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# Dexras1 Potentiates Photic and Suppresses Nonphotic Responses of the Circadian Clock

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## Summary

**Circadian rhythms of physiology and behavior are generated by biological clocks that are synchronized to the cyclic environment by photic or nonphotic cues. The interactions and integration of various entrainment pathways to the clock are poorly understood. Here, we show that the Ras-like G protein Dexras1 is a critical modulator of the responsiveness of the master clock to photic and nonphotic inputs. Genetic deletion of Dexras1 reduces photic entrainment by eliminating a pertussis-sensitive circadian response to NMDA. Mechanistically, Dexras1 couples NMDA and light input to $G_{i/o}$ and ERK activation. In addition, the mutation greatly potentiates nonphotic responses to neuropeptide Y and unmasks a nonphotic response to arousal. Thus, Dexras1 modulates the responses of the master clock to photic and nonphotic stimuli in opposite directions. These results identify a signaling molecule that serves as a differential modulator of the gated photic and nonphotic input pathways to the circadian timekeeping system.**

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## Introduction

Living organisms exhibit daily rhythms in behavior and physiology that are driven by a biological oscillator whose function is to synchronize these activities with the cycles of the natural environment (Dunlap, 1999). Mammalian circadian organization is a hierarchy of oscillators and nonoscillating tissues with a "master" clock, or pacemaker, situated in the suprachiasmatic nucleus (SCN) (Ralph et al., 1990). Synchronization or entrainment of the master clock with environmental cycles (Zeitgebers) occurs through daily resetting in response to photic and nonphotic cues, notably the daily changes in light intensity. Photic information is relayed to the SCN from specialized retinal ganglion cells via the retinohypothalamic tract (RHT) (Hannibal, 2002). The SCN is also responsive to cues that are nonphotic in nature, such as social interaction, food intake, or exposure to novel environments. A projection from the thalamic intergeniculate leaflet (IGL) via a geniculohypothalamic tract (GHT) is considered the major nonphotic input to the SCN (Harrington, 1997).

Within SCN pacemaker cells, resetting signals are conveyed to the molecular circadian clock by neurochemical pathways that are triggered by neurotransmitters of the RHT (glutamate, PACAP), GHT (neuropeptide Y [NPY], GABA), and others (Harrington, 1997; Hannibal, 2002). In vivo and in vitro, glutamate agonists (GLU, N-methyl-D-aspartate [NMDA]) and NPY produce photic- and nonphotic-like responses, respectively (Mrosovsky, 1996a; Hannibal, 2002). Neurotransmitters that are implicated in circadian timekeeping exert their effects through cognate receptors expressed within the SCN, which include G protein-coupled receptors (GPCRs) specific for different subtypes of G proteins and non-GPCRs such as NMDA receptors. The cell signaling events that are actuated by these receptors alter the expression of "circadian clock proteins" (e.g., PER1 and PER2 in mammals) and hence clock timing mechanisms (Maywood and Mrosovsky, 2001).

Importantly, photic and nonphotic responsiveness show distinct temporal patterns. Phase response curves (PRCs), which describe the responsiveness of a circadian system to stimuli as a function of the circadian phase at which they are given, are species specific (Daan and Pittendrigh, 1976; Mrosovsky, 1996a), and their characteristic shapes are thought to be genetically encoded into the core molecular clock mechanism. The amplitudes of photic and nonphotic responses are labile, however, and are context and history dependent. In particular, photic and nonphotic responses are mutually antagonistic. For instance, in the golden hamster, light attenuates the phase-shifting effects of nonphotic stimuli (Biello and Mrosovsky, 1995), and vice versa (Ralph and Mrosovsky, 1992). Together, responses to photic

and nonphotic inputs are integrated to produce stable temporal relationships among behavioral, physiological, and environmental cycles.

Although the entrainment mechanisms of circadian systems have been modeled as simple chemical responses of the core molecular cycles, input pathways are also under rhythmic control (Roenneberg and Merrow, 2000). Recently, Dexras1/AGS1 (activator of G protein signaling 1), a member of the Ras superfamily of small G proteins, has been shown to exhibit circadian rhythms of expression in the SCN, with a peak in the early subjective night, when light has the greatest effect on the central clock (Panda et al., 2002; Ueda et al., 2002; Takahashi et al., 2003). These expression data suggested that Dexras1 may have a role in the photic input pathway to ensure that light only produces circadian resetting at night and that the central clock controls its own input through this pathway.

Dexras1 was first identified as a dexamethasone-inducible gene (Kemppainen and Behrend, 1998) and was later discovered to activate G protein-MAPK signaling pathways, even in the absence of GPCR stimulation (Cismowski et al., 1999). Dexras1 is able to serve as a guanine nucleotide exchange factor (GEF) for $G\alpha_{i/o}$ subtypes of G protein $\alpha$ subunits (Cismowski et al., 2000). Subsequent in vitro studies demonstrated that MAPK activation resulting from GPCR stimulation can be attenuated by Dexras1 (Graham et al., 2002). Hence, Dexras1 may play a dualistic, context-dependent role in $G_{i/o}$ protein signaling. Another study indicated that Dexras1 is activated by neuronal nitric oxide synthase (nNOS), a downstream effector of NMDA receptor signaling (Fang et al., 2000). However, the downstream consequences of NMDA receptor/nNOS-dependent activation of Dexras1 are as yet undetermined.

To test the in vivo function of Dexras1, we generated *dexras1*$^{-/-}$ mice. We report the surprising finding that Dexras1 not only is essential for normal photic responses but also is a negative regulator of nonphotic input to the central pacemaker. *dexras1*$^{-/-}$ mice exhibited the following: (1) impaired entrainment to 24 hr light-dark cycles; (2) reduced responsiveness (phase shifts) to pulses of light given in the early night; and (3) smaller phase shifts to NMDA in vitro. In contrast to reducing the response to photic stimuli, *dexras1*$^{-/-}$ animals exhibited enhanced responses to chronic and acute nonphotic manipulations in vivo, as well as to NPY in vitro. Unexpectedly, the behavioral rhythms of *dexras1*$^{-/-}$ mice also exhibited a greater tendency to dissociate into multiple components in constant light. These findings identify Dexras1 as a critical molecular regulator of multiple input pathways to the mammalian circadian clock, modulating the responsiveness of the central pacemaker to photic, nonphotic, and potentially pacemaker-pacemaker coupling signals.

## Results

### Generation of *dexras1*$^{-/-}$ Mice

The *dexras1* gene was disrupted in murine embryonic stem (ES) cells using a targeting vector in which nucleotides encoding amino acids 13–227, encompassing the GTP binding and hydrolysis domain as well as the effector loop (Graham et al., 2001), were deleted (Supplemental Figure S1A at http://www.neuron.org/cgi/content/full/43/5/715/DC1). The targeting construct was electroporated into ES cells. Two G418-resistant cell lines heterozygous for the mutation at the *dexras1* locus were used to generate chimeric mice, which were backcrossed to C57BL/6J for five generations to obtain heterozygous *dexras1*$^{+/-}$ mice. The intercross of *dexras1*$^{+/-}$ mice produced homozygous *dexras1*$^{-/-}$ mice, as confirmed by Southern blot analysis (Supplemental Figure S1B at http://www.neuron.org/cgi/content/full/43/5/715/DC1). Dexras1 mRNA transcripts were undetectable in brain tissue of *dexras1*$^{-/-}$ mice by RT-PCR and Northern blot analyses (Supplemental Figures S1C and S1D at http://www.neuron.org/cgi/content/full/43/5/715/DC1). *dexras1*$^{-/-}$ mice were born at the expected Mendelian frequency, were fertile, and appeared healthy. Histological analysis of all tissues revealed no overt structural or morphological deficits in adult *dexras1*$^{-/-}$ animals (data not shown). Moreover, *dexras1*$^{-/-}$ mice were indistinguishable from wild-type controls in most tests of behavior (see the Supplemental Data at http://www.neuron.org/cgi/content/full/43/5/715/DC1 for a list of the behavioral assays performed).

### Dexras1 Controls the Responsiveness of the Circadian Clock to Photic Entrainment

Previous studies indicated that Dexras1 is rhythmically expressed within the SCN, reaching peak levels in the early subjective night, a time when the circadian system is most responsive to photic input (Panda et al., 2002; Ueda et al., 2002; Takahashi et al., 2003). We therefore speculated that Dexras1 may participate in the signal transduction pathway(s) that conveys photic information to the SCN clock.

