphine (25 mg/kg in saline) or saline, with or without a light pulse (4 lx, 10 min) 1 h later. The mice initially received injections at circadian time (CT) 15 (where CT12 is the onset of the subjective night, by convention) and light pulses at CT16 (to induce maximal phase delay shifts). The mice then received injections at CT22 and light pulses at CT23 (to induce maximal phase advance shifts). The four treatment conditions at each circadian phase were counterbalanced for order. An infrared viewer was used to permit handling of mice in constant dark.

To evaluate the effects of morphine-induced hyperactivity at CT22 on phase advances to light pulses at CT23, separate groups of mice received either morphine ($N = 20$ mice) or saline ($N = 19$ mice) followed by light, and were confined for 6 h to a small wire enclosure ($5 \times 10 \times 10$

cm) that prevented the expression of drug-induced cage circling and wheel running activity. Half of the mice were naive to injections and these were divided nearly equally between groups.

Phase shifts in response to injections with and without light pulses were quantified using a computer algorithm for detecting the onset of the active phase of each circadian day (the first 10 min bin exceeding 50 wheel revolutions following an interval of 240 min in which this threshold was not exceeded; occasional onsets that were detected at times that were clearly not the beginning of the subjective night were deleted). Separate regression lines were fit to activity onsets for 10 days preceding each treatment, and for days 6–16 following each treatment (the first 6 days were omitted to ensure that phase advance shifts were complete). Regression lines were extrapolated to the day following each treatment, and the displacement of the two lines was recorded as the phase shift in minutes. The slope of the regression lines was used to derive the period (τ) of the free-running rhythms. Differences among treatments in phase shifts, τ changes and activity levels were evaluated by repeated measures ANOVA and paired or independent t -tests. Results are presented as group means $\pm$ S.E.

3. Results

3.1. Phase shifts to morphine, saline and light

Morphine and saline injections at CT22 did not induce significant phase shifts (Fig. 1A, Fig. 2A). Light pulses at CT23 following saline injections induced small phase ad-

