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Low amplitude entrainment of mice and the impact of circadian phase on behavior tests

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Abstract

A tremendous increase in the use of genetically engineered mice as experimental animals has led to increased scrutiny of mouse models generally and mouse behavioral paradigms specifically. Although mice are nocturnal, for practical reasons, most experimental procedures, including behavioral studies, are conducted during their inactive, sleep phase. Accumulating evidence indicates that myriad behavioral, cellular and biochemical processes fluctuate with circadian rhythmicity; however, time of day at which experiments are conducted is rarely controlled. The impact of circadian phase on the reliability of experimental results has received little attention and the present data are conflicting. This study addressed two questions. First, will laboratory mice in a typical animal care facility entrain to a low amplitude light cycle using bright/dim rather than light/dark cycles? A positive answer will make reversing photocycle easy to implement in any facility as dim light suitable for animal husbandry and behavioral testing can substitute for darkness during work hours. By monitoring home cage wheel running, we examined the effectiveness of a dim/bright photocycle as a zeitgeiber. We found that mice subjected to dim/bright photocycles effectively entrained such that their subjective night and activity onset coincided with the beginning of the dim light period, suggesting a potential strategy for standardization and management of circadian phase in nocturnal animals. In a second experiment, we asked what effect circadian phase has on behavioral performance in commonly used mouse behavioral tests. We found no main effect of circadian phase on outcome in open field activity, elevated plus maze emotionality, water maze spatial memory, novel object exploration and hyperactivity in response to amphetamine; however, we observed occasional interactions between circadian phase and both strain and sex that were neither consistent nor systematic. These data suggest that the tests examined here are relatively impervious to circadian phase. In general, testing mice during their active phase is more suitable for behavioral studies; a reversed dim/bright photocycle potentially offers one practical strategy for managing rodents' circadian cycles.

Keywords: Behavior; Circadian; Entrainment; Amphetamine; Activity; Mouse

1. Introduction

As the use of genetically engineered mice becomes more widespread in behavioral neuroscience, close scrutiny of the reliability and validity of commonly employed behavioral paradigms is critical. Recent work by Crabbe et al. and Wahlsten et al. [1,2] garnered considerable attention when the authors reported that the site at which mouse behavioral testing was conducted contributed significantly to variation in test outcomes, although the source of this site variation could not be determined. Subsequently, there has been increased interest in standardization of behavior testing, although the feasibility and desirability of standardization are controversial [1–8]. Nonetheless, the recent attention given assessing and improving the reliability of mouse behavioral tests highlights its importance.

Circadian phase at time of testing–whether mice are tested during their active or inactive phase–may represent an inadequately controlled variable [9]. Commonly, mice are maintained on a 12:12 light–dark cycle where their inactive (light) phase coincides with business hours. It is not uncommon for mice to be tested during this time. It is reasonable to ask whether waking up mice and testing them during their sleep cycle may result in different outcomes than testing them during

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their normal, waking active phase. From a biochemical perspective, we would anticipate that circadian phase would have an effect on testing as many biochemical processes and parameters fluctuate with a circadian rhythm [10–20]. Despite this, we could find little systematic examination of the effect of circadian phase on commonly used mouse behavioral tests. The question of the impact of circadian phase on the reliability of experimental results is not limited to behavioral studies, but is potentially a concern in biochemical and cellular studies as well.

Maintaining mice on a reverse cycle and working with them during their active, dark period is practically difficult. One unexplored strategy is to place mice on a reverse cycle where dim light sufficient to facilitate work with the animals substitutes for darkness. Early work established that mice will entrain to low amplitude light cycles. Investigating small nocturnal mammals in the arctic, Swade and Pittendrigh [21] found that, during the summer solstice when no darkness occurs, mice continue to entrain to fluctuations in the intensity of light from day to night. Further, this ability was not unique to arctic mammals as mice from temperate zones entrained equally well. This early study found (a) that the absolute intensity of light did not appear to matter as much as the ratio between high and low intensity in the cycle and (b) that a ratio as low as 2.5 between the maximum and minimal light intensities would entrain mice. We could find no investigations of the potential application of this finding to the husbandry of nocturnal laboratory animals and the management of their circadian cycles to better facilitate behavioral work with rodents.

This study addresses two questions. First, will laboratory mice in a typical animal care facility entrain to a low amplitude light cycle using bright/dim rather than light/dark cycles? Second, what effect, if any, does circadian phase have on performance in a battery of mouse behavioral tests that are standard components of transgenic phenotype screening? We tested mice during either their active or inactive phase in the open field (OF), the elevated plus maze (EPM), the Morris water maze (WM), a novel object exploration test (NovObj) and an amphetamine challenge (AMPH). We test two of the most commonly used laboratory strains, C57BL/6 and 129S1/SvImJ, the latter being a common genetic background in genetically altered mice.

2. Methods

2.1. General

48 mice (24 129S1/SvImJ (129), 24 C57BL/6 (B6), divided equally by sex) were received at 4 weeks of age from Jackson Laboratories (Bar Harbor, ME) and placed in individual cages for the duration of the experiment. All mice, males and females, were singly housed in one colony room in Animal Care Systems polycarbonate cages with corn cob bedding and isolated air circulation. The cages were changed every 2 weeks and the wheels were cleaned once per month, requiring a 1-day absence of wheels while they were washed and reassembled. No enrichment was provided other than the wheel. Both testing and housing were in non-shared rooms minimizing entries and disturbance. No controlled background noise was provided. Non-shared rooms minimize noise but are not soundproof and thus normal activity of animal care staff can be heard throughout the workday although muted. The animal facility is maintained at a constant room temperature between 21 and 23 °C with humidity between 35% and 55%. Baseline activity cycles were recorded for 6 days in constant dim lighting, approximately 15 lx. Reverse cycle entrainment was begun on day 7, using 12:12 dim/bright with bright lights on at 18:00 and off at 6:00. Bright light was approximately 100 lx. Mice were allowed to entrain on this dim/bright light cycle for 23 days before behavior testing began. The mice were divided into two groups: an AM (active phase) test group and a PM (inactive phase) test group. All testing was conducted within the window of 17–19 h ZT (AM group) or 5–7 h ZT (PM group) with the exception of the water maze which was conducted within the windows of 16–20 and 4–8 h ZT, respectively. Within these windows of test times, animals were selected for testing without regard to order. Immediately prior and throughout the testing period, mice were maintained in constant dim light (15 lx) and tested under similar illumination conditions in order to control for masking effects. Mice were protected from light in all movements from colony room to behavior suites to prevent phase-shifting light pulses. At the completion of all testing except the amphetamine challenge, mice were maintained for 1 month on a standard light/dark light cycle with lights on at 0600 and lights off at 1800. At the end of this month, a 3-day constant dim probe trial was conducted. Subsequently, mice were returned to L/D schedule. Two days prior to and during the amphetamine challenge, mice were again maintained in constant dim lighting as during other behavior testing. Mice were maintained on laboratory chow diet with food/water freely available.