Wild-type and *dexras1*$^{-/-}$ littermate mice were maintained on a schedule of 12 hr light-12 hr dark (LD 12:12), with adjustments made to the light intensity at intervals of 1–2 weeks. Notably, in our experimental design, photic entrainment was examined under standard laboratory conditions (i.e., bright light, 400 lux) as well as more ecologically relevant conditions, using dim, twilight-level light intensities ($<$10 lux). Activity rhythms (wheel running) of both genotypes were entrained initially to a light intensity of 40 lux (Figures 1A–1D). As the light was subsequently dimmed to 10, 5, and, eventually, less than 1 lux, five out of six wild-type littermates remained entrained to the lowest light intensity (Figures 1A and 1C). One wild-type mouse was entrained by 5 lux but not $<$1 lux light (data not shown). In contrast, the ability of all tested *dexras1*$^{-/-}$ mice to entrain in dim light was diminished or absent entirely (Figures 1B and 1D). In line with this, *dexras1*$^{-/-}$ animals exhibited markedly premature onsets of nocturnal activity with respect to dark onset (referred to as phase angle of entrainment), as the entraining light intensity was reduced (Figure 1E). These findings indicate that Dexras1 plays a critical role in vivo in setting the timing of the circadian clock with respect to the LD cycle during entrainment.

### Dexras1 Controls the Nighttime Sensitivity of the Circadian Clock to Light

Since *dexras1*$^{-/-}$ mice were not entrained by low-intensity light when maintained on a full photoperiod, we

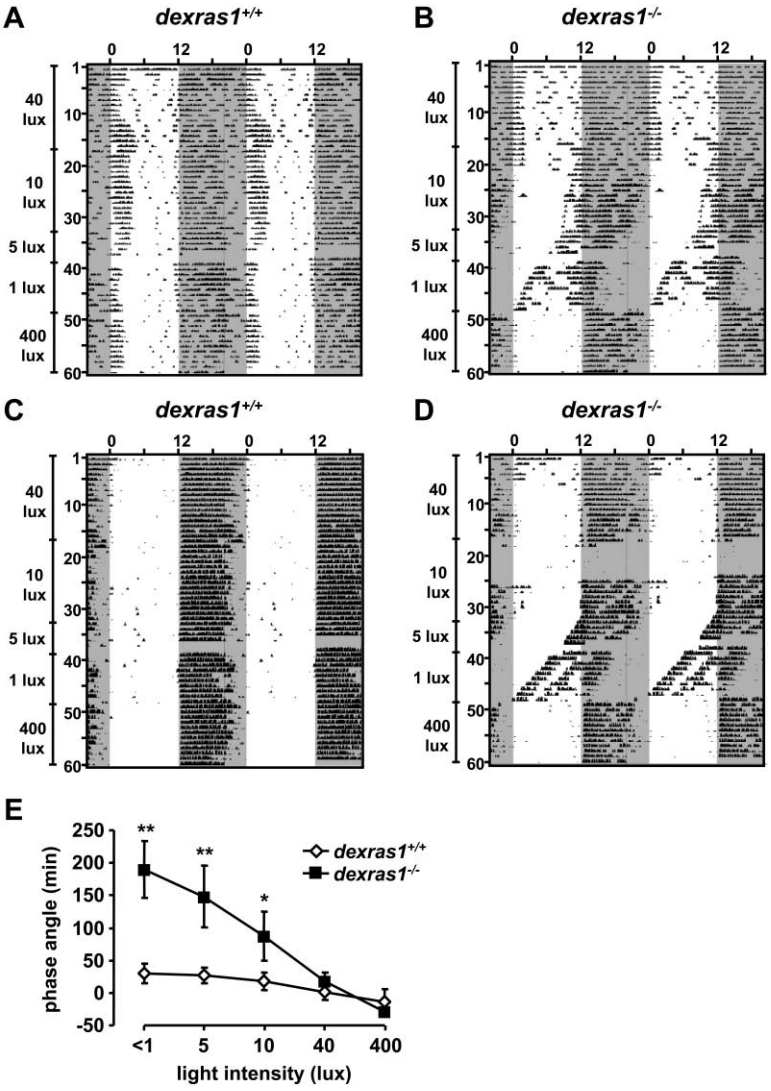


Figure 1. Reduced Sensitivity to Photic Entrainment in *dexras1*$^{-/-}$ Mice

(A–D) Representative actograms of wheel-running activity in *dexras1*$^{+/+}$ (A and C) and *dexras1*$^{-/-}$ (B and D) mice. For the first 16 days, mice were exposed to a 12 hr light:12 hr dark (LD) cycle in which the light intensity was maintained at 40 lux. The light intensity was sequentially reduced to 10 lux (on day 17), 5 lux (on day 33), and 1 lux (on day 40). On day 49, mice were returned to a 12:12 LD schedule of 400 lux. Periods of darkness are shaded in gray. The x axis (top) indicates the Zeitgeber (ZT) time. The y axis (left) indicates the nth day of the experiment. Light intensity (lux) is indicated to the left of the y axis.

(E) Graphical representation of the phase angle of entrainment (min) as a function of light intensity (lux) for all animals that exhibited stable entrainment to the LD cycle. Fisher's least significant difference (LSD). *$p < 0.05$, **$p < 0.01$ versus same-treated wild-type.

speculated first that Dexras1, by participating in the signal transduction pathway(s) that conveys photic information to the SCN clock, regulates the responsiveness of the clock to light. We therefore examined the phase-shifting effect of nocturnal light exposure in entrained mice (Aschoff, 1965; Mrosovsky, 1996b; Refinetti, 2001). Mice received a single light pulse at Zeitgeber time (ZT) 14, 2 hr after onset of the dark phase of their final LD cycle, and subsequently were released into constant darkness (DD). At a low intensity of 10 lux (Figures 2A–2D, red asterisk), light pulses resulted in phase delays in wild-type mice ($-33.6 \pm 13.6$ min) (Figures 2A and 2C) but not in *dexras1*$^{-/-}$ animals ($15.5 \pm 7.9$ min; $p = 0.017$) (Figures 2B and 2D). At 40 lux (Figures 2A–2D, yellow asterisk), however, the induced phase shifts were not statistically different between wild-type ($-33.6 \pm 18.0$ min) (Figures 2A and 2C) and *dexras1*$^{-/-}$ mice ($-4.8 \pm 20.0$ min; $p = 0.370$) (Figures 2B and 2D). Thus, our data, summarized in Figure 2E, indicate that Dexras1 plays an essential role in modulating the responsiveness of the circadian system to nocturnal light.

### Photic Responsiveness of the SCN Is Influenced by Dexras1-Mediated Activation of the MAPK Pathway

The effects of light on the circadian clock, including behavioral phase shifts and induction of gene transcription, have been shown to depend on the MAPK pathway (Dziema et al., 2003; Coogan and Piggins, 2003). Given previous studies indicating that Dexras1 may influence MAPK activity in vitro (Cismowski et al., 1999; Graham et al., 2002), we analyzed whether genetic inactivation of Dexras1 indeed affects light-induced MAPK activation in vivo. To analyze the response of the MAPK pathway to light, the SCN tissue of wild-type and *dexras1*$^{-/-}$ mice that had been exposed to a 10 min light pulse at ZT15 was probed for the phosphorylated, active forms of ERK1 and ERK2 (referred to as p-ERK). Basal levels of p-ERK at ZT15 in the dark were comparable in the SCN of wild-type and *dexras1*$^{-/-}$ mice (Figures 3A and 3B). Light-induced p-ERK expression was significantly attenuated in the SCN of *dexras1*$^{-/-}$ mice compared with that of wild-type controls at all light intensities tested