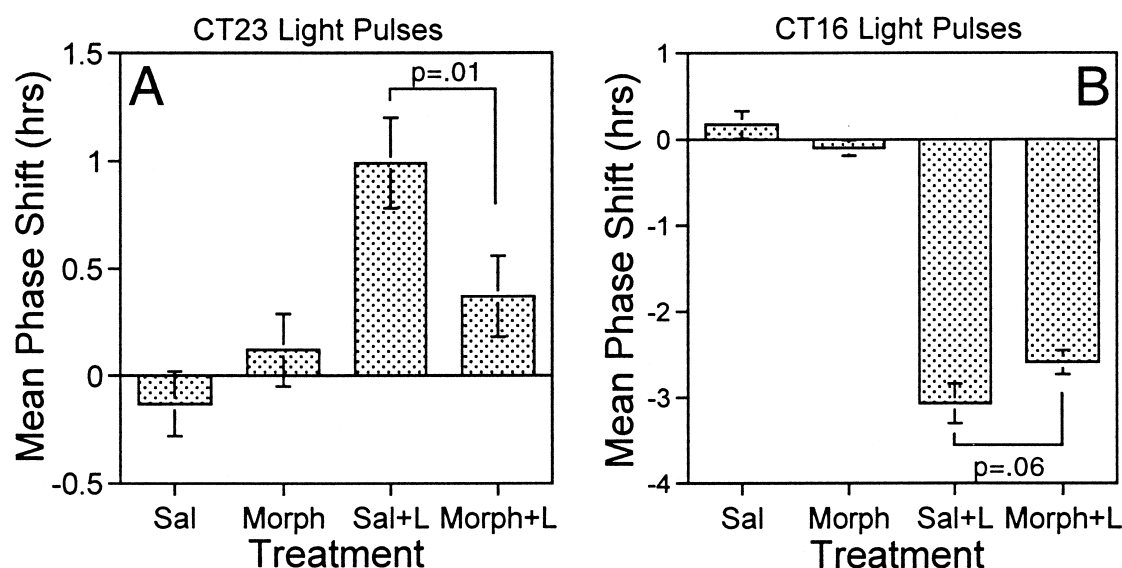


Fig. 1. (A) Group mean ($\pm$ S.E.) phase shifts to injections of saline (sal, CT22), morphine (Morph, CT22), saline (CT22) plus light (CT23; Sal + L) and morphine (CT22) plus light (CT23; Morph + L). (B) Group mean phase shifts to injections of saline (sal, CT15), morphine (Morph, CT15), saline (CT15) plus light (CT16; Sal + L) and morphine (CT15) plus light (CT16; Morph + L). Morphine was delivered i.p. at 25 mg/kg, and the lights pulses were 4 lx for 10 min.

