3-1-2007

Behavioral and Neurochemical Sources of Variability of Circadian Period and Phase: Studies of Circadian Rhythms of *npy*-/- Mice

Mary E. Harrington
Smith College, mharring@smith.edu

Penny Molyneux
Smith College

Stephanie Soscia
Smith College

Cheruba Prabakar
Smith College

Judy McKinley-Brewer
Smith College

See next page for additional authors

Am J Physiol Regul Integr Comp Physiol 292: R1306–R1314, 2007.
First published November 2, 2006; doi:10.1152/ajpregu.00383.2006.

Behavioral and neurochemical sources of variability of circadian period and phase: studies of circadian rhythms of *npy−/−* mice

Mary Harrington, Penny Molyneux, Stephanie Soscia, Cheruba Prabakar, Judy McKinley-Brewer, and Gurprit Lall

Neuroscience Program, Smith College, Northampton, Massachusetts

Submitted 2 June 2006; accepted in final form 17 October 2006

Harrington M, Molyneux P, Soscia S, Prabakar C, McKinley-Brewer J, Lall G. Behavioral and neurochemical sources of variability of circadian period and phase: studies of circadian rhythms of *npy−/−* mice. *Am J Physiol Regul Integr Comp Physiol* 292: R1306–R1314, 2007. First published November 2, 2006; doi:10.1152/ajpregu.00383.2006.—The cycle length or period of the free-running rhythm is a key characteristic of circadian rhythms. In this study we verify prior reports that locomotor activity patterns and running wheel access can alter the circadian period, and we report that these treatments also increase variability of the circadian period between animals. We demonstrate that the loss of a neurochemical, neuropeptide Y (NPY), abolishes these influences and reduces the interindividual variability in clock period. These behavioral and environmental influences, from daily distribution of peak locomotor activity and from access to a running wheel, both act to push the mean circadian period to a value < 24 h. Magnitude of light-induced resetting is altered as well. When photoperiod was abruptly changed from a 18:6-h light-dark cycle (LD18:6) to LD6:18, mice deficient in NPY were slower to respond to the change in photoperiod by redistribution of their activity within the prolonged dark and eventually adopted a delayed phase angle of entrainment compared with controls. These results support the hypothesis that nonphotic influences on circadian period serve a useful function when animals must respond to abruptly changing photoperiods and point to the NPYergic pathway from the intergeniculate leaflet innervating the suprachiasmatic nucleus as a circuit mediating these effects.

suprachiasmatic nuclei; nonphotic cue

CIRCADIAN RHYTHMS are biological rhythms that can be synchronized to a variety of environmental stimuli, characterized by an endogenous period or cycle length. In rodents circadian rhythm precision can be measured either as day-to-day variability in one individual or as animal-to-animal variability within a group of individuals. Both measures of variability are related to average period, with variability increasing the farther the average is from 24 h (38). The master circadian pacemaker for locomotor activity rhythm in mammals is located in the suprachiasmatic nuclei (SCN) of the hypothalamus. Isolated SCN explants and even individual SCN neurons can exhibit circadian rhythms in vitro (1). Day-to-day variability is greater in recordings from individual SCN neurons compared with SCN explants, which have variability similar to that seen in wheel-running activity recordings (16). Because isolated SCN explants were shown to express similar mean period and similar variability in period as observed in behavioral recordings, the level of day-to-day variability in behavioral rhythms might be a property of the SCN. On the other hand, the variability of period might be a property of the multioscillator system, in that one study reports a weak negative correlation between the period of behavioral rhythms and the period of SCN rhythm in vitro (1).

The SCN receives several important inputs (32). Photic information is relayed to the SCN via the retinohypothalamic tract. Input from the intergeniculate leaflet (IGL), which utilizes neuropeptide Y (NPY) among other neurotransmitters, is thought to largely mediate nonphotic influences on the SCN but is also implicated in modulating photic responses, in particular, effects of constant light on circadian period.

Both photic and nonphotic stimuli interact to modulate circadian period. Constant light generally lengthens circadian period of nocturnal animals and can induce arrhythmicity or "splitting" of the 24-h rhythm into two components coupled ~12 h apart (39). The time of day of peak locomotor activity or the availability of a running wheel can modify free-running period in constant darkness (8, 46, 47). Both photic and nonphotic cues can induce abrupt shifts in phase of free-running rhythms when presented as brief stimuli (22). It is likely that photic-nonphotic interaction plays a key role in entrainment to the natural environmental light-dark (LD) cycle. Entrainment is accomplished by modulation of circadian period by photic and/or nonphotic cues, and it appears these inputs may converge on the circadian clock genes *per1* and *per2*. Nonphotic inputs can alter light-induced gene expression, with the *per2* gene showing especially long-lasting changes (2, 29). Regulation of entrainment is especially important in seasonal responses to natural variation in photoperiod. Prior studies suggest an important role for nonphotic inputs in modulating seasonal responses (12, 19, 30, 31, 42).

In this study, we compared circadian rhythms in mice deficient for NPY (*npy−/−*) with those in wild-type (WT) mice. We found replicable changes in variability within these groups, as well as changes in mean period, photic resetting, and light-induced *per2* mRNA. We explain these differences as attributed to nonphotic influences and discuss how these behavioral and environmental influences increase variability in circadian period and allow speedier responses of locomotor activity to altered photoperiods.

MATERIALS AND METHODS

Animals

Experiment 1 used 129S6 WT and 129S6 *npy−/−* mice. Experimental animals were bred in house with littermates from homozygous breeding pairs (provided by Dr. Richard Palmiter, Department of Biochemistry, University of Washington, Seattle, WA; Refs. 11, 28).

Address for reprint requests and other correspondence: M. E. Harrington, Neuroscience Program, Smith College, Northampton, MA 01063 (e-mail: mharring@email.smith.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Experiment 2 used the same 129S6 *npy*−/− mice but further back-crossed for 15 generations onto a C57BL/6 background at Taconic Farms (Germantown, NY; B6.129-*npy*^{tm1} N15), provided by E. Maratos-Flier (Harvard Medical School, Boston, MA; Ref. 41). Experimental animals were bred in house from heterozygous parents and consisted of animals lacking the *npy* gene and their WT littermate controls. Mice were weaned at 3 wk and housed under a 12:12-h LD cycle (LD12:12) unless otherwise noted. Food and water were available ad libitum.

In *experiment 1*, because of constraints in colony size, both males and females were tested. Age of mice varied, ranging from 3 wk to 4 mo. In *experiment 2*, only males at 8 ± 1 wk of age at the start of the experiment were used.

The experiments presented in this article were all reviewed and approved by the Smith College Institutional Animal Care and Use Committee.

Locomotor Activity Assessment

Mice were housed in cages fitted with running wheels (15 cm in diameter). Wheel revolutions or locomotor activity was recorded with Clocklab computer software, with 1-min sampling epochs (Actimetrics, Evanston, IL). In *experiment 1*, mice were initially placed in LD12:12 for 7 days. Animals were placed under constant conditions of dim red light (DD) for 7 days (15 W; Coastar). On the eighth day under DD, animals were exposed to a brief light pulse (150 lx) at circadian time (CT)16 (4 h after activity onset) for 15 min. The experiment ended on *day 15* of DD.