2.2. Activity monitoring

Mice were singly housed each with a 4.5-in. wire mesh wheel (Mini Run-a-Round, Pets International, Ltd., Elk Grove Village, IL). Two counter-balanced magnets (Digi-key, Thief River Falls, MN) were placed on 3/8 in stainless steel strips attached to the wheel (McMaster Carr Supply Co, Chicago, IL). The wheel was situated in the cage such that a magnetic switch closes (Digi-key) at every pass of a magnet. Data were collected using Vitalview acquisition software, QA-4 activity input modules and DP-24 data ports (Mini-mitter Co., Sun River, OR), and analyzed using Clocklab (Actimetrics, Wilmette, IL). The wheels remained in the cages and data were collected every 5 min throughout the duration of the experiment. Wheel activity was monitored at all times except during testing, biweekly cage changes and periodic wheel washing. Actograms were constructed using Clocklab. Onsets were initially identified automatically using Clocklab software. These were then reviewed manually and corrected where appropriate (testing periods sometimes interfered with automated identification of onsets). Phase angle of entrainment was calculated as the difference between the onset of the photophase that provided less illumination (either dark or dim light) and onset of activity

identified above. Circadian time (CT) of testing was calculated as follows: Clocklab statistical module was used to estimate each animal's free-running period (tau) using minimally a 7 consecutive day segment during a constant light period. The difference in hours between activity onset and time of testing was multiplied by 24/tau to arrive at the number of circadian hours elapsed between activity onset and testing. This number was added to CT12 (i.e., CT12 = activity onset) to determine CT at time of testing.

2.3. Test procedures

For each test, a single testing apparatus as described below was used to test all mice, both male and female. The tests were conducted in the following sequence: the open field and elevated plus maze were done on consecutive days. Following the open field and elevated plus maze, the 129 and B6 strains were tested separately on the water maze with a 2- and 5-week interval, respectively. Both strains were tested together on the novel object test, after 3 and 7 weeks following the water maze for the B6 and 129 mice, respectively. The amphetamine challenge was conducted subsequent to the L/D entrainment segment of the experiment, approximately 8 weeks following the novel object test. Table 1 lists the order of experimental segments/tests and the age of the mice at the beginning of each.

2.3.1. Open field (OF)

Each mouse was placed in an acrylic open field chamber 40 cm long × 40 cm wide × 37 cm high (Med Associates, St. Albans, VT). The floor of the chamber is white, the sides transparent. Each chamber was surrounded by black drop cloth obscuring views beyond the chamber. Illumination of open field was set to 16 lx as measured 1 cm above the floor of the chamber. No background noise was provided. Infrared beams recorded the animal's location and path (locomotor activity) as well as the number of rearing movements (vertical activity). Data were collected in 1- or 3-min bins during 30-min trials. The chambers were cleaned between all trials.

2.3.2. Elevated plus maze (EPM)

The elevated plus maze contained four equal length arms made of acrylic and measuring 30 cm in length and 7 cm width elevated 55 cm above the floor. Two arms had 15-cm walls enclosing all but the top and the other two arms remained open. All components were black. Animals were placed in the central platform and allowed to explore the maze for 5 min. Mice were recorded by a video camera placed above the mice and the following parameters were scored: total entries (number of entries into any arm), open arm entry index (number of entries into the open arms divided by the total number of entries) and open arm time ratio (time spent in open arms divided by total time spent in the arms). Illumination was 16 lx. An entry was scored when all four limbs entered the box. Timing of the duration of an open arm entry started upon entry and was terminated when one limb left the arm. The maze was cleaned between all trials. The elevated plus maze was repeated approximately 1 month after the first test session for all mice following the same procedure.

Table 1
Order of experimental procedures and age of mice (days) at start of each procedure

Experimental procedure	Age (days)
Habituate/baseline	28
Dim/bright photoperiod	34
Constant dim probe	57
Open field/elevated plus maze	60
Water maze visible trials (all mice)	76
Water maze hidden platform (129)	77
Water maze hidden platform (B6)	97
Novel object	126
Light/dark photoperiod	139
Constant dim probe	175
Amphetamine challenge	183

2.3.3. Morris water maze

A white acrylic pool 100 cm diameter was filled so that the water line was approximately 15 cm below the lip of the pool. Water was maintained between 22 and 24 °C. For the hidden platform trials, 2 pints of nontoxic white tempera paint (Reeves and Poole Group, Toronto, Canada) were stirred in. Movements were recorded and analyzed using EthoVision software (Noldus, Wageningin, Netherlands). Lighting was approximately 15 lx. In visible trials, a circular platform (diameter 8 cm) was at water level with an orange ping-pong ball attached as a flag. Mice were initially placed on the platform for 10 s. Three subsequent trials were run with the platform moved to a new location at each trial. If the mice did not find the platform within 60 s, the trial was terminated and the mouse was put on the platform for 10 s before beginning the next trial. The three trials for each mouse were averaged to yield a single visible platform latency measure. For hidden trials, the same platform was submerged 1 cm below the water level was placed in one location and not moved for the duration of the training trials. Cues were placed on the walls approximately 8 in. above the lip of the pool. Cues were white construction paper cut-outs of a circle, star and square, approximately 30 cm across. For probe trials, the hidden platform was removed. In protocol 1 (129 mice only), four consecutive training trials were conducted for each mouse for 4 consecutive days (i.e., four trials on each of 4 days, total 16 trials). Immediately prior to the first trial, the mouse was placed on the invisible platform for 30 s. A trial was terminated when the mouse found the platform or at 60 s. If the mouse did not find the platform, it was placed there. Mice remained on the platform between trials for 30 s on day 1 and 20 s on training days 2–4. A probe trial was conducted on day 5 in which the platform was removed and mice were given a single 1-min trial. In protocol 2 (C57BL/6J), mice were provided one 60-s training trial 1× per day. Every 3 training days were followed by a probe trial; the entire sequence was repeated three times. Two mice were excluded from the water maze experiment (one would only float, the other showed severe swimming deficits).

2.3.4. Novel object exploration

The same apparatus used in the open field was used for these tests. Mice were placed in the open field chambers and allowed

30 min to habituate. A small red object (5 cm × 2.5 cm) was then taped to the center of the chamber and activity was monitored for another 30 min. Data were collected in 1-min bins and analyzed in 10-min blocks starting with the 10 min prior to placement of the object as baseline.

2.3.5. Amphetamine challenge

Amphetamine (or equal volume of saline) was administered at 1.5 mg/kg through IP injection at either 11:00 or 23:00. After injection, mice were returned to their cages and wheel running activity was monitored. For analysis, the 5-min bins used for data collection were collapsed into 30-min blocks representing the average of the six data points comprising each block.