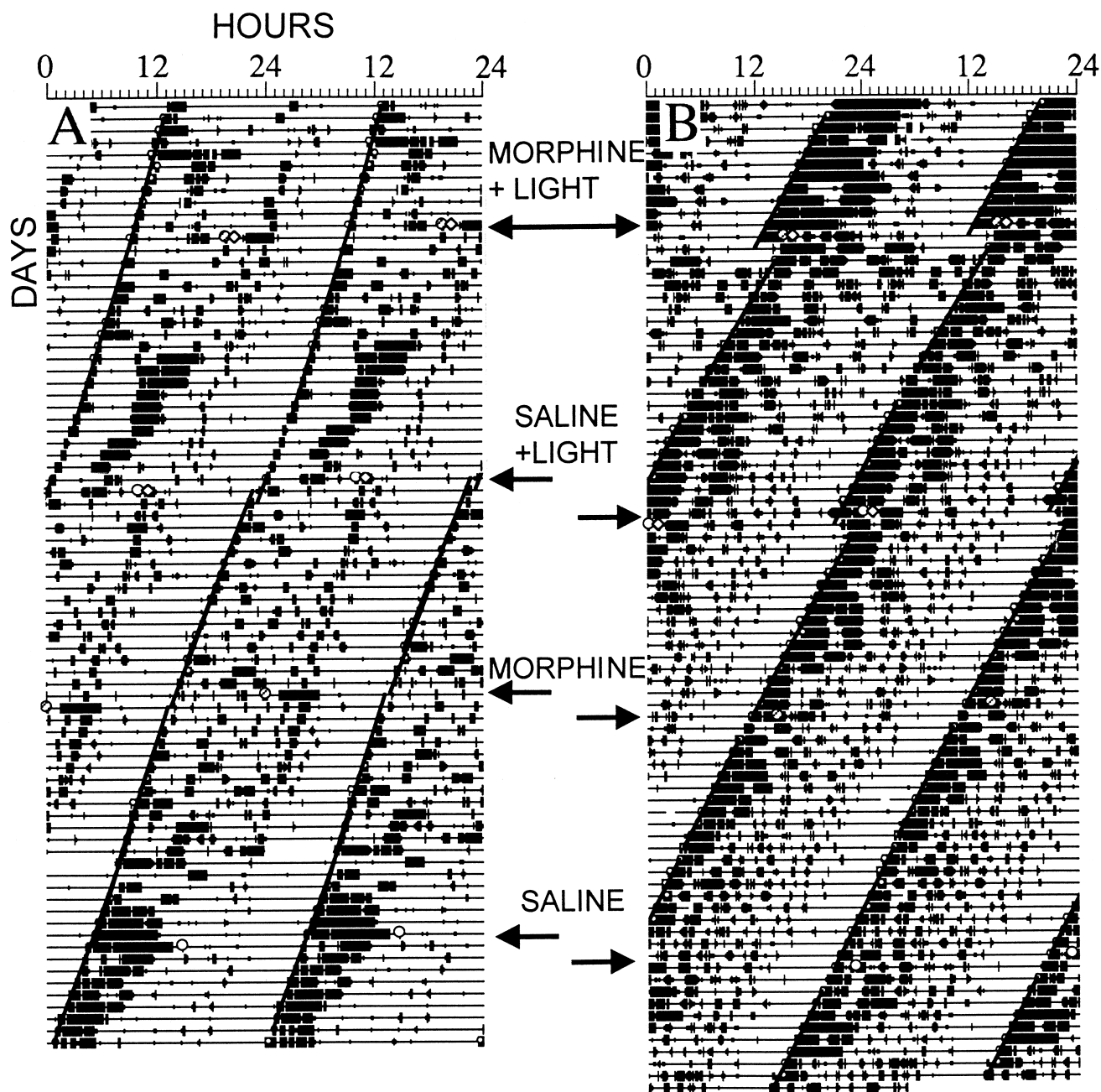


Fig. 2. Representative activity records from a mouse receiving morphine or saline injections, with or without light pulses. Time within a day is plotted from left to right in consecutive 10-min bins. Each day is double-plotted, i.e., consecutive days are aligned left-to-right and top-to-bottom. Time bins in which wheel revolutions occurred are represented by vertical deflections. Bins in which no revolutions occurred are represented by a single point. The time of light pulses is indicated by a diamond symbol, and injections by a circle. Small circles represent activity onsets selected by computer, and heavy bars represent regression lines fit by computer. (A) Injections at CT22 with or without light pulses at CT23. In this example, no shift was registered to morphine and light, and a small phase advance (60 min) was registered to saline and light. Morphine and saline alone produced little or no change in phase. (B) Injections at CT15 with or without light pulses at CT16. In this example, light pulses with morphine or saline produced large delay shifts of approximately 2.5 h. Morphine and saline alone produced no detectable shifts.

vance shifts averaging 0.99 ± 0.21 h (e.g., Fig. 2A). Morphine injections attenuated the group mean phase advance to light pulses at CT23 by 63% (0.37 ± 0.13 h; $t = -2.81$, $p < 0.01$; e.g., Fig. 2A). Attenuation (i.e., smaller phase shifts to light in the morphine condition) was evident in 15 of 21 mice.

Morphine and saline injections at CT15 also did not induce significant phase shifts (Fig. 1B, Fig. 2B). Light pulses at CT16 following saline injections induced phase delay shifts of 3.07 ± 0.23 h (e.g., Fig. 2B). Morphine attenuated the group mean phase delay shift by 16% (2.59 ± 0.14 h; $t = 1.97$, $p = 0.064$). Attenuation was evi-

dent in 15 of 21 mice (this subset was not identical to the subset exhibiting attenuation to CT23 light pulses).

3.2. Effects of morphine and saline injections on activity

Visual inspection confirmed that the mice were hyperactive after morphine injections at both CT15 and CT22. During the first 1–2 h after the injections, the mice exhibited bouts of running around the perimeter of the cage, interspersed with short bouts of eating, drinking and grooming. Group mean wheel running was not significantly increased during the first 3 h after the injections, but was substantially increased during the second 3 h. Running during this time block was enhanced by 229% ($t = 2.57$, $p = 0.02$, $N = 19$, two data points lost due to switch failure) following CT15 injections that preceded CT16 light pulses, and by 111% ($t = 4.81$, $p < 0.001$; $N = 19$, two data points lost due to switch failure) following CT22 injections that preceded CT23 light pulses, relative to the matched saline–light pulse conditions.

During this second 3-h time block after morphine injections, mice that exhibited attenuation of phase shifts to light at CT16 ran significantly more than mice that did not exhibit shift attenuation (2805 ± 647 wheel revs in attenuators vs. 738 ± 264 revs in non-attenuators, $t = 2.96$, $p < 0.01$). In addition, there was a significant positive correlation between the degree of shift attenuation (expressed as the difference in phase shift size between the morphine + light and the saline + light conditions) and the level of drug-induced activity during this 3-h time point (expressed as the difference in 3-h cumulative wheel revolutions

between morphine + light and saline + light conditions) ($r = 0.48$, $p = 0.05$; Fig. 3B).