In *experiment 2*, mice were housed in cages with running wheels for the first part of the study. Daily locomotor activity of the mice was also monitored for a portion of *experiment 2* with passive infrared detectors (model K-940, Visonic, Bloomfield, CT) installed over each cage at a distance of 20 cm from the cage top. The passive infrared motion detector works by sensing abrupt changes in position of the animal against the cooler background of its environment. If a sufficiently large position change occurs, the detector momentarily closes a switch; such closures are counted to compile an actogram. Each detector had an LED indicator, which is normally turned on during the switch closure interval; these indicators were disabled by resetting an internal jumper so that the emitted light would not exert an unwanted influence on the animal.

In *experiment 2*, mice were exposed to LD12:12 until *day 23*, when they began the first episode of constant darkness (DD1) interrupted by a 4-h light stimulus on *day 35* timed for each animal to fall at CT14–CT18. The LD cycle was resumed on *day 44*. The second episode of constant darkness (DD2) began on *day 60*, with a 15-min light pulse at CT16 on *day 70*, and the LD cycle resumed on *day 91*. Motion sensors were installed on *day 149*. The third episode of constant darkness (DD3) began on *day 161*. Wheels were removed on *day 175*, allowing measures of the response to constant darkness in the absence of the wheel (DD4). An LD18:6 cycle began on *day 192* and was changed to LD6:18 on *day 218* by ending the light period 6 h early on *day 217* and beginning the light period 6 h after the normal start time for the previous cycle on *day 218*. Total duration of the study was 241 days (34.4 wk). Standard overhead room lighting used for entrainment and maintenance averaged 50 lx (General Electric F40SP35). Light pulses in *experiment 2* were 450 lx (Sylvania F40DSGN50 bulbs). Dim red light was not present continuously during dark periods in *experiment 2*, but only used for routine checks of the animals.

Behavioral Analysis

Activity onset was determined with ClockLab, with the default window settings of 6 h off and 6 h on. Occasionally, the program selected as an onset a time that appeared well outside of the expected range, and in those instances the onset time for that day was deleted or edited to a bout of activity if the record showed an unambiguous onset bout. Measures of period as reported here are from regression lines fit to the activity onsets, but, because of the element of subjective "eye-fit" given this occasional editing, χ^2 periodograms were also calculated on the same data. Periodogram estimates of circadian period always showed high correlations with regression-line estimates ($r > 0.93$ in all samples). The mean time of activity onset over a period of 7–10 days in an LD cycle was calculated to assess phase angle of entrainment. The free-running period for each individual animal was computed from the days under DD preceding light pulse treatments, or before and after wheel removal, using 7 days in *experiment 1* and 10-day segments in *experiment 2*. We assessed the amplitude of the circadian component from a Fourier analysis using these same segments of DD. Phase shifts to light were measured by comparing predicted activity onset for the day after the light pulse from extrapolated lines fit to activity onsets 7 days immediately before pulse and 7 days after pulse starting from the day following the pulse. All calculations and figures were derived from Clocklab software. Animals were considered reentrained once the activity onset occurred with a stable phase relationship to dark onset. All results are reported as means ± SE.

In Situ Hybridization

WT 129S6 and 129S6 *npy*−/− mice ($n = 20$; 13 males, 7 females) were housed under LD12:12. At zeitgeber time (ZT)16, half of the animals ($n = 10$) were exposed to a 15-min light pulse (200 lx), while the other half ($n = 10$) remained in the dark. At ZT17.5 (1.5 h after exposure), the animals were overdosed with halothane and killed. The brains were removed and stored at −80°C with tools that were RNase free. The brains were sectioned on a cryostat, and SCN slices (20 μm) were collected on Superfrost slides. The slices were evaluated for *per2* mRNA by in situ hybridization.

The brain tissue was fixed in 4% formalin in 1× phosphate-buffered saline, rinsed in 2× saline sodium citrate (SSC), and treated with 1× triethanolamine with 0.25% acetic anhydride (2). Radiolabeled [^{35}S]-cRNA probes were transcribed from a 480-bp mouse per2 fragment (GenBank No. AF035830 nt9-nt489) inserted in pCRII vector (Invitrogen), a gift from Lauren Shearman and S. Reppert

Table 1. *Behavioral measures from npy−/− and wild-type mice in experiments 1 and 2*

Measure	*Experiment 1 npy−/−*	*Experiment 1* WT	*Experiment 2 npy−/−*	*Experiment 2* WT
Phase delay to 15-min LP at CT16	−1.47±0.11* (n=9)	−2.56±0.24 (n=6)	−1.52±0.13*(n=10)	−2.08±0.18 (n=11)
Phase delay to 4-h LP at CT14			−2.55±0.31 (n=10)	−2.67±0.13 (n=11)
Free-running period in DD1	24.13±0.03*† (n=9)	23.69±0.13 (n=6)	23.93±0.03*(n= 9)	23.73±0.08 (n=12)
Free-running period in DD2			23.84±0.02*† (n=10)	23.62±0.06 (n=12)
Free-running period in DD3			23.99±0.04*(n= 9)	23.76±0.06 (n=12)
Free-running period in DD3 after wheel removal			23.95±0.02*† (n= 9)	23.66±0.05 (n=12)

Values (in h) are means ± SE for n animals, WT, wild-type mice; *npy*−/−, mice deficient in neuropeptide Y; LP, light pulse; CT, circadian time; DD1, 1st episode of constant darkness; DD2, 2nd episode of constant darkness; DD3, 3rd episode of constant darkness. **npy*−/− and WT groups show statistically significant differences in values ($P < 0.05$); †*npy*−/− and WT groups show statistically significant differences in variability ($P < 0.05$).

(Harvard Medical School). The probe was applied to the tissue at 1×10^6 cpm/25 μl buffer, after which it was allowed to hybridize for ~16–20 h. Posthybridization of the SCN tissue involved 50% formamide washes at 52°C, RNase treatment (50 μg/ml), numerous SSC rinses, and ethanol dehydration. The slides were air dried, exposed to film for 3 days, and then emulsion dipped. This process allowed us to scan for any spatial differences in expression between the groups during our analysis. Levels of *per2* mRNA in the SCN were quantified with SCION imaging software.

RESULTS

Behavioral Results

Experiment 1 (129S6 background). Under LD12:12, *npy*−/− and WT mice showed similar phase angles of entrainment. In DD, however, the *npy*−/− mice had significantly longer free-running rhythms than the WT mice, and when exposed to 15-min light pulses at CT16 *npy*−/− mice showed significantly smaller phase shifts (see Table 1 and Fig. 1). The two groups did not statistically differ by age, and age was not correlated with free-running period.