2.4. Statistical analyses

Data were analyzed using StatView 5.0.1. ANOVA was used for data collected in a single trial and repeated measure ANOVA was used when data were collected in multiple trials. Analysis was performed using both the full data set and a restrictive data set where mice, which were not within a 2-h window in either direction of either CT18 or CT6 at the time of testing were excluded.

3. Results

3.1. Entrainment in low amplitude light cycles

Running wheels placed in the home cages of individually housed mice were used to monitor activity cycles (see Methods). To test the effectiveness of low amplitude dim/bright (Dm/B) light cycles as a zeitgeiber, we reversed the phase of the mice using 12:12 Dm/B photocycles with 15 lx intensity from 06:00 to18:00 and approximately 100 lx from 18:00 to 6:00. Upon arrival from shipping, mice were allowed 6 days of constant dim light (Dm/Dm) to verify activity onset prior to

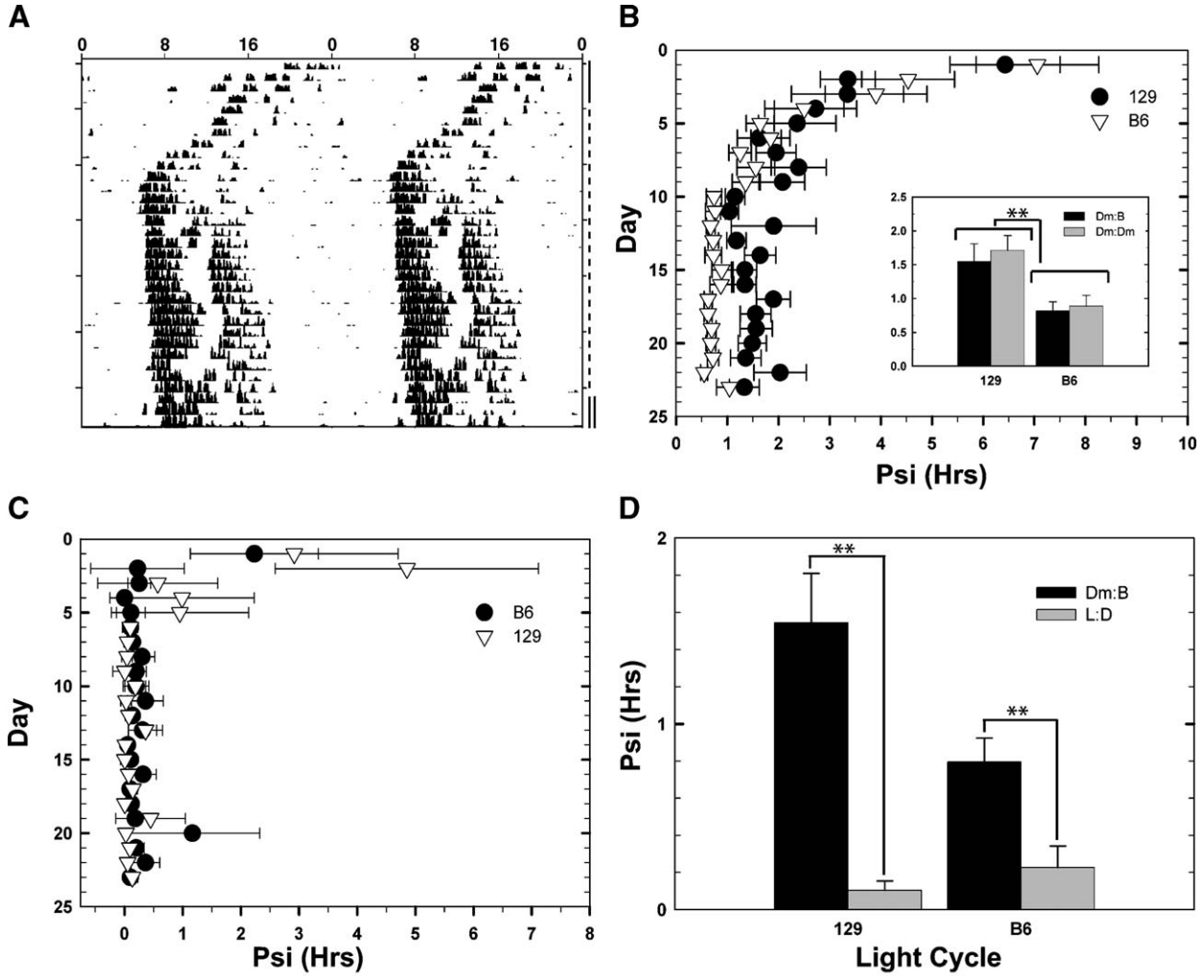

Fig. 1. Entrainment of mice using standard and low amplitude light cycles: (A) representative actogram showing initial baseline (solid bar to right of graph), phase reversal using dim:bright light cycle (dashed bar) and 3 days constant dim (double bar). (B) Mean (±S.E.M.) psi (absolute values) for mice during phase reversal using dim/bright light cycles (129 mice, filled circles; B6 mice, open triangles). $N=45$. Inset: mean (±S.E.M.) psi values for last 3 days of dim/bright (Dm/B) and first 3 days of constant dim (Dm/Dm). (C) Mean (±S.E.M.) psi values for mice during phase reversal using standard light/dark photoperiod. $N=11$. (D) Mean (±S.E.M.) psi values for last 3 days of phase reversal using either dim/bright or light/dark light cycles. $^{**}p<0.01$.

photoperiod manipulations (18:00 onset). Animals were then exposed to a Dm/B cycle for 23 days followed by constant dim light. Fig. 1A shows a representative actogram of one animal exposed to Dm/Dm followed by Dm/B. Generally, mice exhibited small (<2 h) phase angles of entrainment (psi value) indicating that the onset of locomotor activity coincided with the onset of the dim light portion of the photocycle. Fig. 1B shows the mean psi value of all animals during the Dm/B phase reversal entrainment period split by strain. The 129 mice entrained more poorly than the B6 mice [$F(1,38)=10.55$, $p=0.002$] (Fig. 1B inset). The average of the psi values for the last 3 days of Dm/B light were comparable to those observed during the first 3 days of constant dim subsequent to phase reversal [$F(1,38)=0.464$, $p=0.5$], indicating that coupling of locomotor activity with the light cycle observed during the Dm/B period reflects entrainment of the animals' endogenous rhythm rather than masking effects of light intensity (Fig. 1B, inset). There were no differences between sexes in phase angle of entrainment [$F(1,38)=0.883$, $p=0.353$, not shown].