In the CT23 light pulse condition, activity levels following morphine were not significantly different between shift attenuators (1497 ± 325 revs) and non-attenuators (2052 ± 509 revs; $t = 0.92$, $p > 0.1$). However, there was a positive correlation between the degree of shift attenuation and the amount of drug-induced activity, although this was statistically no more than a trend (Pearson $r = 0.39$, $p = 0.1$; Fig. 3A).

Activity levels did not differ between saline and morphine conditions in any other time block of the injection day, or during the 5 days immediately preceding and immediately following injections.

3.3. Phase shifts to morphine and light following activity restriction

To further evaluate the role of hyperactivity in the attenuation of photic phase shifting by morphine, mice were confined to a small wire enclosure for 6 h following saline or morphine injections at CT22, and exposed to light at CT23. Resulting phase shifts did not differ significantly between groups (0.68 ± 0.14 h vs. 55 ± 0.20 h; independent $t = 0.34$, $p > 0.1$).

3.4. τ changes

Occasional changes in τ were evident following injections with and without light pulses at both circadian phases.

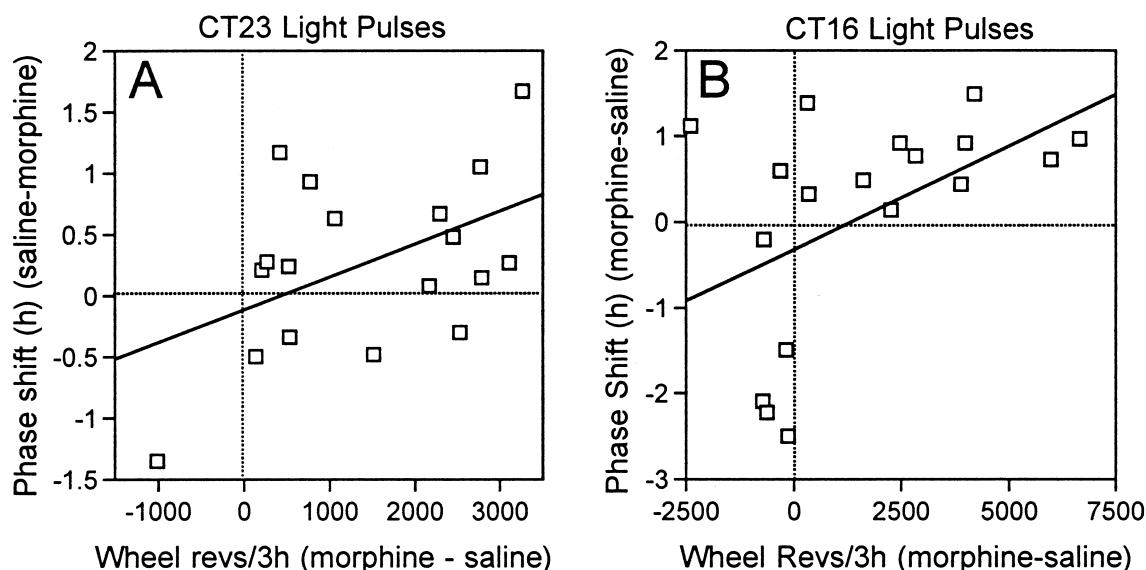


Fig. 3. Scatterplots illustrating the relationship between drug-induced activity (expressed as the difference between the amount of wheel running during the second 3-h time block following the morphine and saline injections that preceded light pulses) and drug-attenuation of photic shifts (expressed as the difference in the size of the phase shifts in the saline + light and morphine + light conditions). In panel A, phase shifts were negative (i.e., CT16 light pulses induce phase delays, which are by convention designated as negative in sign), thus, shifts to saline + light were subtracted from shifts to morphine + light, so that a positive value means that the shifts were smaller following morphine injections. In panel B, phase shifts were usually positive (i.e., CT23 light pulses induce phase advances), and shifts to morphine + light were subtracted from shifts to saline + light, so that positive values again mean that shifts were smaller following morphine injections.