Experiment 2 (C57BL/6 background). Similarly to *experiment 1,* when housed under LD12:12, *npy*−/− mice did not differ from WT mice in time of activity onset or time of peak activity. The *npy*−/− mice did show significantly fewer wheel revolutions during the dark phase compared with WT mice [*npy*−/− mice: 11,368 ± 2,136 vs. WT: 17,341 ± 2,004, $t(19) = 2.45$, $P = 0.02$] but did not differ in the number of revolutions in the light period or in the total daily revolutions

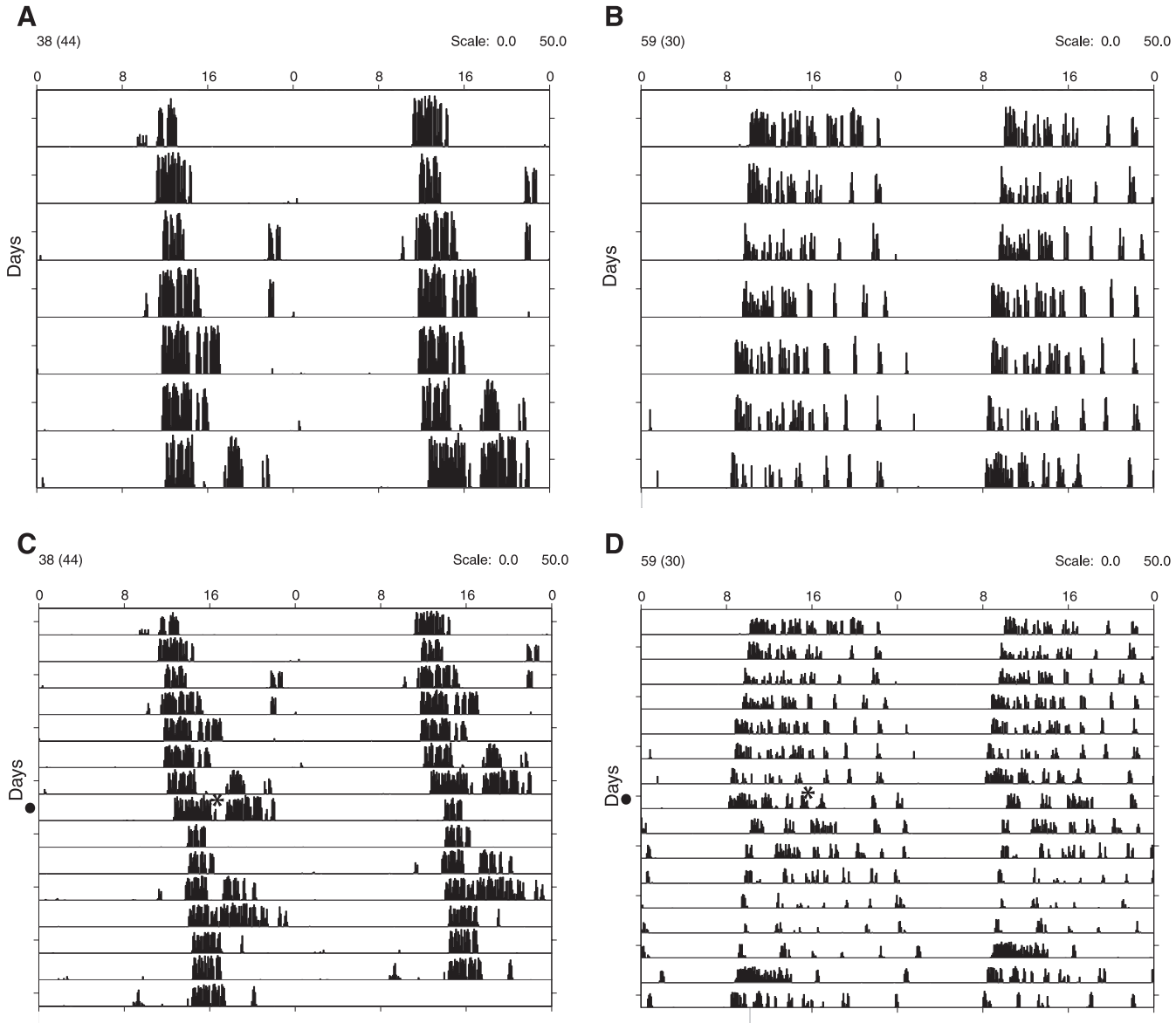


Fig. 1. Actograms from mice in *experiment 1*. *A*: neuropeptide Y (NPY)-deficient (*npy*−/−) mouse in constant darkness. The free-running period for this animal is 24.11 h. *B*: wild-type (WT) mouse in constant darkness. The free-running period for this animal is 23.69 h. *C*: response to 15-min light pulse. The day of the light pulse is denoted with a black dot and the time of the pulse with a star. The phase shift for this animal is −1.26 h. *D*: response of a WT mouse when presented with light at CT16. The phase shift for this animal is −2.35 h. Actograms show days on the *y*-axis and hours on the *x*-axis. Activity levels are marked by black bars. This actogram is double plotted, showing the subsequent day both below and adjacent to the previous day.

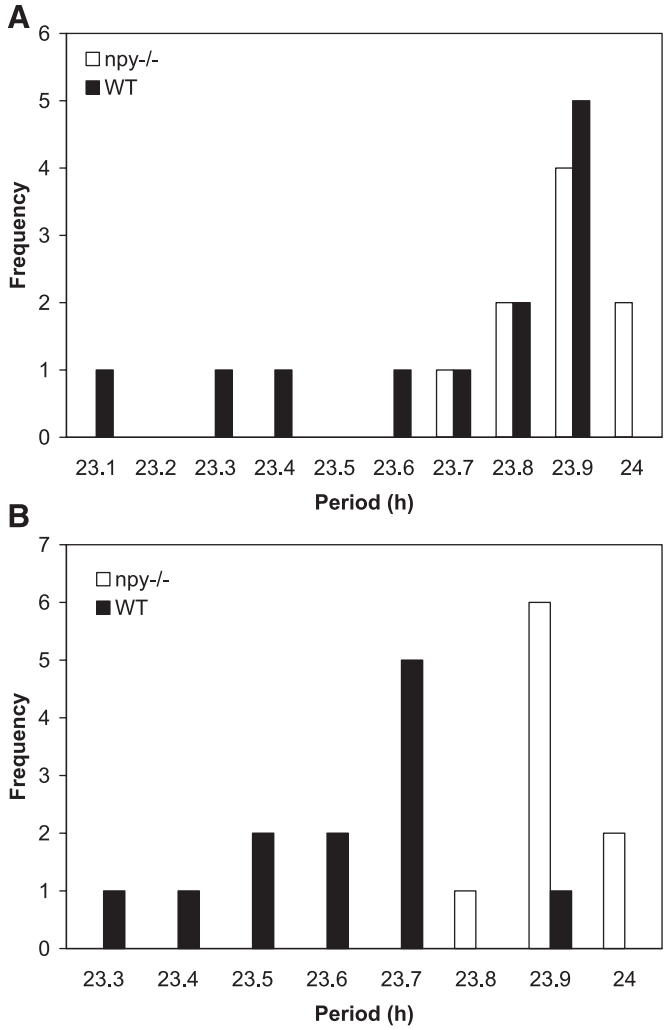


Fig. 2. Frequency histograms showing distribution of measures of free-running period (h) in constant darkness for *npy*−/− mice and WT mice. *A*: 1st exposure to constant darkness in *experiment 2*, age 8 wk. *B*: final exposure to constant darkness in *experiment 2*, after removal of the running wheels, age 31 wk. See Table 1 for statistical summary.

(assessed over 7 days under LD12:12). There was no difference between the groups in the phase delay to light exposure from CT14 to CT18, but we replicated our previous study showing a decreased phase delay to a 15-min light pulse at CT16 (see Table 1).