At the end of the experimental period, the light cycle was again reversed using a standard alternating 12:12 light/dark (L/D) cycle. Fig. 1C shows the mean psi value of all animals during the first 23 days of this L/D phase reversal. Notably, the average onset of locomotor activity was more tightly coupled to the onset of darkness than the onset of dim light in the previous phase reversal, with an average of 0.5 versus 1–2 h [$F(1,50)=11.74$, $p=0.001$], respectively (Fig. 1D). Nonetheless, mice did effectively entrain to the low amplitude light cycle; that is, in the temporal organization of their locomotor behavior, animals oriented their circadian system in dim–bright light cycles such that their subjective night corresponded to the dim light portion and their subjective day to the bright light portion of the light cycle.

3.2. Distribution and stability of circadian phase during testing sessions

For behavior testing, mice were divided into two test groups, designated "active" and "inactive." Mice were maintained in constant dim light (Dm/Dm) throughout the testing periods and tested in the same light intensity as their Dm/Dm colony room to isolate phase effects and avoid confounds with masking effects. Although the methodological aim was to conduct testing at CT6 (mid-inactive phase) and CT18 (mid-active phase), we anticipated a gradual shift would in phase would occur (attributable to free running of the circadian system) as time in Dm/Dm passed. To verify that animals were being tested during either their active or inactive phase, we calculated the circadian time (CT) at which the tests were conducted for each animal during the first and last days of each test series (Fig. 2A). We observed a bimodal distribution with the active period testing time clustering around CT18 and the inactive period testing time clustering around CT6. For each test series, we observed a gradual change in the CT at which testing occurred. This drift was minimal except in the case of the second Morris water maze (WM) protocol, which, because it occurred over a period of 2 weeks, the circadian system of individual animals drifted by as much as 6 h. Thus, at the end of the WM series, two groups of animals could be distinguished as being tested at CT12 and CT0. To examine the distribution of shifts in activity onset, we calculated a pseudo "phase angle of entrainment" for activity onset with reference to the time of light onset during the photoperiod to which the mice had entrained prior to the constant dim light, which we denote "psi∗". All psi∗ values for all animals during each day of testing (excluding the amphetamine challenge which was conducted in a single day) were plotted in a scattergram (Fig. 2B); a histogram of the distribution (above plot) indicated an approximately normal distribution. Together, these data indicate that animals were tested at the middle of either their active or inactive phase and that deviations were normally distributed suggesting that, in the aggregate, the mice were sampled representatively in both the active and inactive phases.

If the range of circadian times at which individual animals were tested were too broad, it might be that an actual circadian effect becomes obscured. To evaluate this possibility, in selective tests (open field, elevated plus maze and novel object exploration), we

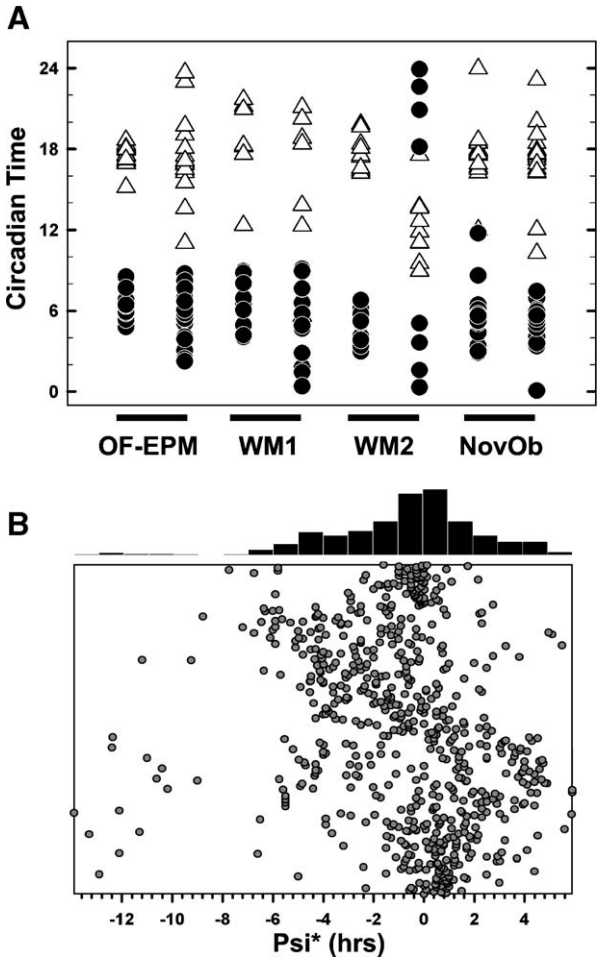

Fig. 2. Circadian time at time of testing: (A) Circadian time calculated for each mouse (open triangles, AM/active phase test group; filled circles, PM/inactive phase group) at time of testing on the first and last days of testing sessions for the open field and elevated plus maze (OF-EPM), both protocols of the Morris water maze (WM1 and WM2) and the novel object exploration test (NovOb). (B) Scattergram of individual psi values from testing days shown in panel A with frequency histogram plotted above.

used a more restrictive subset of the data in which mice which were greater than 2 h from either CT18 or CT6 were excluded from the analysis. The results from this analysis were identical to the analysis including all animals and consequently we present the full data set analysis.

3.3. Effects of circadian phase on measures of locomotor activity and 'emotionality': the open field and elevated plus maze

Perhaps the single most commonly used behavioral test is the open field, generally used to test locomotor activity. Consistent with the findings of Valentinuzzi et al. [22], we found that circadian phase had no effect on locomotor activity in the open field test (Fig. 3A). Although the test effectively discriminated between strains [$F(1,40)=29.55$, $p<0.0001$], neither phase [$F(1,40)=0.032$, $p=0.858$] nor phase by strain interaction [$F(1,40)=0.001$, $p=0.976$] contributed significantly to variation within this test. There were no significant differences between sexes [$F(1,40)=0.672$, $p=0.417$]. Analysis of rearing behavior yielded similar results (data not shown), discriminating between strains [$F(1,35)=59.631$, $p<0.0001$] with no significant circadian phase effect [$F(1,35)=0.747$, $p=0.3934$]. Additionally, we analyzed time spent in corners and center (data not shown) and found no effects of circadian phase [center time, $F(1,40)=2.22$, $p=0.145$; corner time, $F(1,40)=3.01$, $p=0.09$], although a main effect for strain was observed [center time, $F(1,40)=16.37$, $p=0.0002$; corner time, $F(1,40)=56.67$, $p<0.0001$]. No effect of sex was observed for either measure [center time, $F(1,40)=1.01$, $p=0.32$; corner time, $F(1,40)=0.043$, $p=0.837$]. Finally, we examined activity in 3-min blocks across the session to ascertain whether or not there might be a difference in habituation to the test chamber. We found no significant effect of circadian phase in habituation [$F(9,351)=1.41$, $p=0.181$].