There was a significant group mean shortening of τ after saline with light pulses at CT16 (0.11 ± 0.03 h, $t = 3.37$, $p = 0.003$), and a significant lengthening of τ after morphine alone at CT15 (0.06 ± 0.03 h, $t = -2.35$, $p = 0.03$). There were no significant group mean τ changes following tests at CT22.

4. Discussion

Two previous studies have demonstrated that, in hamsters, phase advance shifts to light pulses can be significantly attenuated by inducing the animals to run in novel wheels just prior to and during the light exposure [13,20]. The present study provides evidence that behavioral inhibition of light-induced phase resetting is also characteristic of the C57BL/6j mouse. Morphine significantly attenuated phase advances to light pulses late in the subjective night. Morphine also induced hyperactivity, which was expressed as continuous locomotion around the perimeter of the cage followed by vigorous wheel running. When locomotor activity was prevented by confining the mice to a small enclosure for 6 h following the injections, morphine had no significant effect on light-induced phase shifts, indicating that attenuation of photic shifting by morphine is mediated by behavioral activation.

Induction of locomotor activity by morphine was robust at both circadian phases tested, but attenuation of photic shifting was significant only for light pulses at CT23. There was only a trend toward attenuation of phase shifts to light at CT16 ($p = 0.06$). Similar results were obtained previously using hamsters; behavioral inhibition was significant for phase advancing light pulses but not for phase delaying light pulses [13]. In the present study, failure to see a significant drug effect in the phase delay condition may be due to individual variability in the amount of running stimulated by the morphine injections. Consistent with this interpretation, there was a significant positive correlation between the amount of drug-induced activity and the degree of attenuation of phase shifts to light. In addition, mice that did not show attenuation exhibited significantly lower levels of wheel running during the first 6 h, and particularly hours 4–6, following morphine treatment. If variability in activating effects of morphine explains the differential attenuation of photic phase delays and phase advances, then we might expect this difference to be absent in future studies that utilize a behavioral procedure, such as forced treadmill running, that provides better control over running intensity and duration.

The relation between attenuation of light-induced shifts and amount of drug-induced wheel running was not as clear in the CT23 light pulse condition. Mice that showed smaller phase shifts to light following morphine (i.e., shift attenuators) did not run significantly more than mice that did not show smaller phase shifts (non-attenuators). There was a positive correlation between the morphine-induced

increase in running and the degree of shift attenuation, but this was statistically only a trend. Failure to observe a strong relation between activity and shift attenuation may be because our measure of activity was limited to wheel running. Visual observations indicated that much of the drug-induced activity, particularly during the first few hours after injections, was in the form of rapid running around the perimeter of the cage. A more general measure of activity that includes this form of locomotion might reveal a stronger relation between activity and shift attenuation at this circadian phase.

Changes in τ following drug or light treatments can complicate the interpretation of phase shifts. In the present study, there was a significant shortening of group mean τ following the CT16 light pulses in the saline condition. A shortening of τ following any treatment would tend to accentuate phase delay shifts, due to the differences in the slope of the regression lines used to quantify phase before and after the injection days. Therefore, the phase delays to light in the saline condition may have been slightly overestimated. This could have contributed to the appearance of a marginal attenuation of phase delay shifts in the morphine condition, because no significant change in τ was evident in that condition. This weakens our confidence in the interpretation, based on a trend in the data, that morphine may be capable of attenuating phase delay shifts to light early in the subjective night. Systematic changes in τ did not occur following injections with or without light pulses at CT23, and thus do not color the interpretation of the significant attenuation of photic advance shifts by morphine late in the subjective night.

It remains to be determined if stronger evidence for behavioral inhibition of photic phase delays can be obtained in mice by utilizing a stronger, more controlled behavioral stimulus (e.g., treadmill running) or a weaker photic stimulus. Our own dose response pilot studies revealed that a 4-lx, 10-min light pulse produces significantly smaller phase delays than a 40-lx, 10-min light pulse, but the shifts induced are nonetheless large, suggesting that a weaker photic stimulus could be employed without reducing the reliability of shifting in the control condition (which was a concern for the CT23 light pulses, due to the small size of phase advances to light in mice).