With three separate measures of free-running period in constant darkness in this study, we found in all instances that the *npy*−/− mice showed longer period rhythms than the WT controls (see Table 1 for summary of estimates of period). Within-group variability was greater for the WT mice than for the *npy*−/− mice (Levene test statistic = 5.183, df = 1, 20, $P = 0.034$; DD2; see Table 1), an effect still observed after removal of the running wheels [DD4; Levene test statistic = 5.332, degrees of freedom (df) = 1,19, $P = 0.032$; see Table 1 and Fig. 2]. The increased variability in circadian period was not associated with altered levels of activity, in that circadian period was not significantly correlated with average daily counts per minute, and average daily wheel revolutions did not differ with genotype (examined for the portions of the experiment labeled DD2 and DD4 in Table 1). The amplitude of the circadian component (0.04 cycles/h) from a Fourier analysis on the DD records did not show differences between the groups.

Analysis of the activity distribution in 30-min bins during the first full day of the first two exposures to DD (i.e., DD1 and DD2) for each individual in *experiment 2* showed an effect of time of peak activity on free-running period for that exposure to DD in WT mice, as previously reported from a sample of 100 mice (8). Free-running period was shorter in mice with peak activity earlier in the subjective night. Our sample of *npy*−/− mice did not show evidence for this relationship (see Fig. 3*A*).

A paired *t*-test indicated that WT mice did significantly lengthen free-running period in response to wheel removal [$t(11)=4.04$, $P = 0.002$], but *npy*−/− mice showed no such significant effect [change in free-running period following removal of the running wheels: *npy*−/− mice 0.04 ± 0.04 h ($n = 9$), WT 0.10 ± 0.02 ($n = 12$); see Fig. 4]. After wheel removal, the free-running period remained significantly different between the two groups (see Table 1).

When the photoperiod was abruptly altered from LD18:6 to LD6:18, *npy*−/− mice took an average of 10.3 days longer to

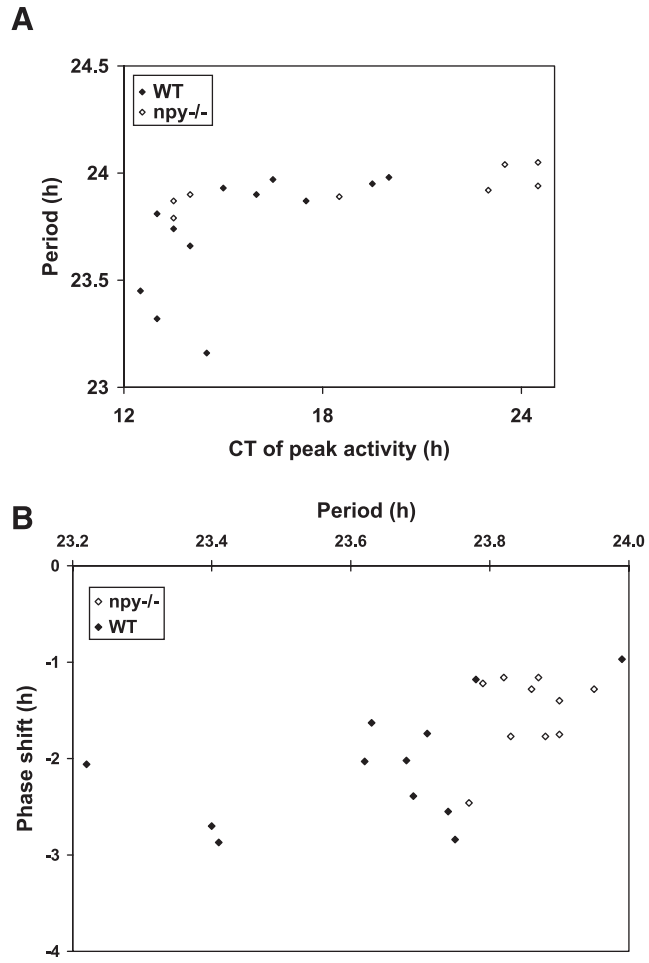


Fig. 3. *A*: scatterplot of the time on the 1st day of constant darkness when peak activity was expressed vs. the free-running period during the subsequent free run in constant dark (DD1). CT, circadian time. *B*: scatterplot showing relationship between magnitude of phase delay shift to a 15-min light pulse at CT16 vs. free-running period in constant darkness preceding the light pulse for *experiment 2*.

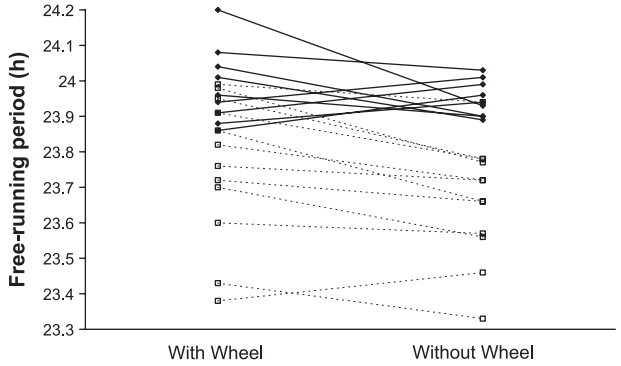


Fig. 4. Change in the free-running period of individual mice as measured by motion sensors either when housed with a running wheel (with wheel) or when the running wheel had been removed (without wheel). Filled symbols, *npy*−/− mice; open symbols, WT mice. Lines connect measures from each individual.

reentrain and to expand their activity period compared with WT mice [days to reentrain: *npy*−/− 18.1 ± 0.8, WT 7.8 ± 1.4 days; $t(19) = 5.84$, $P < 0.001$]. The *npy*−/− mice showed a more negative phase angle of entrainment under LD6:18, starting activity several hours after dark onset even once reentrained [see Fig. 5; time of activity onset relative to dark onset: *npy*−/− −1.0 ± 0.3 h vs. WT 0.2 ± 0.2 h; $t(19) = 2.62$, $P = 0.017$]. There was no difference between the *npy*−/− and WT mice in phase angle of entrainment to LD18:6.

Although body weight was not the focus of our study, we noted that mice differed in body weight at 40 wk of age [*npy*−/− 40 ± 2 g vs. WT 34 ± 1.2 g, $t(19) = 2.41$, $P = 0.01$].

In Situ Hybridization

The level of *per2* induced in both *npy*−/− and WT mice (129S6 background) that were exposed to both light and dark stimuli was measured by quantifying the density of *per2* mRNA in the SCN. A statistically significant difference was found between both groups (see Fig. 6). *per2* mRNA levels were higher in the animals exposed to light compared with those exposed to the dark [$F(3,13) = 7.96$, $P = 0.003$]. In addition, the WT mice showed higher levels of *per2* induction compared with *npy*−/− mice [$t(8) = 2.63$, $P = 0.03$].

DISCUSSION

Nonphotic influences of locomotor activity and wheel-running activity were shown to alter both average circadian period and variability of circadian period. Mice deficient in NPY showed a circadian period more tightly controlled near 24 h. A concurrent effect may be a diminished behavioral response to changes in photoperiod, as a timely response to abrupt expansion of the dark phase appears to depend on integrity of nonphotic influences. Phase angle of entrainment was altered only under the short photoperiod of LD6:18, but not under LD12:12 or LD18:6.