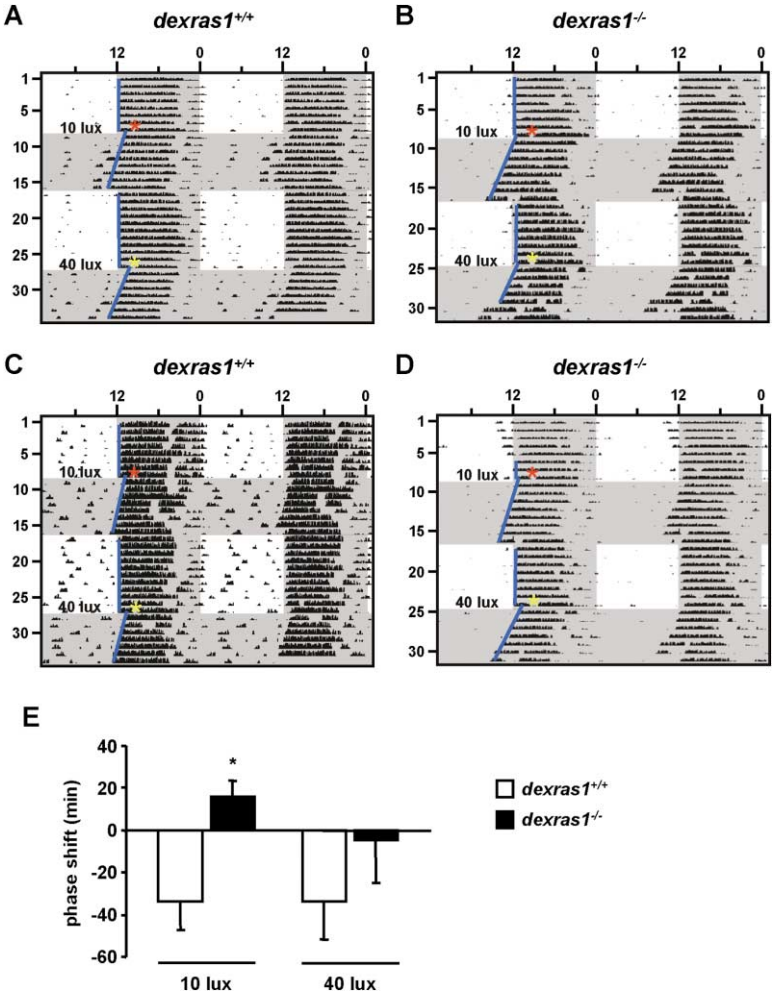


Figure 2. The Phase-Delaying Effect of a Brief Light Pulse in the Night Is Attenuated in the Absence of Dexras1

(A–D) Representative actograms of wheel-running activity in *dexras1*$^{+/+}$ (A and C) and *dexras1*$^{-/-}$ (B and D) mice. Mice were entrained to a 12:12 LD schedule (400 lux) for 8–11 days. On their final LD cycle, the mice received a 15 min light pulse of 10 lux (red asterisk) at ZT14 and were maintained in DD for 7–8 days. Animals were reentrained, and the procedure was repeated with a light pulse of 40 lux (yellow asterisk). Activity onsets are indicated by blue lines.

(E) Quantification of light-induced phase shifts. Values are presented as mean ± SEM phase shift. n = 5–6 per group. Student's t test. *p < 0.05 versus same-treated wild-type.

(Figures 3A and 3B). In line with our behavioral data on photic entrainment, the attenuation of ERK activation was greater at lower intensities of light (4 and 10 lux). Thus, Dexras1 plays a critical role in modulating the responsiveness of the circadian system to photic input and in regulating the sensitivity of the MAPK pathway to light-induced activation.

### Dexras1 Regulates the Phase-Shifting Effects of NMDA on the SCN Rhythm by a MAPK-Dependent Mechanism

NMDA receptor signaling within the SCN is a principal mediator of the phase-resetting effects of light on the central pacemaker (Colwell et al., 1991; Ding et al., 1994; Shibata et al., 1994). Moreover, NMDA has been shown to induce Dexras1 activation in vitro (Fang et al., 2000). We therefore tested whether the deficit in photic entrainment exhibited by *dexras1*$^{-/-}$ mice is due to a reduction in NMDA receptor-mediated signal transduction within the SCN. Using brain slices isolated from wild-type and *dexras1*$^{-/-}$ mice, we assessed the phase-shifting effects of NMDA on SCN neuronal firing rhythms in vitro.

In SCN slices prepared from wild-type mice, NMDA applied at ZT15 induced a phase delay in firing rhythms in a dose-dependent fashion (1–100 μM) compared with the time of maximal firing of untreated wild-type SCN neurons (Figure 4A; see Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/43/5/715/DC1 for raw data). Untreated SCN neurons of *dexras1*$^{-/-}$ mice exhibited comparable peak times as untreated wild-type slices (mean $ZT_{peak}$ [hr] of untreated wt versus *dexras1*$^{-/-}$ SCN: 5.89 ± 0.21 versus 6.01 ± 0.10, $p > 0.05$). However, the magnitude of NMDA-induced phase delay was significantly diminished in *dexras1*$^{-/-}$ SCN slices compared with that in same-treated wild-type controls at all doses tested (Figure 4A). These data provide genetic evidence that Dexras1 has a physiological role in NMDA receptor signaling and NMDA-induced phase shifting of the SCN clock.

Next, we investigated the functional relevance of diminished ERK activation on the reduced photic responsiveness of *dexras1*$^{-/-}$ mice. Perfusion of wild-type and *dexras1*$^{-/-}$ SCN slices with U0126, a selective blocker of the upstream kinase MEK1/2, did not alter the firing rhythms of SCN neurons of either genotype on the subsequent day (Figure 4B, lanes 1 and 2; see Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/43/5/715/DC1 for raw data). NMDA-induced phase shifts were not affected by vehicle treatment and were comparable to slices treated with NMDA alone in the two genotypes

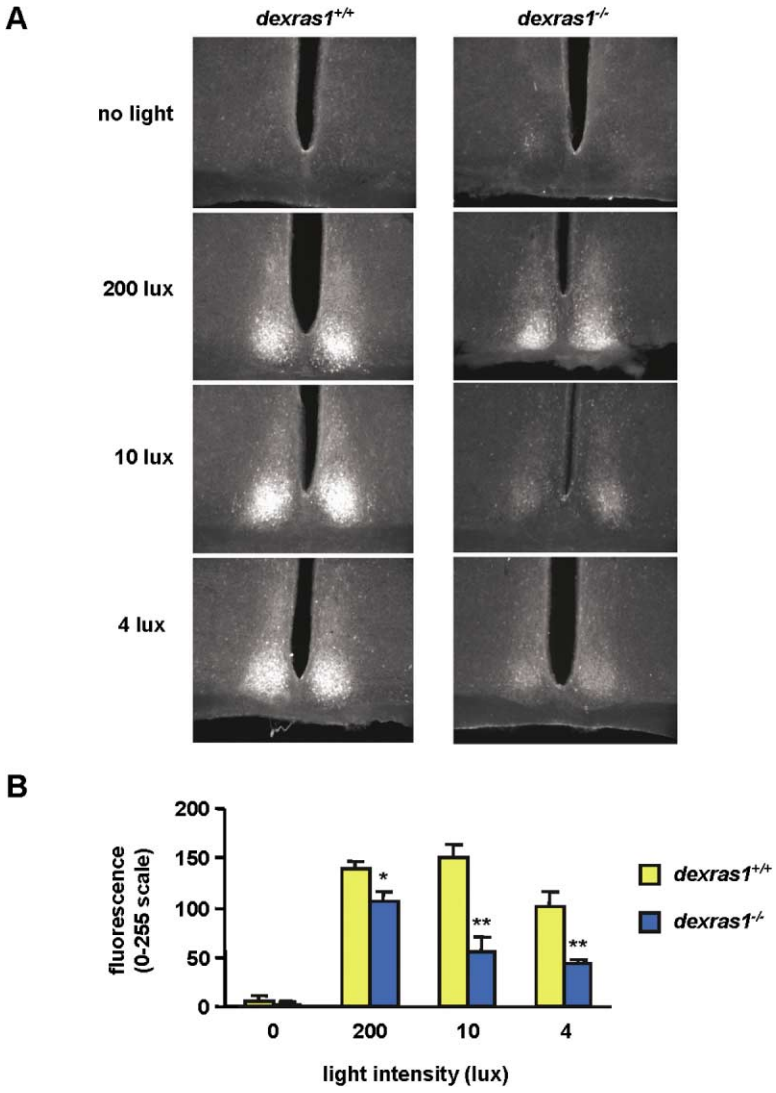


Figure 3. Disruption of Dexras1 Attenuates Light-Induced ERK Activation in the SCN

Animals were exposed to light (200, 10, or 4 lux) for 10 min at ZT15. (A) At the end of the light treatment, animals were sacrificed, and brain sections were processed using immunofluorescent labeling for the phospho-activated form of ERK. ERK activation was significantly reduced in the SCN of *dexras1*$^{-/-}$ mice relative to wild-type controls at all light intensities tested (4, 10, and 200 lux). (B) Quantitation of pERK expression in the SCN. Data are presented as mean ± SEM fluorescent signal. n = 3–6 per group. Student's t test. *$p < 0.05$, **$p < 0.01$ versus same-treated wild-type.