We previously reported that morphine alone late in the subjective night induced a non-significant trend toward small phase delays [10]. In the present study, we again failed to detect significant phase shifts to morphine either early or late in the subjective night. These results indicate that the attenuation of phase advances to light and the lack of significant attenuation of phase delays to light were not a trivial consequence of competing or compensating shifts directly induced by the drug.

The neural mechanisms that mediate behavioral inhibition of light-induced phase shifts remain to be elucidated in mice. In hamsters, behavioral inhibition of phase advances can be blocked by pretreating subjects with the

general 5HT1 antagonist metergoline, suggesting that serotonin transmission mediates behavioral inhibition of light input to the SCN circadian pacemaker [13]. There is a convergence of evidence consistent with this hypothesis. Microdialysis studies confirm that extracellular serotonin (5HT) levels in the hamster SCN increase during wheel running [5]. Photic activation of SCN single unit activity or *c-fos* expression can be inhibited by 5HT in rats and hamsters [11,21,26]. Photic resetting of behavioral rhythms in hamsters can be inhibited by 5HT agonists with affinity for presynaptic 5HT1b receptors and postsynaptic 5HT1a and 5HT7 receptors [19,21]. Ablation of 5HT afferents to the SCN in mice is associated with enhancement of light-induced phase shifts [2]. A reasonable prediction based on these results is that inhibition of light-induced phase advances by morphine or behavioral activity in mice will be blocked by lesions or antagonists directed at 5HT afferents or receptors in the SCN.

One discrepancy in the evidence for serotonergic mediation of behavioral inhibition of photic resetting is that 5HT agonists can inhibit both phase delays and phase advances to light, whereas good evidence for behavioral inhibition has so far been limited to phase advances. This could simply be a matter of the drug doses having exceeded the 'behavioral' doses in the studies conducted to date. It is also possible that endogenous 5HT release may normally be high early in the night, thereby limiting behavioral enhancement of inhibitory serotonergic tone on photic inputs to the SCN pacemaker. In vivo measures of 5HT release in the SCN support this interpretation [5].

A role for neuropeptide Y in behavioral attenuation of photic resetting should also not be ruled out, given the evidence that injection of this peptide into the SCN area can inhibit phase advances to light but is less effective against phase delays [24], and that antiserum to NPY can enhance phase advances to light [1]. Dose response studies of 5HT, NPY and behavioral activation in competition with light in mice are needed.

Differential sensitivity of the phase delay and phase advance portions of the light pulse PRC to behavioral inhibition, if confirmed, could also follow as a consequence of the different postsynaptic intracellular transduction pathways that are thought to mediate light-induced phase delays and advances [e.g., Ref. [4]]. Neural correlates of locomotor activity may limit phase advances more readily than delays because their postsynaptic effects differentially intersect these transduction pathways in photically responsive SCN neurons.

A significant morphine-induced elevation of wheel running activity was not evident until the second 3-h time block after the injections, beginning 2 h after the light pulses. This might be taken to suggest that drug-induced activity attenuates light-induced phase shifting by effects on the intracellular transduction pathways that mediate shifting of pacemaker cells, rather than on the immediate transmission of photic information to the pacemaker cells.

It also suggests that light-induced shifting is not complete for at least 2 h after the light pulse. However, the activity restriction procedure, which prevented shift attenuation, included the first 6 h after drug application. It thus remains to be tested whether attenuation of phase shifts to light is mediated exclusively by delayed wheel running, or whether intense general cage activity expressed immediately following the morphine injections also contributes.

Behavioral inhibition of photic resetting has now been demonstrated in two mammalian species, and analogous effects have been reported in preliminary form in scorpions [6]. The adaptive significance, if any, of inhibitory interactions between photic and non-photoc inputs to the circadian pacemaker is not obvious. Nonetheless, this phenomenon would appear to have significant implications for the design of schedules of light exposure and exercise to facilitate resetting of the circadian clock in humans.

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