Although running wheels are commonly used in studies of circadian rhythms, their presence can alter circadian period (8), an effect that disappears after ablation of the IGL (24). Simply the size of the running wheel can have a significant effect; mice with larger wheels showed greater total amounts of activity and shorter circadian periods, along with greater phase delay shifts to light (5). We saw a small effect of wheel absence on circadian period in our WT mice, but the *npy*−/− mice did not show a change in period when the wheels were removed. Studies of nonphotic effects on rhythms often examine the phase-shifting effect of a subjective day treatment such as presentation of a novel running wheel, but mice appear to be resistant to behaviorally elicited nonphotic influence (3), although they can show pharmacologically induced nonphotic phase resetting (6, 27). Instead, we measured another type of nonphotic effect previously described in mice. Locomotor activity can alter free-running rhythm period depending on when in the subjective night the mouse is most active (8). In our study, we observed such nonphotic effects in WT mice (see Fig. 3*A*), but *npy*−/− mice did not demonstrate a similar relationship between an early subjective night time of peak

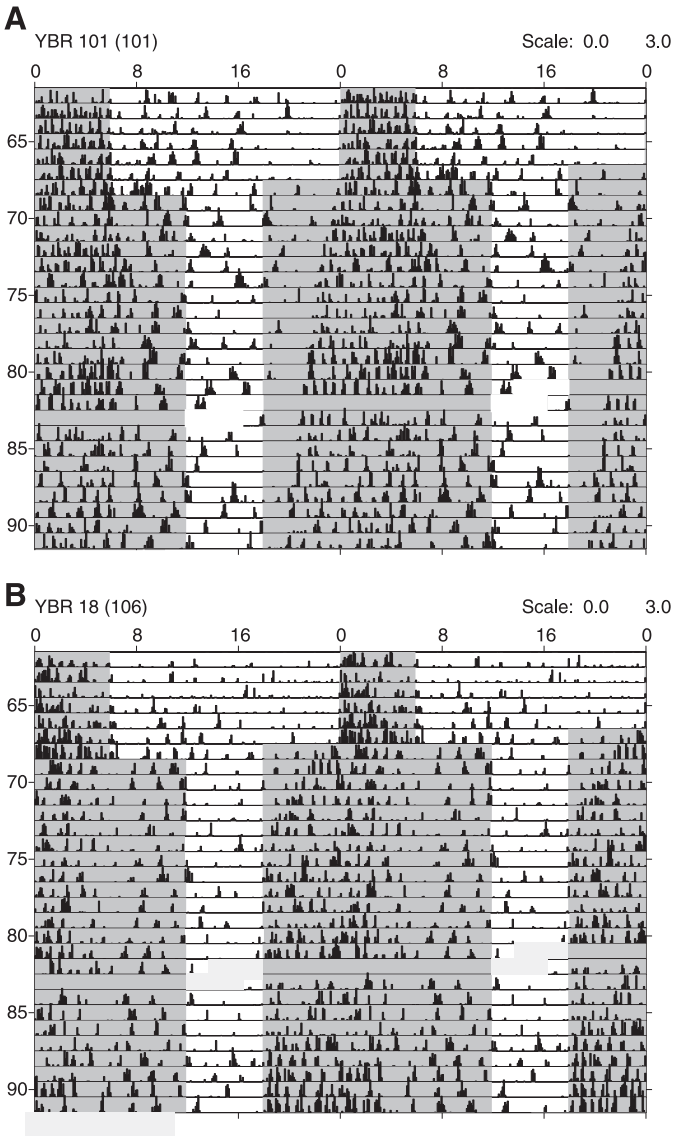
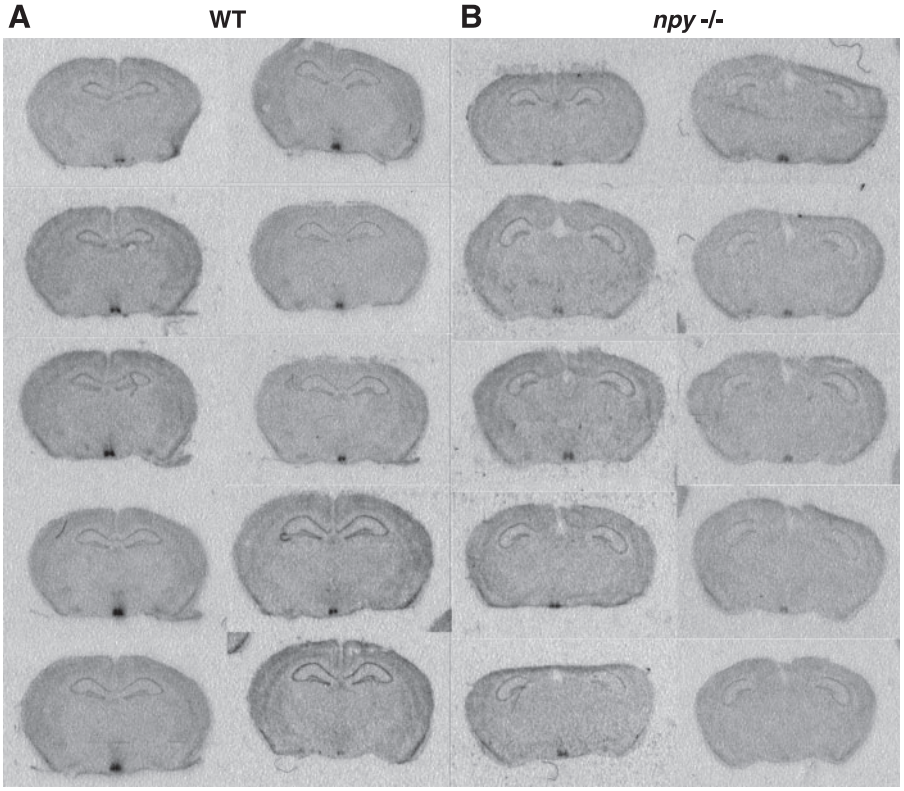


Fig. 5. Activity records from motion sensors as mice transition from LD 18:6 to LD 6:18. *A*: *npy*−/− mouse. *B*: WT mouse. The break in the record indicates a day when the computer was not taking in data. Shading indicates times when room lights were off except for the time when the computer was malfunctioning on *days 81–82*. On these days the light cycle was as on the previous days and was not affected by the computer problems.



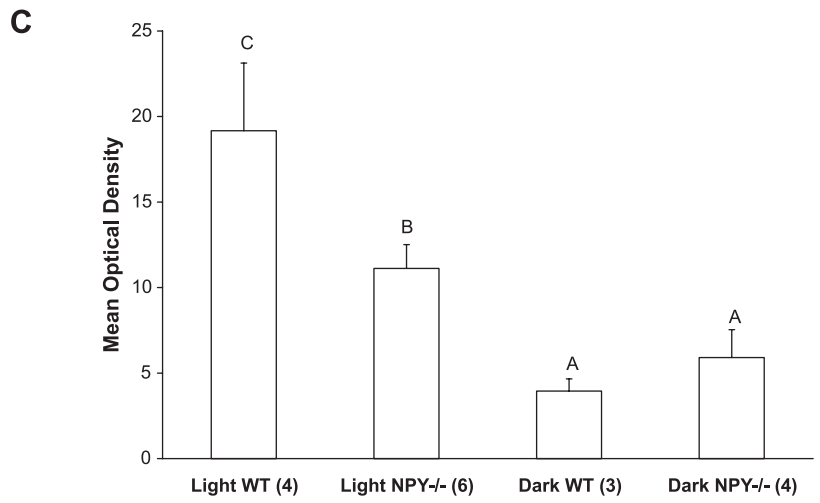


Fig. 6. Mice deficient in NPY showed reduced light-induced *per2* mRNA in the suprachiasmatic nuclei (SCN). *A*: sections through the brain showing the SCN and surrounding areas from a light-exposed WT mouse. *B*: similar series of sections from a light-exposed *npy*−/− mouse. *C*: summary of quantified results. Groups A, B, and C (over bars in *C*) are significantly different from each other ($P < 0.05$).

daily activity and shorter circadian period. Our results suggest that these effects of locomotor activity on period may be mediated by NPY.