(Figure 4B, lanes 3 and 4). Although NMDA alone induced a smaller phase delay in *dexras1*$^{-/-}$ SCN neurons compared with wild-type controls, MEK1/2 blockade resulted in a complete inhibition of the phase delay elicited by NMDA application at ZT15, irrespective of genotype (Figure 4B, lanes 5 and 6). Thus, Dexras1 is essential for the maximal activation of ERK1/2 by NMDA in SCN slices and for activation of the ERK signaling pathway in response to light in vivo. Importantly, our electrophysiological data indicate that the deficit in photic responsiveness of *dexras1*$^{-/-}$ mice is mediated at the level of the SCN and that the absence of Dexras1 significantly attenuates NMDA receptor signaling.

### Dexras1-Dependent $G_{i/o}$ Signaling Is Essential for Maximal Responsiveness of the SCN to Photic Stimuli

Previous studies reported that Dexras1 possesses GEF activity for $G_{i/o}$ proteins, promoting their activation (Cismowski et al., 1999, 2000). We therefore examined the effect of the $G_{i/o}$ inhibitor pertussis toxin (PTX) on the rhythmic firing of wild-type and *dexras1*$^{-/-}$ SCN neurons. Static incubation with PTX did not affect the basal firing rhythms of either wild-type or *dexras1*$^{-/-}$ SCN neurons (Figure 4C, lanes 1 and 2; see Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/43/5/715/DC1 for raw data). Moreover, a static incubation step per se had no significant effect on NMDA-induced phase delays in wild-type or *dexras1*$^{-/-}$ slices (Figure 4C, lanes 3 and 4). In *dexras1*$^{-/-}$ SCN slices, PTX pretreatment did not alter the potency of NMDA to phase shift firing rhythms (Figure 4C, lane 6). In contrast, PTX reduced the effectiveness of NMDA to induce phase delays in wild-type SCN slices (Figure 4C, lane 5). Importantly, PTX eliminated the difference in the magnitude of NMDA-induced phase delays between wild-type and *dexras1*$^{-/-}$ SCN (Figure 4C, lanes 5 and 6). These data indicate that NMDA-induced phase shifts are mediated by two separable pathways: a PTX-sensitive, $G_{i/o}$ protein-dependent component and a PTX-insensitive, $G_{i/o}$ protein-independent component. The absence of Dex-

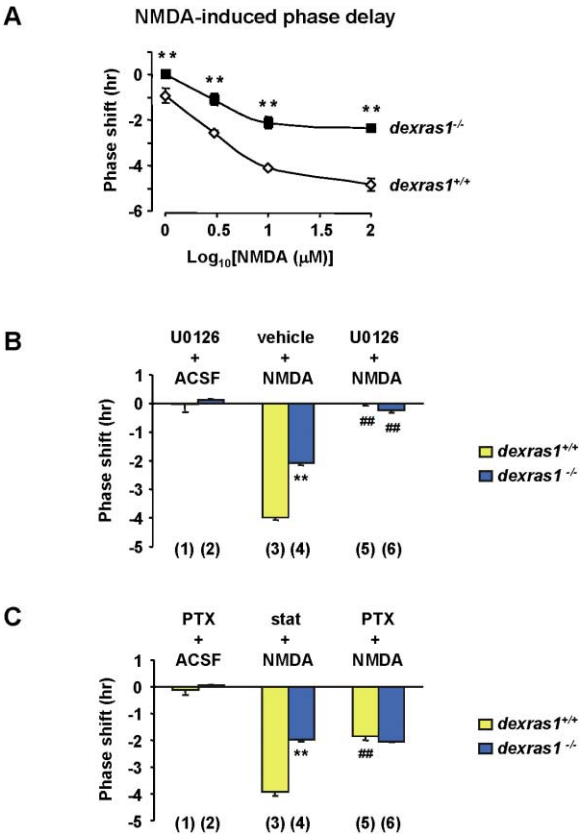


Figure 4. Attenuation of NMDA-Induced Phase Shift in the Absence of Dexras1 Is Mediated by ERK- and $G_{i/o}$ Protein-Dependent Mechanisms

(A) Dose response curve of NMDA-induced phase delays. *dexras1*$^{+/+}$ and *dexras1*$^{-/-}$ SCN neurons were treated with 0, 1, 3, 10, or 100 μM NMDA at ZT15 on the first day in vitro, and firing rates of individual neurons were recorded on the second day at different ZT. Phase shifts were calculated as the difference in ZT of peak firing between the experimental and untreated control (0 μM NMDA) slices of the same genotype. (B) The effect of the MEK1/2 blocker U0126 on NMDA-induced phase shifts. *dexras1*$^{+/+}$ (lanes 1, 3, and 5) and *dexras1*$^{-/-}$ (lanes 2, 4, and 6) SCN slices were treated with 20 μM U0126 and ACSF at ZT15 (lanes 1 and 2), vehicle and 10 μM NMDA at ZT15 (lanes 3 and 4), or 20 μM U1026 and 10 μM NMDA at ZT15 (lanes 5 and 6), on the first day in vitro. Phase shifts were calculated relative to untreated (0 μM NMDA) *dexras1*$^{+/+}$ SCN slices ($ZT_{peak}$ = 5.89 ± 0.21). (C) The effect of pertussis toxin (PTX) on NMDA-induced phase shifts. *dexras1*$^{+/+}$ (lanes 1, 3, and 5) and *dexras1*$^{-/-}$ (lanes 2, 4, and 6) SCN slices were treated with 1 μg/ml PTX (static incubation) and ACSF at ZT15 (lanes 1 and 2), ACSF static (stat) incubation and 10 μM NMDA at ZT15 (lanes 3 and 4), or 1 μg/ml PTX and 10 μM NMDA at ZT15 (lanes 5 and 6), on the first day in vitro. Phase shifts were calculated relative to untreated (0 μM NMDA) *dexras1*$^{+/+}$ SCN slices ($ZT_{peak}$ = 5.89 ± 0.21). Values are presented as mean ± SEM phase shift. n = 3–4 per group. Neuman-Keuls test. **$p < 0.01$ versus same-treated wild-type. ##$p < 0.01$ versus vehicle- or static-treated slices of the same genotype.

ras1 abrogates the PTX-sensitive component. Thus, a Dexras1-$G_{i/o}$ pathway contributes to the phase-shifting actions of NMDA on SCN neurons. These data demonstrate that $G_{i/o}$ proteins are an essential component of a NMDA receptor-mediated photic input pathway into the circadian clock.

## The Absence of Dexras1 Shortens the Circadian Period

In our initial experiments, the advance of phase angle of entrainment in lower light intensities might have been due in part to an increase in the frequency of the circadian oscillator, leading to a shortened period. In constant darkness (DD), *dexras1*$^{-/-}$ mice (Supplemental Figures S4B and S4D at http://www.neuron.org/cgi/content/full/43/5/715/DC1) displayed a significantly shorter circadian period than that of wild-type mice (Supplemental Figures S4A and S4C at http://www.neuron.org/cgi/content/full/43/5/715/DC1) (period [hr] of wt versus *dexras1*$^{-/-}$ mice: 23.71 ± 0.08 versus 23.28 ± 0.11, $p = 0.008$). Both wild-type and *dexras1*$^{-/-}$ mice that were kept for up to 60 days in DD conditions remained rhythmic (data not shown). These data indicate that, while Dexras1 influences period length, it is not required for the generation and maintenance of circadian rhythms.