The elevated plus maze is commonly used to assess "emotionality", generally considered a measure of anxiety. Conceivably, mice removed from their home cage during a period of inactivity and sleep might be either more or less responsive to a measure of emotionality than mice removed to a testing environment during a waking, active period. However, we found no difference between mice tested during their active or inactive periods on the elevated plus maze (Fig. 3B). As in the open field, our results easily discriminated between strains [$F(1,34)=12.54$, $p=0.0012$], but circadian phase did not contribute significantly to variation within this test [$F(1,34)=0.024$, $p=0.877$]. There were no significant differences by sex [$F(1,34)=0.027$, $p=0.869$]. Within the B6 male group, there is an apparent difference by circadian phase (i.e., sex × strain × CT), but this is not statistically significant [$F(1,35)=0.028$, $p=0.867$]. Fig. 3B (inset) shows the results of a repeated EPM maze conducted approximately 1 month following the first test. Although a repeated EPM may evoke a different response from the mice than the initial test due to habituation and therefore does not constitute a replication, the repeated test shows the same pattern between groups as the initial test but without the apparent difference in the B6 males.

3.4. Effects of circadian phase on learning and exploration: the Morris water maze and novel object exploration

The Morris water maze is a commonly used measure of learning in rodents. In this test, we tested the two strains separately using a different protocol for each strain. The 129S1/SvImJ mice were given intensive training (four consecutive 1-min trials) for 4 days and then administered a test probe to assess how well they have learned. As can be seen in both the latency to finding the platform during training trials and time spent in the platform quadrant on the probe trials (Fig. 4A), there are no significant differences between mice tested during their active or inactive phase [latency, $F(1,17)=0.207$, $p=0.655$; probe trials, $F(1,17)=1.39$, $p=0.255$]. There were no significant differences between sexes [not shown, latency, $F(1,17)=0.333$, $p=0.571$; probe trials, $F(1,17)=0.002$, $p=0.961$]. We also examined swim velocity and

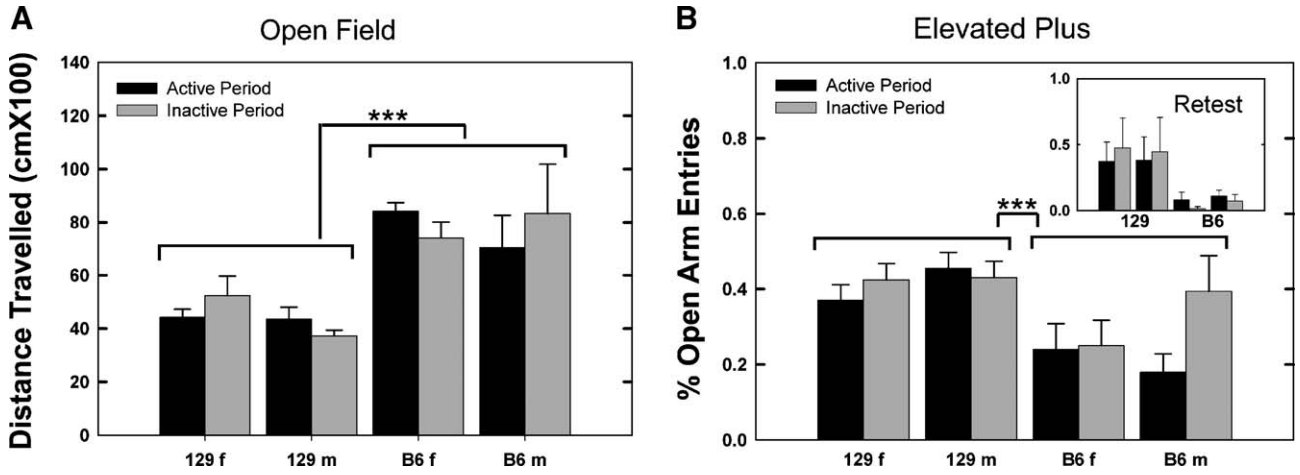

Fig. 3. Effect of circadian phase on open field and elevated plus maze: (A) mean (±S.E.M.) distance traveled in the open field, $N=48$ and (B) mean (±S.E.M.) open arm entries as percentage of total entries in the elevated plus maze, $N=45$, from mice tested during subjective day (dark bars) and subjective night (light bars). Inset: mean (±S.E.M.) open arm entries as percentage of total entries in a repeat test on the elevated plus maze (groups are arranged in same order as panel B). ***$p<0.0001$.