The period length of the free-running rhythm was significantly greater, and phase delays to 15-min light pulses were reduced, in *npy*−/− mice relative to the WT control mice. These two findings are likely to be closely related, as prior studies have shown that period length and phase shift amplitude change in a coordinate manner (Ref. 4; see Fig. 7*B* in present study). Studies conducted on IGL-ablated mice (25, 35), hamsters (36), and rats (24) show increased period in constant darkness, similar to our results with *npy*−/− mice (but see Refs. 7, 33). On the other hand, prior studies of the effects of IGL lesions on light-induced phase shifts have produced variable results across species. It is interesting that the study showing no effect of IGL lesions on light-induced shifts used longer-duration light pulses (1 h; Ref. 40) while studies finding smaller advance shifts in IGL-lesioned hamsters used shorter pulses (15 min; Refs. 15, 36). A recent study indicates that IGL-lesioned hamsters show reduced phase advances to short-duration (5 min) light pulses but no alteration in responses to longer-duration pulses (34). We did not observe differences between *npy*−/− and control mice when using long-duration light pulses but did see reduced phase shifts when using shorter-duration light pulses, supporting the idea that this input is particularly relevant for responses to brief light stimuli. NPY can block photic phase shifts in mice in vitro (43), leading to an expectation of increased photic phase shifts in the *npy*−/− mice, an expectation not supported by our data.

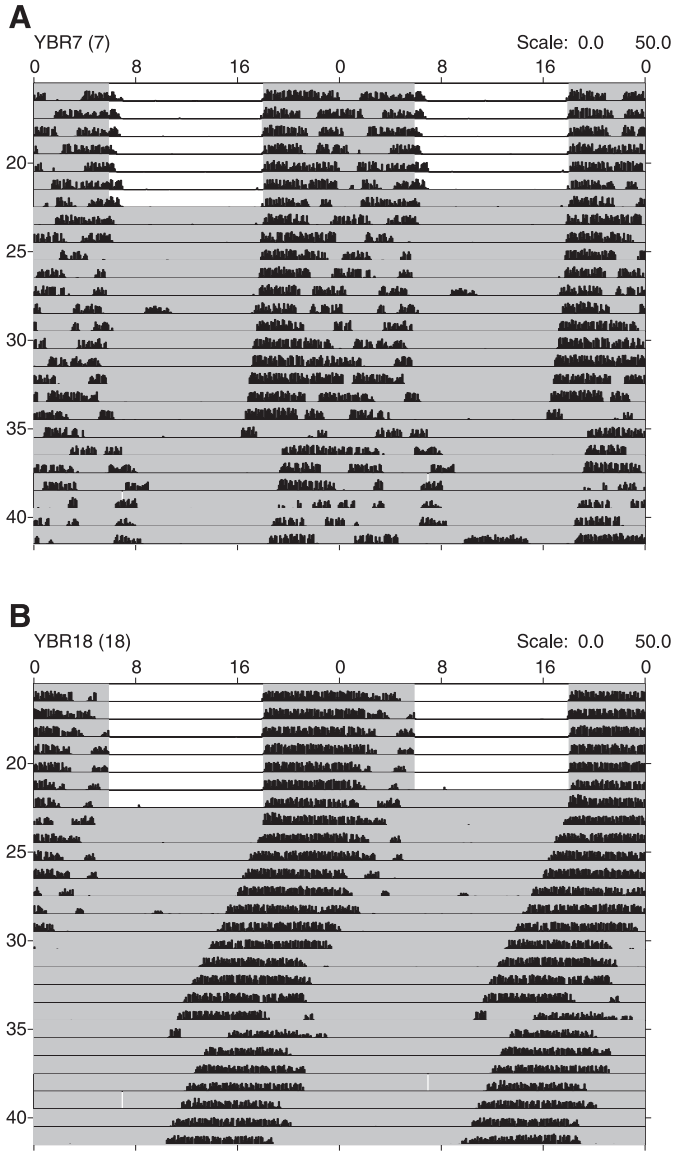


Fig. 7. Actograms from *experiment 2* showing initial entrainment to 14:10-h light-dark cycle (LD14:10) (time of lights on is shown as a gray background), free-running rhythm in constant darkness, and response to a 4-h light pulse on *day 35*. *A*: *npy*−/− mouse with a free-running period of 23.87 h and a phase delay of −3.26 h in response to the light stimulus. *B*: WT mouse with a free-running period of 23.32 h and a phase delay of −2.74 h. Mice were given fresh cages during the subjective day on *days 28* and *42*.

Phase shifts to light have been shown to induce the expression of a number of clock-related genes, notably the Period genes *per1* and *per2*. In the present study we have shown that *npy*−/− mice show diminished light-induced *per2* mRNA relative to WT controls. The *per2* gene was implicated in several studies of NPY and nonphotic phase resetting (2, 29, 48). It is not clear if the reduction in light-induced *per2* in *npy*−/− mice indicates a particularly close relationship between NPY and light-induced *per2* gene expression or is simply a reflection of the decreased-magnitude phase shift. Other studies support the idea that *per1* may be critical for nonphotic phase resetting, as, for example, suppression of *per1* can induce nonphotic phase resetting (13) and NPY can alter light-induced *per1* (2, 29). Further work is necessary to clarify the relative importance of *per1* and *per2* in these pathways.

The *npy*−/− mice showed free-running periods closer to 24 h than WT controls, with less within-group variability, mirroring results from other species indicating reduced variability in period as it approaches 24 h (37). Mice with deletion of the prion protein gene showed similar effects on circadian period, with period being less variable and closer to 24 h under constant darkness (see Fig. 1 in Ref. 45). Although several papers link prion disease with alterations in NPY (see Ref. 17 for a review), there is not much more than a common link to circadian rhythms and sleep to connect these two proteins. It is possible that circadian system plasticity is constrained so that changes in period that bring period closer to 24 h also reduce variability, perhaps to compensate for the potential instability in phase angle of entrainment when period is close to 24 h (37). We expect that neuronal coupling mediates circadian rhythm precision, given prior work demonstrating a concordance between precision of period in an SCN explant and precision of wheel-running rhythms (16). Our study examined variability within a group of animals, in contrast to the prior study with explants. It would be interesting to determine whether SCN explants from *npy*−/− mice showed reduced variability in circadian period. It could be possible that NPY might reduce coupling between SCN neurons. Perhaps the observation that IGL-ablated hamsters rarely show split rhythms when housed under constant light (14, 15) indicates that this pathway is important for flexibility in coupling of component oscillators within the circadian system.