A shorter free-running period, as exhibited by *dexras1*$^{-/-}$ mice, may result from the following: (1) a direct change in core clock mechanisms; or (2) enhanced effects of nonphotic inputs (e.g., locomotor activity) to the circadian clock. In support of the latter hypothesis, when wild-type and *dexras1*$^{-/-}$ animals with similar levels of activity were compared, the free-running period of *dexras1*$^{-/-}$ mice was consistently shorter than that of matched wild-type controls (Supplemental Figure S4E at http://www.neuron.org/cgi/content/full/43/5/715/DC1). There was a strong negative correlation between activity and period length in both wild-type ($r = -0.60$) and *dexras1*$^{-/-}$ mice ($r = -0.65$), but there was a significant difference between the effect of activity on circadian period in *dexras1*$^{-/-}$ animals compared with wild-type controls ($p = 0.004$). The total level of locomotor activity was similar between wild-type and *dexras1*$^{-/-}$ mice ($p = 0.64$). These data are consistent with the notion that the period shortening in *dexras1*$^{-/-}$ mice is due to enhanced responsiveness to nonphotic cues rather than an increase in the level of nonphotic stimulation per se.

## *dexras1*$^{-/-}$ Mice Have an Increased Responsiveness to Nonphotic Stimuli

Our results demonstrate that Dexras1 is an important modulator of photic responsiveness of the circadian system. However, as a GEF for $G_{i/o}$ proteins, Dexras1 may also influence nonphotic signaling. We therefore tested *dexras1*$^{-/-}$ mice in two distinct behavioral paradigms that assess the influence of nonphotic stimuli on circadian rhythms.

In the first experimental paradigm, nonphotic feedback stimulation was reduced or enhanced by the locking or unlocking, respectively, of the running wheel (Edgar et al., 1991). When the wheel was immobilized, circadian rhythms were measured using drinking behavior. Period length of wild-type mice was not significantly altered by the locking of the running wheel (period [hr] before versus after wheel locking: 23.71 ± 0.06 versus 23.87 ± 0.06, $p > 0.05$) (Figures 5A and 5C). Likewise, unlocking of the wheel had no effect on the period in wild-type mice (period [hr] before versus after wheel

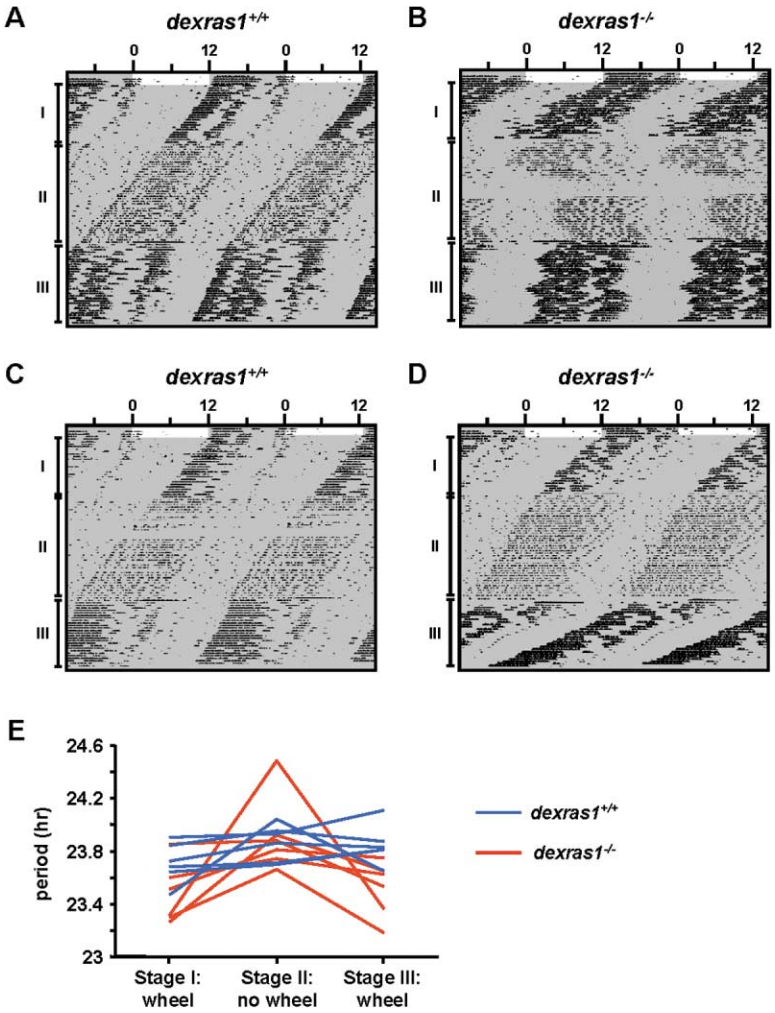


Figure 5. Nonphotic Effects of Daily Exercise Are Enhanced in the Absence of Dexras1

(A–D) Representative actograms of *dexras1*$^{+/+}$ (A and C) and *dexras1*$^{-/-}$ (B and D) mice. Mice were initially maintained on a 12:12 LD cycle (400 lux). They were subsequently released into constant darkness (DD) with free access to the running wheel (stage I). After 3 weeks, the running wheels were locked in position, and daily rhythms were assessed by drinking behavior (stage II). Six weeks later, the running wheels were unlocked, and daily rhythms were once again assessed by wheel-running activity (stage III). Test stages are indicated to the left of each actogram (Roman numerals).

(E) Graphical representation of the period length of individual *dexras1*$^{+/+}$ (blue) and *dexras1*$^{-/-}$ (red) mice at each stage of the experiment. Wheel locking and unlocking had a significant effect on the period length of *dexras1*$^{-/-}$ mice but not that of wild-type controls (Fisher's LSD; see the Results section for additional statistics).

unlocking: 23.87 ± 0.06 versus 23.85 ± 0.06, $p > 0.05$) (Figures 5A and 5C). These data indicate that the nonphotic effects of running activity are small in wild-type mice. Strikingly, the period of *dexras1*$^{-/-}$ mice was significantly increased by immobilization of the wheel (period [hr] before versus after wheel locking: 23.47 ± 0.09 versus 23.92 ± 0.12, $p < 0.01$) and was significantly decreased after the wheel was unlocked (period [hr] before versus after wheel unlocking: 23.92 ± 0.12 versus 23.52 ± 0.09, $p < 0.01$) (Figures 5B and 5D). A comparison between genotypes indicated that the period length was statistically indistinguishable between wild-type and *dexras1*$^{-/-}$ mice when the wheel was immobilized [wt versus KO: 23.87 ± 0.06 versus 23.92 ± 0.12; $F(1, 10) = 0.427$, $p > 0.05$]. However, period length was shorter in the *dexras1*$^{-/-}$ mice relative to wild-type controls when the wheel was freely accessible [period (hr) of wt versus KO mice after wheel unlocking: 23.85 ± 0.06 versus 23.52 ± 0.09; $F(1, 10) = -2.835$, $p < 0.01$]. These data, summarized in Figure 5E, indicate that the nonphotic effects of spontaneous activity (wheel running) are enhanced in *dexras1*$^{-/-}$ mice, and this, in turn, contributes to their shorter free-running period.

In the second experimental paradigm, we measured novelty-induced phase shifts in response to acute nonphotic pulses. Consistent with previous findings, wild-type mice did not phase shift in response to a single 3 hr bout of novel wheel exposure administered between ZT6 and ZT9 in the mid-daytime (Figures 6A, 6C, and 6E). In stark contrast, loss of Dexras1 resulted in a gain-of-behavior phenotype, with all *dexras1*$^{-/-}$ mice studied exhibiting significant and strong phase advances (Figures 6B, 6D, and 6E). The total amount of running on the novel wheel was comparable between the two genotypes (data not shown). The dramatic phase-shifting response of *dexras1*$^{-/-}$ mice to a novel wheel pulse deserves particular emphasis. Mice were hitherto considered to be much less responsive than golden hamsters to nonphotic manipulation, particularly an acute, behavior-based stimulus that produces a discrete phase shift in responsive species (Reebs and Mrosovsky, 1989a). In contrast, 3 hr novel wheel pulses administered in the late night (between ZT20 and ZT23) did not produce significant phase shifts in either wild-type (−0.21 ± 0.16 hr) or *dexras1*$^{-/-}$ (0.25 ± 0.26 hr) mice, indicating that the enhanced nonphotic responsiveness of *dexras1*$^{-/-}$ mice is phase restricted (data not shown). Taken together, our data show that Dexras1 plays a critical