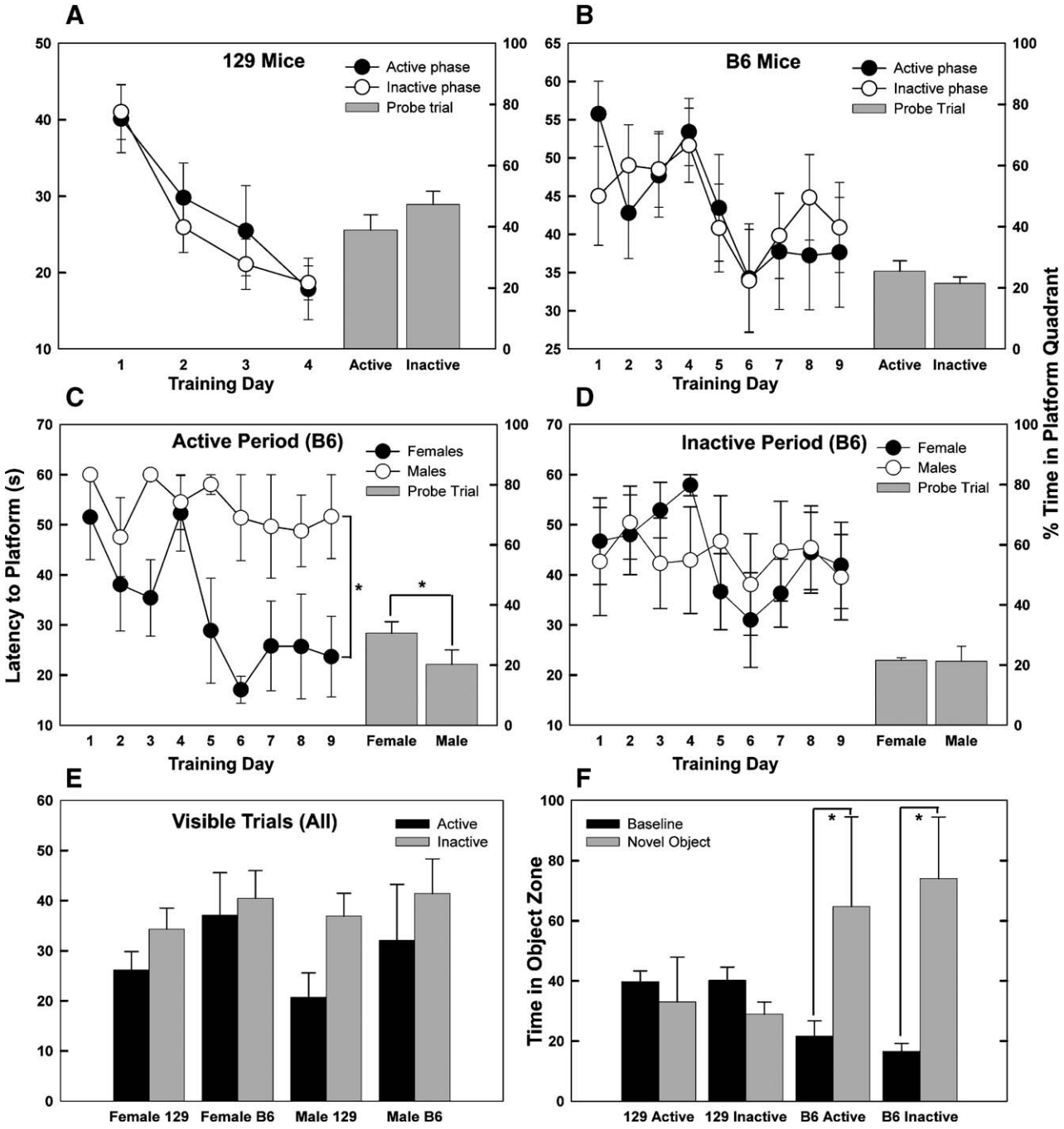

Fig. 4. Effect of circadian phase on Morris water maze performance and novel object exploration: (A) 129 mice trained for 4 days, four trials per day, $N=21$. Mean (±S.E.M.) latency to platform during training trials (line graph, left axis) and mean (±S.E.M.) percentage time spent in platform quadrant during probe trials (bar graph, right axis). (B) B6 mice trained for 9 days, one trial per day with a probe trial after every 3 days, $N=21$. Mean (±S.E.M.) latency to platform during training trials (line graph, left axis) and mean (±S.E.M.) time spent in platform quadrant during probe trials (bar graph, right axis). (C and D) Mean (±S.E.M.) latency to platform during training trials and percentage time spent in platform quadrant during probe trials between male and female B6 mice for active (C) and inactive (D) phase groups. (E) Mean (±S.E.M) latency to visible platform for all mice, $N=42$. (F) Novel object exploration for all mice, $N=39$. Baseline represents mean (±S.E.M) of activity 10 min prior to object placement. Novel object represents mean (±S.E.M) of activity 30 min after object placement calculated as average activity of three 10-min blocks. $^*p<0.05$.

time spent on the perimeter. We found no effect of circadian phase [velocity, $F(1,17)=0.502$, $p=0.488$; perimeter, $F(1,333)=2.99$, $p=0.085$] nor sex [velocity, $F(1,17)=4.07$, $p=0.06$; perimeter, $F(1,333)=1.06$, $p=0.305$] for either measure.

We considered the possibility of a ceiling effect in that the training was too intensive to detect subtle differences in learning between mice at different points in their circadian phase. Consequently, with the C57BL/6J mice, we used a much more stringent training protocol where mice received a single 1-min trial each day for 3 consecutive days with a probe trial administered on the fourth day. This cycle was repeated three times. As seen in both latency to find the platform across

training trials and the final probe trial (Fig. 4B), no effect of circadian phase can be observed [latency, $F(1,18)=0.017$, $p=0.899$; probe trials, $F(1,16)=0.427$, $p=0.523$]. Notably, during the probe trials (only final probe shown), the mice did not exceed more than 25% of the time in the platform quadrant suggesting that on this restrictive training protocol, the mice did not learn the task. However, the mice tested during their active and inactive phase performed equally poorly. Thus, we observed no main effects of circadian phase on performance in the Morris water maze. Although there was no main effect of sex on probe trials [$F(1,16)=0.161$, $p=0.694$], we did observe a sex effect on training trials [$F(1,18)=7.45$, $p=0.014$] and an interaction between circadian phase and sex on both training and probe trials [training, $F(1,18)=7.98$, $p=0.011$; probe trials, $F(1,16)=4.56$, $p=0.049$]. Fig. 4C shows the active phase mice split by sex where clearly the females learned the task while the males did not. A similar sex effect was not observed in the inactive phase mice (Fig. 4D). On measures of velocity and time spent on the perimeter (data not shown), we again found no effect of circadian phase [velocity, $F(1,17)=1.42$, $p=0.251$; perimeter, $F(1,14)=2.69$, $p=0.123$]. There was no main effect for sex on time spent in the perimeter [$F(1,14)=2.25$, $p=0.156$]; however, there was an effect of sex on velocity with the females swimming faster [$F(1,17)=6.12$, $p=0.024$].

Prior to the invisible platform trials, we conducted visible platform trials. We found a significant effect of circadian phase on latency to finding the visible platform [$F(1,34)=4.55$, $p=0.04$] with mice tested during their inactive phase taking longer to find the platform (Fig. 4E). There were no significant effects of strain [$F(1,34)=3.59$, $p=0.067$] nor sex [$F(1,34)=0.159$, $p=0.692$] nor circadian phase by sex or strain interactions [CT × sex, $F(1,34)=0.642$, $p=0.429$; CT × strain, $F(1,34)=0.447$, $p=0.508$]. As we used a bright orange ping-pong ball as a flag marking the visible platform, we considered that mice during their inactive phase might be more averse to novel stimuli. Consequently, we conducted a novel object exploration test (Fig. 4F). We found no difference in responses to a novel object between mice tested during their active or