Knockout mouse studies have other complications not found in IGL lesion studies. A loss of cell function might occur if those cells happen to depend on NPY for development. Because NPY acts as a neuromodulator in feeding, anxiety, and seizure-related systems to name a few, other systems are also affected by the lack of NPY (10, 28). Development of new animal models, similar to those that allow targeted downregulation of NPY expression in the adult mouse arcuate (26, 44), might be helpful in further studies of circadian effects of NPY. The background strain of the mice can also have major influences on the loss of the target gene function. The *npy*−/− mouse on a mixed 129S6 background shows little change in body weight, while the same animal back-crossed further onto a C57BL/6 background shows late-onset obesity (41), the latter result confirmed in *experiment 2* in the present study. One strength of our study is that the alterations in circadian rhythms were observed in both male and female mice of varied ages and also in two different background strains, suggesting that these are relatively robust findings.

The measurement of seasonal changes in day length, or changes in photoperiod, depends on changes in phase relation of the LD cycle and the internal circadian clock (9). Hamsters show an increase in the number of NPY mRNA-containing cells in the IGL when housed in a short photoperiod (20). Mice lacking NPY were slow to respond to an abrupt change in photoperiod as measured by changes in their pattern of locomotor activity. The slower reentrainment is unlikely to be entirely passive. Before exposure to the photoperiod change, the *npy*−/− mice showed a circadian period of 23.95 h. If they were simply free running during the photoperiod change, we would expect an advance of 3 min a day, or 1 h over 20 days. Instead, the mice had shifted by ~6 h in 18 days, on average,

indicating that they were responding to the light cycle, albeit more slowly than control mice. A role for the IGL in the response to shortened photoperiod has been demonstrated for both Siberian and Syrian hamsters (12, 19, 30, 31, 42). Similar to *npy*−/− mice in the present study, IGL-ablated Siberian hamsters were slow to expand duration of activity as dark period increased in a simulated natural photoperiod and were less likely to molt to a winter-type pelage (12). IGL-ablated hamsters also showed reduced hibernation response after a transition to cold temperatures and a short photoperiod (31).

One possible mechanism by which IGL ablation alters photoperiodic response would be via the loss of nonphotic effects on rhythms. A brief presentation of a novel running wheel can alter the phase angle of entrainment of activity rhythms of hamsters housed under a LD cycle, and this effect is stronger when hamsters are housed in a shorter photoperiod (21). Thus one possible interpretation of our results is that mice lacking NPY lack activity feedback effects on rhythms and this is associated with slowed circadian reentrainment and altered phase angle of entrainment to a short photoperiod.

Other studies indicate a role for NPY innervation of the hypothalamus in control of endocrine events. One study reported that a single nonphotic stimulus on the day of proestrus can delay the estrous cycle of female hamsters (49). IGL neurons innervate many of the same targets as the SCN, including neuroendocrine neurons of the hypothalamus (18). The functional role of these direct connections to neuroendocrine neurons is as yet unknown.

The abrupt change in photoperiod used in these laboratory studies is very different from the more gradual change observed in nature. Further experiments should attempt to determine the role of nonphotic inputs following gradual changes in photoperiod better mimicking external cycles. For example, Siberian hamsters exposed to a decreasing simulated natural photoperiod showed more positive phase angle of entrainment of activity onset relative to light offset compared with IGL-ablated hamsters (12). Interestingly, we saw a similar difference in phase angle of entrainment when comparing the *npy*−/− and WT mice, similar to findings reported for Syrian hamsters (23, 40).

Our results support the hypothesis that nonphotic influences on circadian period serve a useful function when animals must respond to changing photoperiods. The influence appears to be particularly important for responses to short photoperiods. We suggest that the observed nonphotic effect to shorten circadian period is associated with increased phase resetting by light, and both these effects help the animal to track seasonal changes in the timing of dawn. Nonphotic inputs might loosen coupling between component oscillators in the circadian system, allowing flexibility and variability in response to changing environmental conditions.

ACKNOWLEDGMENTS

We thank Kate Dahlgren, Jeanne McKeon, and Shirin Hastings for technical assistance.

A portion of this work was submitted in partial fulfillment of an undergraduate honors thesis (S. Soscia).

GRANTS

This work was supported by National Science Foundation Grant 0234203 (to M. E. Harrington), National Institute of Mental Health Grant MH-59166 (to Eric Bittman), and the Smith College Tomlinson Fund (to S. Soscia). S. Soscia and C. Prabakar were supported by summer fellowships from the Howard Hughes Medical Institute and the Frances Baker Holmes Fund in Psychology.

REFERENCES

1. **Aton SJ, Block GD, Tei H, Yamazaki S, Herzog ED.** Plasticity of circadian behavior and the suprachiasmatic nucleus following exposure to non-24-hour light cycles. *J Biol Rhythms* 19: 98–207, 2004.
2. **Brewer JM, Yannielli PC, Harrington ME.** Neuropeptide Y differentially suppresses *per1* and *per2* mRNA induced by light in the suprachiasmatic nuclei of the golden hamster. *J Biol Rhythms* 17: 28–39, 2002.
3. **Cheng HY, Obrietan K, Cain SW, Lee BY, Agostino PV, Joza NA, Harrington ME, Ralph MR, Penninger JM.** Dexras1 potentiates photic and suppresses nonphotic responses of the circadian clock. *Neuron* 43: 715–728, 2004.
4. **Daan S, Pittendrigh CS.** A functional analysis of circadian pacemakers in nocturnal rodents. II. The variability of phase response curves. *J Comp Physiol* 106: 253–266, 1976.
5. **Deboer T, Tobler I.** Running wheel size influences circadian rhythm period and its phase shift in mice. *J Comp Physiol [A]* 186: 969–973, 2000.
6. **Ebihara S, Goto M, Oshima I.** The phase-shifting effects of pentobarbital on the circadian rhythm of locomotor activity in the mouse: strain differences. *Brain Res* 454: 404–407, 1988.
7. **Edelstein K, Amir S.** The role of the intergeniculate leaflet in entrainment of circadian rhythms to a skeleton photoperiod. *J Neurosci* 19: 372–380, 1999.
8. **Edgar DM, Kilduff TS, Martin CE, Dement WC.** Influence of running wheel activity on free-running sleep/wake and drinking circadian rhythms in mice. *Physiol Behav* 50: 373–378, 1991.
9. **Elliott JA.** Circadian rhythms and photoperiodic time measurement in mammals. *Fed Proc* 35: 2339–2346, 1976.
10. **Erickson JC, Clegg KE, Palmiter RD.** Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. *Nature* 381: 415–421, 1996.
11. **Erickson JC, Hollopeter G, Palmiter RD.** Attenuation of the obesity syndrome of ob/ob mice by the loss of neuropeptide Y. *Science* 274: 1704–1707, 1996.
12. **Freeman DA, Dhandapani KM, Goldman BD.** The thalamic intergeniculate leaflet modulates photoperiod responsiveness in Siberian hamsters. *Brain Res* 1028: 31–38, 2004.
13. **Hamada T, Antle MC, Silver R.** The role of Period1 in non-photic resetting of the hamster circadian pacemaker in the suprachiasmatic nucleus. *Neurosci Lett* 362: 87–90, 2004.
14. **Harrington ME, Rusak B.** Ablation of the geniculo-hypothalamic tract alters circadian activity rhythms of hamsters housed under constant light. *Physiol Behav* 42: 183–189, 1988.
15. **Harrington ME, Rusak B.** Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms. *J Biol Rhythms* 1: 309–325, 1986.
16. **Herzog ED, Aton SJ, Numano R, Sakaki Y, Tei H.** Temporal precision in the mammalian circadian system: a reliable clock from less reliable neurons. *J Biol Rhythms* 19: 35–46, 2004.
17. **Hokfelt T, Broberger C, Zhang X, Diez M, Kopp J, Xu Z, Landry M, Bao L, Schalling M, Koistinaho J, DeArmond SJ, Prusiner S, Gong J, Walsh JH.** Neuropeptide Y: some viewpoints on a multifaceted peptide in the normal and diseased nervous system. *Brain Res Brain Res Rev* 26: 154–166, 1998.
18. **Horvath TL.** An alternate pathway for visual signal integration into the hypothalamo-pituitary axis: retinorecipient intergeniculate neurons project to various regions of the hypothalamus and innervate neuroendocrine cells including those producing dopamine. *J Neurosci* 18: 1546–1558, 1998.
19. **Jacob N, Vuillez P, Lakdhar-Ghazal N, Pevet P.** Does the intergeniculate leaflet play a role in the integration of the photoperiod by the suprachiasmatic nucleus? *Brain Res* 828: 83–90, 1999.
20. **Jacob N, Vuillez P, Moller M, Pevet P.** Photoperiodic dependent changes in the number of neurons containing mRNA encoding neuropeptide Y in the intergeniculate leaflet of the Syrian hamster. *Brain Res* 813: 160–166, 1998.
21. **Janik D, Godfrey M, Mrosovsky N.** Phase angle changes of photically entrained circadian rhythms following a single nonphotic stimulus. *Physiol Behav* 55: 103–107, 1994.
22. **Johnson CH, Elliott JA, Foster R.** Entrainment of circadian programs. *Chronobiol Int* 20: 741–774, 2003.