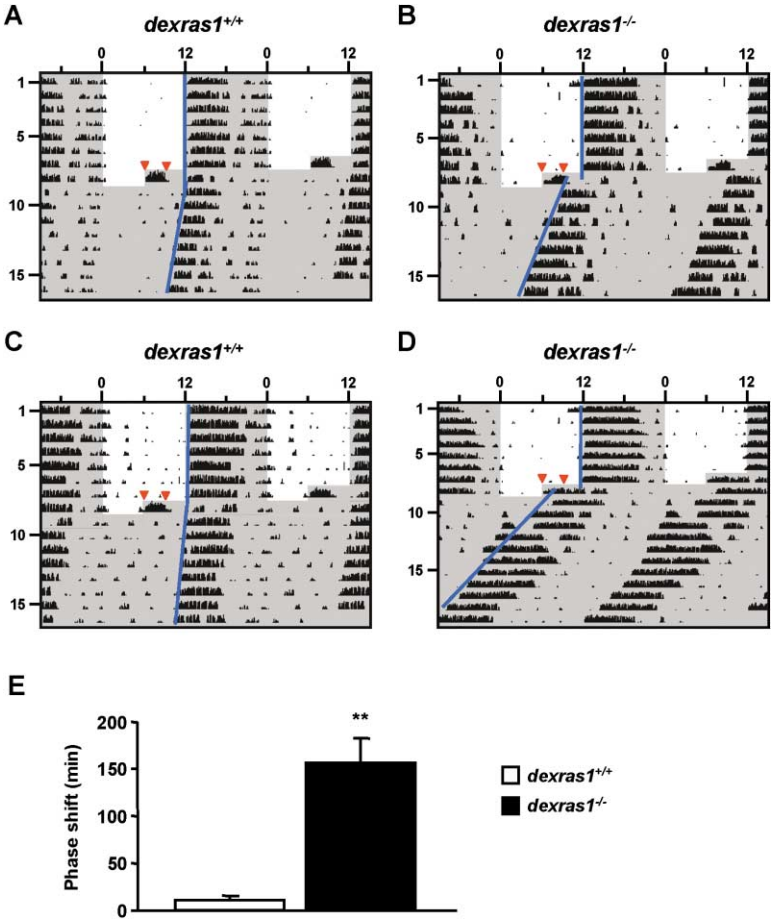


Figure 6. The Phase-Advancing Effect of Dark and Novel Wheel Exposure Is Potentiated in the Absence of Dexras1

(A–D) Representative actograms of *dexras1*$^{+/+}$ (A and C) and *dexras1*$^{-/-}$ (B and D) mice. Animals were entrained to a 12:12 LD cycle (400 lux). On day 8, mice were confined to a novel running wheel between ZT6 and ZT9 and maintained in darkness during the 3 hr wheel pulse (interval between the red arrowheads). Animals were then returned to their home cages and maintained in DD for at least 1 week. Activity onsets are indicated by blue lines.

(E) Quantification of novel wheel-induced phase shifts. Values are presented as mean ± SEM phase shift. n = 5–6 per group. Student's t test. **$p < 0.01$ versus same-treated wild-type.

role in suppressing the responsiveness of the circadian system to nonphotic stimuli.

### The Absence of Dexras1 Sensitizes the SCN to the Antagonistic Effects of NPY on NMDA-Induced Phase Shifts

We next addressed the mechanisms underlying the increased nonphotic effects in *dexras1*$^{-/-}$ mice. In various species, nonphotic-like effects can be elicited by neuromodulators, including NPY, serotonin, or GABA (Shibata et al., 1992; Lall and Biello, 2003). All subtypes of NPY receptors, including those implicated in circadian timekeeping by the SCN (Y1, Y2, and Y5), signal through $G_{i/o}$ proteins. As Dexras1 is also coupled through $G_{i/o}$ proteins, we asked whether the influence of NPY on the firing rhythms of SCN neurons was altered by the absence of Dexras1. Application of 0.2 ng NPY alone at ZT15 did not significantly alter the time of peak firing of either wild-type or *dexras1*$^{-/-}$ SCN neurons, relative to untreated controls (Figures 7A and 7B). In SCN slices from wild-type mice, the phase shift induced by the coapplication of NMDA and 0.2 ng NPY at ZT15 was similar to that elicited by NMDA alone (Figure 7C). In contrast, in *dexras1*$^{-/-}$ SCN slices the phase-shifting effects of NMDA were significantly attenuated by 0.2 ng NPY (Figure 7D). Preliminary results suggest that higher doses of NPY (2 ng and 20 ng) completely abrogated the phase-delaying effect of NMDA (10 μM), irrespective of genotype (data not shown). Thus, the absence of Dexras1 enhances the sensitivity of the SCN to the antagonistic effect of NPY on NMDA-induced phase shifts.

The mutually antagonistic effects of NMDA and NPY receptors may be due to their input into the common downstream substrate $G_{i/o}$. To analyze this hypothesis, we examined the effect of PTX on NPY-mediated attenuation of NMDA phase delays. As expected, PTX and NPY in combination did not alter the firing rhythms of SCN slices from wild-type and *dexras1*$^{-/-}$ mice, relative to untreated controls (Figures 7E and 7F). In wild-type slices, PTX attenuated the phase delay induced by NMDA in combination with NPY to values observed in slices treated with PTX and NMDA (Figure 7G). Importantly, in *dexras1*$^{-/-}$ SCN slices, PTX abolished the antagonistic effect of NPY on NMDA-induced phase delays (Figure 7H, compare with Figure 7D) and returned the time of peak firing to values observed following NMDA treatment alone. These data (Figure 7I) show that the absence of Dexras1 increases the sensitivity of SCN neurons to the antagonistic effect of NPY on NMDA-induced phase shifts. Inhibition of $G_{i/o}$ signaling reverts these effects, suggesting that this molecular antagonism is the consequence of enhanced sensitivity of activation of $G_{i/o}$ proteins by NPY receptors. Thus, Dexras1 plays an essential role, not only in mediating the $G_{i/o}$-dependent component of NMDA-induced phase shifts, but also in modulating the $G_{i/o}$-dependent, opposing influence of NPY on NMDA-induced phase delays.

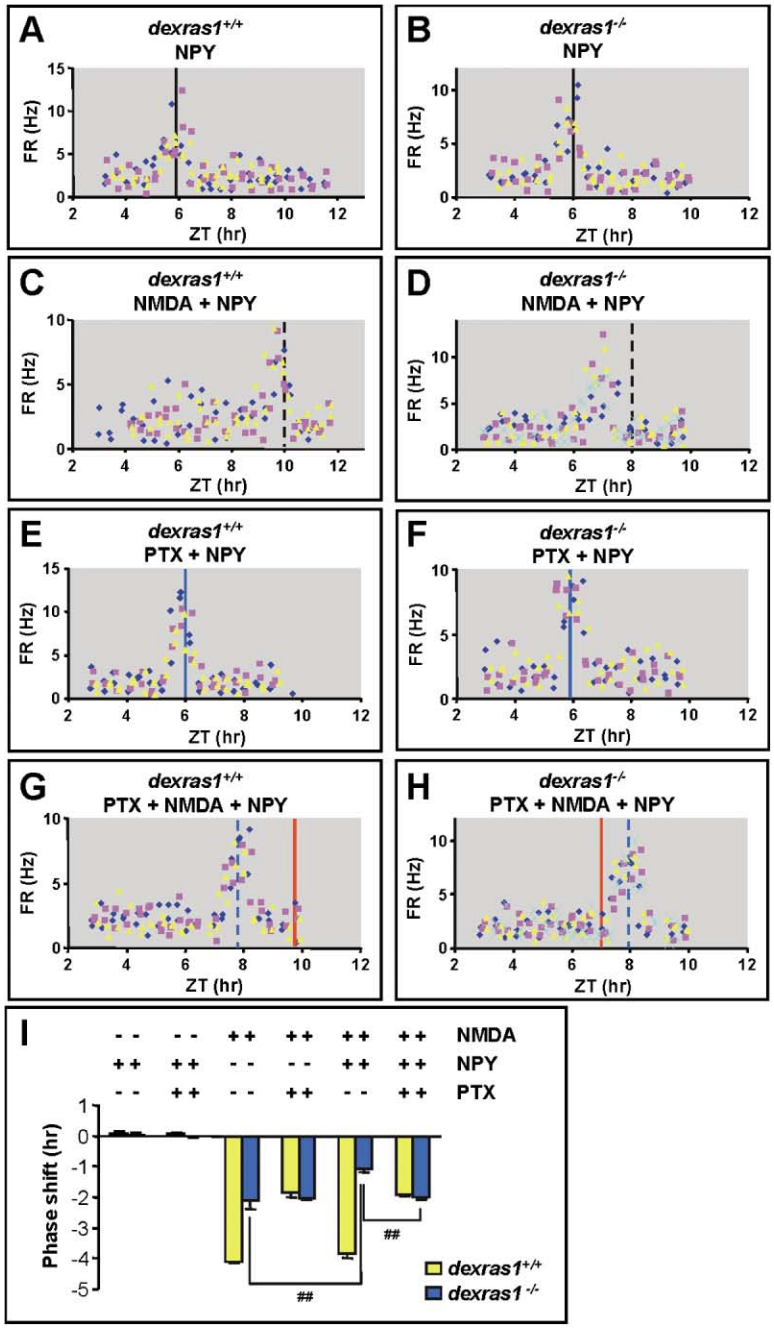


Figure 7. NMDA-Induced Phase Shifts Are More Sensitive to NPY Blockade in the Absence of Dexras1