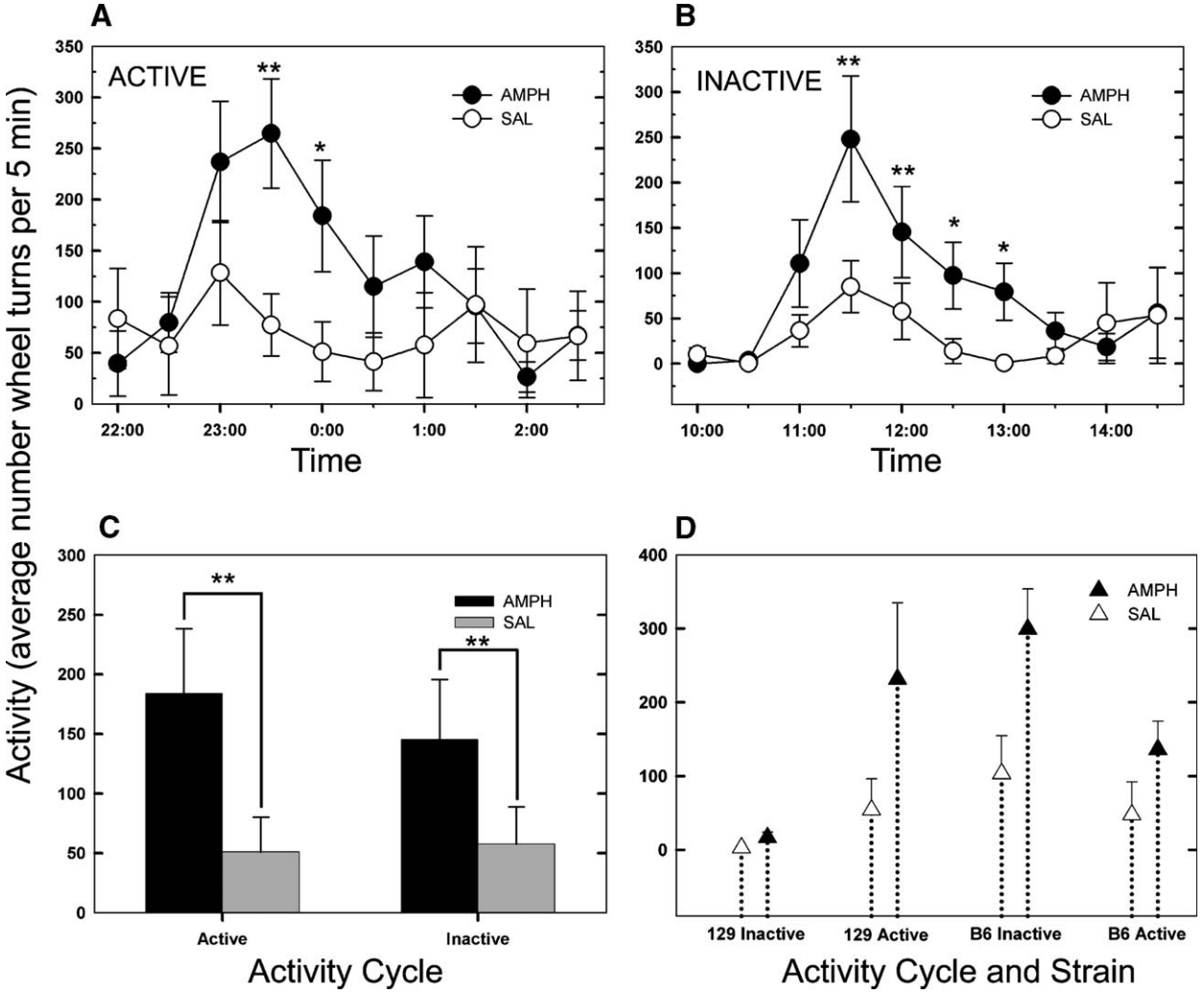

Fig. 5. Effect of circadian phase on amphetamine response: Activity response to 1.5 mg/kg amphetamine challenge (filled symbols) administered during active (A) and inactive (B) phases with saline control (open symbols), $N=45$. Arrowhead marks time of injection. (C) Comparison of amphetamine response between active and inactive phase mice 1 h post-injection (amphetamine, black bars; saline, gray bars), combined analysis of 129S1/SvImJ and C57BL/6 mice. (D) Comparison of amphetamine response between active and inactive phase mice 1 h post-injection (amphetamine, filled triangles; saline, open triangles) by strain. $^{*}p<0.05$, $^{**}p<0.01$.

inactive phase [object × CT, $F(1,31)=0.191$, $p=0.665$] nor any differences by sex [object × sex, $F(1,31)=1.96$, $p=0.172$]. A strain difference was observed where the B6 mice clearly responded to the novel object, while the 129 mice did not [strain × object, $F(1,31)=7.24$, $p=0.011$]; however, there was no interaction between circadian phase and strain in response to the novel object [object × CT × strain, $F(1,31)=0.299$, $p=0.588$].

3.5. Effects of circadian phase on response to amphetamine challenge

Mice were administered either amphetamine (AMPH) or saline (SAL) at either CT5 or 17 and returned to their home cages to monitor wheel running activity. A clearly observed increase in wheel running activity, greater than saline-injected groups, can be seen in response to the amphetamine injection administered during both the inactive and active phase (Fig. 5A,B, *p*-values indicated in figure). However, as shown in Fig. 5C, the magnitude of the response did not significantly vary according to circadian phase during which the challenge occurred [$F(1,41)=0.137$, $p=0.713$], despite the fact that the baseline activity level was very different between the two groups. We observed a statistically significant interaction [$F(1,37)=10.16$, $p=0.0029$] between strain and circadian phase (Fig. 5D). Interestingly, amphetamine appears to have had little impact on 129 mice during their inactive phase where it appears they largely went back to sleep. In contrast, the locomotor enhancing effects of amphetamine were less pronounced with the B6 mice during their active phase.

4. Discussion

The objectives in this study were to examine the effectiveness of low amplitude light cycles as zeitgeibers in a laboratory setting and to investigate the impact of circadian phase on commonly used mouse behavioral tests. The present data indicate that mice entrain to low amplitude dim/bright light cycles. It appears they do not entrain as tightly to a dim/bright schedule as a light/dark schedule and that there are strain differences. In this study, we observed that the 129 mice did not entrain as well as the B6 mice to the dim/bright photocycle but showed no difference on the light/dark photocycle. Nonetheless, both strains did adapt their activity cycles to synchronize with the dim/bright light cycle with a phase angle of entrainment of less than 2 h. Although expected based on earlier observations [21], we are not aware that anyone has tested this in a typical large animal facility where there is uncontrolled extraneous noise and other disruptions (frequent entry, cage changes, health checks). It may have been that low amplitude light cycles are only weak zeitgeibers, which our data suggest, and would not entrain within this environment. This, however, is not the case. Although entrainment is not as strong as with light/dark photocycles, mice clearly entrain to the low amplitude photocycles. Additionally, we are not aware that low amplitude entrainment has been specifically tested with these two laboratory strains of mice, among the most common in use today.

With respect to the effect of circadian phase on outcome in commonly used behavioral tests, on the whole, it made little difference whether mice were tested during their active, waking period or disturbed during their inactive sleep period. Except for the visible trials in the water maze, we observed no main effect of circadian phase on any of these tests. This cannot be attributed to a lack of sensitivity as the tests detected strain differences, as shown in the open field and elevated plus maze.

It is possible that the constant dim light housing and testing conditions used during the testing period exerted a constant masking effect which obscured or dampened circadian effects on behavior; however, this is unlikely as the animals' activity cycles observed in running wheel behavior were normal and similar to standard light/dark conditions. Moreover, the dim light level used, 15–20 lx, is not associated with significant behavioral inhibition. Alternative experimental designs, such as housing mice in constant dark during testing, introduce other confounds, such as possible masking effects arising from *relative* changes in illumination as mice are removed from dark housing to dim (but brighter) testing conditions. To compensate for this and do all tests in dark conditions defeats the practical aspect of the experiment, which is to study the effect of circadian phase on tests *as commonly administered*. Among possible (and practically feasible) designs, the present study minimizes confounding masking effects to the greatest extent. Thus, we conclude that the present data indicate that these particular tests are largely robust with respect to circadian phase.