23. **Johnson RF, Moore RY, Morin LP.** Lateral geniculate lesions alter circadian activity rhythms in the hamster. *Brain Res Bull* 22: 411–422, 1989.
24. **Kuroda H, Fukushima M, Nakai M, Katayama T, Murakami N.** Daily wheel running activity modifies the period of free-running rhythm in rats via intergeniculate leaflet. *Physiol Behav* 61: 633–637, 1997.
25. **Lewandowski MH, Usarek A.** Effects of intergeniculate leaflet lesions on circadian rhythms in the mouse. *Behav Brain Res* 128: 13–17, 2002.
26. **Luquet S, Perez FA, Hnasko TS, Palmiter RD.** NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 310: 683–685, 2005.
27. **Marchant EG, Mistlberger RE.** Entrainment and phase shifting of circadian rhythms in mice by forced treadmill running. *Physiol Behav* 60: 657–663, 1996.
28. **Marsh DJ, Hollopeter G, Kafer KE, Palmiter RD.** Role of the Y5 neuropeptide Y receptor in feeding and obesity. *Nat Med* 4: 718–721, 1998.
29. **Maywood ES, Okamura H, Hastings MH.** Opposing actions of neuropeptide Y and light on the expression of circadian clock genes in the mouse suprachiasmatic nuclei. *Eur J Neurosci* 15: 216–220, 2002.
30. **Menet J, Vuillez P, Jacob N, Pevet P.** Intergeniculate leaflets lesion delays but does not prevent the integration of photoperiodic change by the suprachiasmatic nuclei. *Brain Res* 906: 176–179, 2001.
31. **Menet JS, Vuillez P, Saboureau M, Pevet P.** Inhibition of hibernation by exercise is not affected by intergeniculate leaflets lesion in hamsters. *Am J Physiol Regul Integr Comp Physiol* 285: R690–R700, 2003.
32. **Morin LP, Allen CN.** The circadian visual system, 2005. *Brain Res Brain Res Rev* 51: 1–60, 2005.
33. **Morin LP, Pace L.** The intergeniculate leaflet, but not the visual midbrain, mediates hamster circadian rhythm response to constant light. *J Biol Rhythms* 17: 217–226, 2002.
34. **Muscat L, Morin LP.** Intergeniculate leaflet: contributions to photic and non-photic responsiveness of the hamster circadian system. *Neuroscience* 1: 305–320, 2006.
35. **Pickard GE.** Intergeniculate leaflet ablation alters circadian rhythms in the mouse. *Neuroreport* 5: 2186–2188, 1994.
36. **Pickard GE, Ralph MR, Menaker M.** The intergeniculate leaflet partially mediates effects of light on circadian rhythms. *J Biol Rhythms* 2: 35–56, 1987.
37. **Pittendrigh CS, Daan S.** A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. *J Comp Physiol* 106: 291–331, 1976.
38. **Pittendrigh CS, Daan S.** A functional analysis of circadian pacemakers in rodents. I. The stability and lability of spontaneous frequency. *J Comp Physiol* 106: 223–252, 1976.
39. **Pittendrigh CS, Daan S.** A functional analysis of circadian pacemakers in rodents. V. Pacemaker structure: a clock for all seasons. *J Comp Physiol* 106: 333–355, 1976.
40. **Redlin U, Vrang N, Mrosovsky N.** Enhanced masking response to light in hamsters with IGL lesions. *J Comp Physiol [A]* 184: 449–456, 1999.
41. **Segal-Lieberman G, Trombly DJ, Juthani V, Wang X, and Maratos-Flier E.** NPY ablation in C57BL/6 mice leads to mild obesity and to an impaired refeeding response to fasting. *Am J Physiol Endocrinol Metab* 284: E1131–E1139, 2003.
42. **Smale L, Morin LP.** Photoperiodic responsiveness of hamsters with lesions of the lateral geniculate nucleus is related to hippocampal damage. *Brain Res Bull* 24: 185–190, 1990.
43. **Soscia SJ, Harrington ME.** Neuropeptide Y attenuates NMDA-induced phase shifts in the SCN of NPY Y1 receptor knockout mice in vitro. *Brain Res* 1023: 148–153, 2004.
44. **Ste Marie L, Luquet S, Cole TB, Palmiter RD.** Modulation of neuropeptide Y expression in adult mice does not affect feeding. *Proc Natl Acad Sci USA* 102: 18632–18637, 2005.
45. **Tobler I, Gaus SE, Deboer T, Achermann P, Fischer M, Rulicke T, Moser M, Oesch B, McBride PA, Manson JC.** Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature* 380: 639–642, 1996.
46. **Yamada N, Shimoda K, Ohi K, Takahashi S, Takahashi K.** Free access to a running wheel shortens the period of free-running rhythm in blinded rats. *Physiol Behav* 42: 87–91, 1988.
47. **Yamada N, Shimoda K, Takahashi K, Takahashi S.** Relationship between free-running period and motor activity in blinded rats. *Brain Res Bull* 25: 115–119, 1990.
48. **Yannielli PC, McKinley Brewer J, Harrington ME.** Is novel wheel inhibition of per1 and per2 expression linked to phase shift occurrence? *Neuroscience* 112: 677–685, 2002.
49. **Young Janik L, Janik D.** Nonphotic phase shifting in female Syrian hamsters: interactions with the estrous cycle. *J Biol Rhythms* 18: 307–317, 2003.