(A–H) Firing rates (FR) of *dexras1*$^{+/+}$ (A, C, E, and G) and *dexras1*$^{-/-}$ (B, D, F, and H) SCN neurons at different ZT recorded on the second day in vitro in slices treated with 0.2 ng NPY alone at ZT15 (A, B, E, and F) or 0.2 ng NPY and 10 μM NMDA at ZT15 (C, D, G, and H), under standard conditions (A–D) or following incubation with 1 μg/ml PTX (E–H). The black line in (A) and (B) indicates the mean time of peak in untreated controls of the same genotype. The black dashed line in (C) and (D) indicates the mean time of peak in slices of the same genotype treated with 10 μM NMDA at ZT15. The blue line in (E) and (F) indicates the mean time of peak in slices of the same genotype treated with 1 μg/ml PTX prior to ACSF application at ZT15. The red line in (G) and (H) indicates the mean time of peak in slices of the same genotype treated with 0.2 ng NPY and 10 μM NMDA at ZT15. The blue dashed line in (G) and (H) indicates the mean time of peak in slices of the same genotype treated with 1 μg/ml PTX prior to 10 μM NMDA application at ZT15.

(I) Graphical summary of the phase-shifting effects of NMDA and NPY and the influence of pertussis toxin on the elicited phase shifts. Phase shifts were calculated relative to untreated (0 μM NMDA) *dexras1*$^{+/+}$ slices ($ZT_{peak}$ = 5.89 ± 0.21). Values are presented as mean ± SEM phase shift. n = 3–4 per group. Neuman-Keuls test. $^{\#\#}p < 0.01$.

### The Absence of Dexras1 Results in Behavioral Instability in Constant Light

All of our data support the notion that the loss of Dexras1 produces a reduced response to light and an enhanced response to nonphotic stimuli. Previous studies have shown that increased locomotor activity leads to period shortening, whereas in constant light (LL), increasing light intensities produce period lengthening (Aschoff, 1960). We predicted, therefore, that period lengthening in LL would be attenuated in *dexras1*$^{-/-}$ mice.

To test this hypothesis, animals were maintained under LL condition (10 lux) for a total of 8 weeks. Six out of eight wild-type mice exhibited a stable, lengthened period (25.15 ± 0.11 hr) and a stable level of locomotor activity throughout the experiment (Figures 8A and 8D). In one wild-type animal, locomotor activity was suppressed and circadian rhythmicity was lost in the last 4 weeks of LL; however, prior to this, the stable period length (24.88 hr) and locomotor activity were comparable to those of the other wild-type controls (data not shown). In the other wild-type animal, there were two overlapping rhythms with a short (23.16 hr) and a long (25.2 hr) period (data not shown).

In contrast, none of the *dexras1*$^{-/-}$ mice exhibited behavior comparable to that observed in the wild-type controls with a stable period. Only one out of eight *dexras1*$^{-/-}$ mice showed robust locomotor activity in a single bout, but the period underwent a continuous

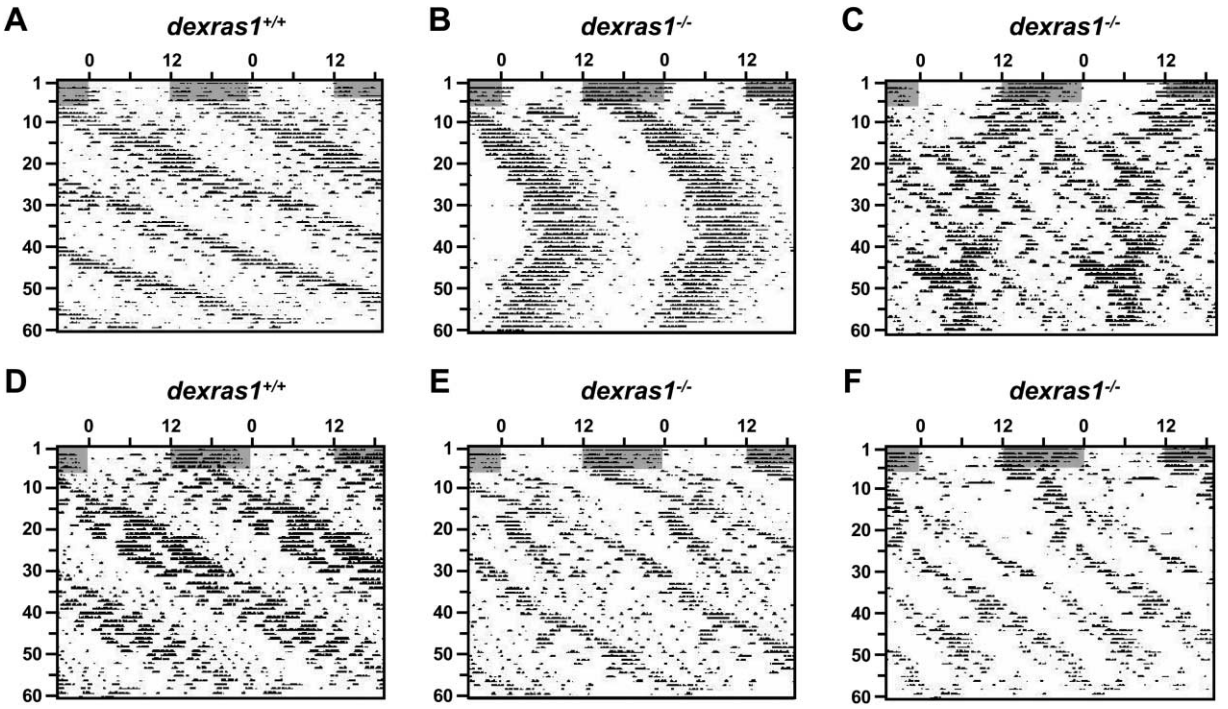


Figure 8. *dexras1*$^{-/-}$ Mice Exhibit Unstable Circadian Locomotor Activity under Constant Light

Representative actograms of wheel-running activity in *dexras1*$^{+/+}$ (A and D) and *dexras1*$^{-/-}$ (B, C, E, and F) mice. The light intensity was set at 10 lux for the constant light (LL) portion of the experiment (days 6 to 60).

shortening over the 8 week period (Figure 8B). This gradual reduction in period was observed in another *dexras1*$^{-/-}$ mouse, but this animal desynchronized, giving rise to a second, minor bout of activity with a period of 24.86 hr (data not shown). Immediately upon transfer to LL, one *dexras1*$^{-/-}$ mouse showed a dramatic suppression of locomotor activity and weak rhythmicity (data not shown). Three out of eight *dexras1*$^{-/-}$ mice showed a highly unstable and erratic period during the 8 weeks in LL (Figure 8C). One *dexras1*$^{-/-}$ mouse exhibited desynchrony: the major activity bout was maintained for 8 weeks with a stable, long period (25 hr), and a minor bout with a shorter period (24.62 hr) appeared between the 10th and 24th days in LL (Figure 8E). Most remarkably, after only 10 days in LL, the last *dexras1*$^{-/-}$ mouse showed a "hamster-like" splitting of locomotor rhythms, defined by the antiphase relationship of the two bouts of equal period (24.8 hr) (Figure 8F). Because of the instability in period length and desynchronization observed in all *dexras1*$^{-/-}$ mice, we felt that reporting a single value for period length in LL would not sufficiently describe the behavior of the mutant animals. These data indicate that Dexras1 is essential for stabilizing the circadian system under constant light condition. Dexras1 is therefore involved in multiple transduction pathways, one of which appears to be required for normal pacemaker-pacemaker communication.