This conclusion, however, needs to be tempered by the occasional observation of circadian interactions with other independent variables, such as sex and strain. For example, in the water maze, we observed an interaction between circadian phase and sex with the B6 mice but not the 129 mice. Within the amphetamine challenge, although there was no main effect of circadian phase, there was an interaction between phase and strain such that the 129 inactive group showed no significant amphetamine response and the B6 active group showed an attenuated response.

In a sense, this makes circadian phase a particularly devilish source of unreliability in behavior testing. While investigators clearly acknowledge and control for strain and sex, because differences arising from these factors are common, potentially large and robust, these same investigators may ignore circadian effects. Our data suggest that often, in fact, phase effects will make no difference; however, our data also suggest an unpredictable interaction may crop up unbeknownst to the investigator. For example, if two researchers test amphetamine response on a transgenic mouse line with a 129 background and one conducts tests during the inactive phase and the other during the active phase, our data suggest they will likely obtain contradictory results.

There has been little systematic investigation of the impact of circadian phase on the outcomes of mouse behavioral testing. Of the available, relevant literature most use rats as subjects. Only one study reviewed [23] addresses the effect of time-of-day on a battery of mouse behavioral tests, focusing specifically on high throughput mouse behavioral phenotyping. This study, however, did not control for lighting effects at time of testing.

It is well established that light can directly influence rodent behavior, a 'masking' effect, so-called because the effect of light can suppress overt expression of circadian rhythms [24,25]. Distinguishing between masking and circadian effects is crucial, as illustrated by a study in which the authors examined the effect of time of day on the activity of C57BL/6J mice in the open field [22]. The authors tested mice at six different times of day and found that mice were more active at the darker times; however, further experiments which controlled for illumination at the time of testing found no circadian phase effects. In the study by Hossain et al. [23], the authors found that testing mice during the dark phase improves the tests' ability to discriminate between phenotypes. However, because the dark phase mice were tested in the dark while the light phase mice were tested in light, it is unclear whether the observed differences in testing resulted from circadian phase effects or test illumination effects. The lack of a circadian effect found in both our data and Valentinuzzi et al.'s [22]–when test illumination was controlled–suggest that the group differences Hossain et al. [23] observed most likely arise from illumination conditions at the time of testing rather than circadian changes in performance.

In another study, Valentinuzzi et al. [26] examined the effect of circadian phase on water maze performance in rats and found that circadian phase did not affect spatial memory or learning; however, the authors observed that rats during the active and inactive phases exhibited different search patterns when seeking the platform and that rats during the inactive phase exhibited greater motivation to escape, using swimming speed as an index of motivation. Differences between this study and ours may reflect subtle differences between mice and rats in response to the water maze. We observed no difference in swimming speed between the two groups (data not shown), suggesting mice during both the active and inactive phase are equally motivated to escape the water. Interestingly, their suggestion that active- and inactive-phase rats may employ a different search strategy may be a plausible explanation for the difference we observed between the two groups on the visible trials, although we did not investigate this possibility. This potential difference in search patterns between active and inactive phase groups, however, did not impact performance on the hidden platform trials.

The lack of an observed circadian phase main effect in these experiments might arise as a consequence of the nature of the tests themselves. All tests examined here comprise high stimulation, low demand tasks that may sufficiently engage the mice such that their arousal and performance is comparable regardless of what phase in their circadian cycle the task is presented. That is, being tossed in a large, cold pool of water (or poked with a needle, or placed on narrow lanes with a precipitous drop in an unfamiliar place) may simply engage a response system not subject to significant circadian fluctuation.

Consistent with this hypothesis, Chaudhury et al. [27] observed a circadian phase effect on learning in the commonly used fear-conditioning paradigm; however, this was only evident when using very low current for training. At any training shock above 0.3 mA, no difference was observed. The Chaudhury study demonstrates that circadian phase does play a role in this type of learning; however, at the level of stimulation used by most behavioral studies (0.5 to 0.8 mA), circadian phase effects are no longer evident. Thus, the fear-conditioning paradigm as commonly employed is robust with respect to circadian phase.

Other behavior tests and paradigms may be susceptible to circadian effects. For example, Gaytan et al. [28] found, consistent with our findings, that in rats the locomotor enhancing effects of amphetamine were not influenced by circadian phase; however, they found that the inhibitory effect of higher doses were influenced by circadian cycles. Moreover, a subsequent study found that sensitization to amphetamine was sensitive to time of day at which the drug was administered [29]. This suggests that other established tests, such as the resident–intruder paradigm or conditioned place preference, as well as emerging testing paradigms, including home cage monitoring and high throughput approaches, need to be examined individually to assess for circadian phase effects. Moreover, new tests are likely to be developed, which might target more refined behavioral phenotypes and/or have less stimulating/disruptive and more demanding characteristics, which may increase the impact of circadian phase effects on outcome. Thus, we would offer a narrow interpretation of our results and not generalize beyond the specific tests examined. With these tests, however, as commonly administered, we demonstrate that circadian phase does not exert any significant main effects on test outcome but that occasional interaction effects with other variables may be encountered.

Identifying and controlling factors that contribute non-experimental variability to behavioral testing can be difficult [7]. Although our present data indicate that some of the most commonly used behavioral tests are largely not affected by circadian fluctuations, it would be imprudent to generalize these results to mouse behavioral work generally, particularly in light of the interaction between circadian phase and other factors we observed in some instances. As examining and controlling the effect of circadian phase in every investigation is impractical, a standard approach to managing the circadian cycles of nocturnal laboratory animals such that they are tested during their active phase would be advantageous. A recently reported study [30] examined the use of sodium lamps to maintain illumination during the dark phase of mice maintained in a reverse cycle. This type of light does not interfere with the animals' dark phase but allows researchers and staff to work comfortably. Our data suggest that this same reverse phase strategy may work equally well with standard white light where dim light substitutes for darkness. Earlier work on low amplitude entrainment indicates that the effectiveness of a light cycle as a zeitgeiber is dependent not on the absolute intensity of the light but on the ratio between its maximum and minimum [21]. Thus, it is likely a level of dim illumination could be established that would be comfortable for people working with the mice without interfering with the animals' dark phase. Moreover, adjusting the ratio between dim and bright light may improve the entrainment resulting in lower phase angles of entrainment than we observed in this study.

Although our concern focuses on behavioral investigation, the effect of circadian phase may be equally important for physiological, cellular and molecular oriented investigations

where circadian biochemical fluctuations may directly impact the processes and systems being studied (for example, see [31–36]). Thus, assessing the impact of this often ignored variable on experimental results and devising a strategy for managing circadian phase represents an important and inadequately examined concern for all investigators using mice.

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