## Discussion

Our results demonstrate a pivotal role for Dexras1 in the operation of the mammalian biological clock. We report that genetic inactivation of Dexras1 results in the following: (1) abnormal photic entrainment of the clock; (2) reduced responsivity of the clock to activation of the photic entrainment pathway in vivo and in vitro; (3) enhanced responsivity to nonphotic stimulation in vivo and in vitro; (4) an altered circadian period in constant dark; and (5) reduced integrity of rhythm generation in constant light. Thus, our experiments have identified an essential signaling molecule that integrates various inputs to the molecular clock, modulates the responsiveness of the master clock to photic and nonphotic stimuli in opposite directions, and provides stabilization of period generation and entrainment.

Our initial experiments were based on existing evidence that Dexras1 is expressed rhythmically in the SCN and peaks at night (Panda et al., 2002; Ueda et al., 2002; Takahashi et al., 2003) and that Dexras1 is activated downstream of NMDA receptor-mediated signaling (Fang et al., 2000). Indeed, *dexras1*$^{-/-}$ mice do not remain synchronized with the experimentally imposed light-dark cycle when the light intensity is reduced, demonstrating a deficit in photic entrainment. This is further substantiated by the finding that brief light pulses are less effective at changing the phase of the circadian system in *dexras1*$^{-/-}$ mice. Our electrophysiological data indicate that the photic entrainment deficit is mediated at the level of the SCN and that loss of Dexras1 attenuates NMDA receptor signaling. At all doses analyzed, NMDA produced a smaller phase delay in the firing rhythms of *dexras1*$^{-/-}$ SCN neurons compared with wild-type controls. Importantly, our in vitro data indicate that the entrainment deficit is not merely the

byproduct of the period shortening that is observed concomitantly in *dexras1*$^{-/-}$ mice, but that photic responsiveness itself is also compromised in the absence of Dexras1. These data are in keeping with the role of glutamate as a principal mediator of photic effects on the central pacemaker (Hannibal, 2002), with NMDA receptors playing an important role in glutamatergic signal transduction within the SCN (Colwell and Menaker, 1992). For instance, intra-SCN injections of NMDA in vivo (Mintz et al., 1999) or NMDA application onto SCN slices in vitro (Ding et al., 1994; Shibata et al., 1994) elicit photic-like phase shifts in locomotor behavior or firing rhythms, respectively, with the resulting phase response curve of NMDA closely resembling that of light. Moreover, NMDA has been shown to induce Dexras1 activation in vitro (Fang et al., 2000). Together, these observations support our conclusion that a NMDA receptor-Dexras1 signaling pathway influences photic responsiveness of the circadian system.

The study by Fang et al. (2000) demonstrated that Dexras1 activity is enhanced by NMDA receptor activation, but the signal transduction mechanisms downstream of a NMDA receptor-Dexras1 pathway remained unexplored. Here, we show that MAPK activation in response to light is severely attenuated in *dexras1*$^{-/-}$ mice. MAPK inhibitors completely abrogate NMDA-induced phase shifts in SCN slices irrespective of genotype, and light-induced ERK activation is severely compromised in the SCN of *dexras1*$^{-/-}$ mice. Pharmacological blockade of $G_{i/o}$ proteins has no effect on NMDA phase shifts in *dexras1*$^{-/-}$ SCN neurons but reduces NMDA phase shifts in wild-type SCN neurons to mutant levels. These data are consistent with our interpretation: (1) NMDA receptors activate $G_{i/o}$ protein-dependent and -independent pathways; (2) Dexras1 couples NMDA receptors to $G_{i/o}$ activation; (3) Dexras1 activates the MAPK pathway; and (4) MAPK activation is essential for photic-like phase shifts, regardless of their dependency for Dexras1. Recently, others have shown that light-induced MAPK activation in the SCN is restricted to the night (Obrietan et al., 1998) and that MAPK inhibitors strongly attenuate photic phase shifts of behavioral rhythms (Butcher et al., 2002; Coogan and Piggins, 2003). In other biological systems, NMDA receptor stimulation has been demonstrated to activate the MAPK pathway in a manner that is sensitive to $G_{i/o}$ inhibition by PTX (Chandler et al., 2001; Perkinton et al., 2002). Together with a biochemical report that Dexras1 has GEF activity for $G_{i/o}$ proteins (Cismowski et al., 2000) and can activate MAPK in vivo (this report) and in vitro (Cismowski et al., 1999), our study identifies a Dexras1-$G_{i/o}$ pathway that couples NMDA receptors and light stimulation to MAPK activation. Disruption of this pathway, for example, by genetic ablation of Dexras1, leads to alterations in photic entrainment.

The circadian system responds not only to the external day-night cycle (photic) but also to nonphotic cues, such as arousal, activity, food, or predators. As an example of the contrasting nature of the effects of photic and nonphotic cues, the maximal responsiveness of the hamster circadian system to these kinds of stimuli occurs in opposite phases of the circadian cycle (Reebs and Mrosovsky, 1989b), and their clock-resetting actions are mutually antagonistic (Ralph and Mrosovsky, 1992; Biello et al., 1997; Maywood and Mrosovsky, 2001). In this report, we have identified a signaling molecule that modulates the responsiveness of the master clock to both photic and nonphotic inputs. We provide genetic evidence that while Dexras1 positively regulates the photic input pathway, the same molecule negatively modulates nonphotic signaling. Not only do changes in activity levels (by locking or unlocking the running wheel) have a more pronounced effect on the free-running period of *dexras1*$^{-/-}$ mice compared with wild-type controls, but presentation of an acute nonphotic stimulus (the novel wheel) in the daytime, in fact, produces a strong phase-advancing response that is not typical of mice in general. In other words, the absence of Dexras1 enhances nonphotic responsiveness of the mouse circadian system.

In keeping with this, we find that the lack of Dexras1 sensitizes the SCN to NPY effects, as lower doses of NPY are able to attenuate NMDA-induced phase shifts in *dexras1*$^{-/-}$ but not wild-type SCN neurons. This NPY effect on *dexras1*$^{-/-}$ SCN is abrogated by PTX, indicating that this is a $G_{i/o}$-mediated process. Thus, it appears that in the absence of Dexras1, $G_{i/o}$ proteins are more effectively coupled to NPY receptor activation. Consistent with our observation, other studies have shown that events downstream of GPCR-dependent $G_{i/o}$ activation, for example, potassium channel or MAPK activation, are attenuated by Dexras1 overexpression in cell lines in vitro (Graham et al., 2002; Takesono et al., 2002). While our data suggest that an altered sensitivity of the NPY system contributes to the enhanced nonphotic effects in *dexras1*$^{-/-}$ mice, Dexras1 may play an additional role in nonphotic signaling. In fact, some or all receptors for NPY, serotonin, and GABA are GPCRs, which activate $G_{i/o}$ proteins, as are the receptors for other neuromodulators with nonphotic-like properties, such as opioids and melatonin (reviewed in Wong, 2003).

Under constant illumination, *dexras1*$^{-/-}$ mice show a plethora of phenotypes, ranging from unstable period length to desynchrony and splitting of locomotor rhythms. Spontaneous splitting under LL has been reported in hamsters but not in mice; even then, only 30% or 60% of hamsters exhibit this behavior under constant illumination (Pickard et al., 1993). Splitting in hamsters is prevented by lesions to the intergeniculate leaflet (IGL), which releases NPY and GABA via the GHT onto the SCN (Pickard et al., 1987; Harrington and Rusak, 1988). It is tempting to speculate that the splitting observed in our *dexras1*$^{-/-}$ mouse is due to enhanced NPY signaling, as our other data would suggest, and that Dexras1 is a stabilizing factor for the circadian clock.

Two major implications arise from our present study. Firstly, based on our genetic evidence, Dexras1 sits at an interface between photic and nonphotic signal transduction in the circadian system: Dexras1 is both a positive regulator of the photic input pathway and a negative modulator of nonphotic signaling. A mechanistic explanation for this duality is based on the effect of Dexras1 on $G_{i/o}$ protein signaling (Cismowski et al., 1999; Graham et al., 2002) (Figure 9). Our data indicate that light or photic-like stimuli (e.g., NMDA) reset the SCN clock by activating a MAPK signaling cascade that itself is dependent on Dexras1-mediated $G_{i/o}$ activation (Figure 9). In contrast, we show that the effects of NPY on the SCN

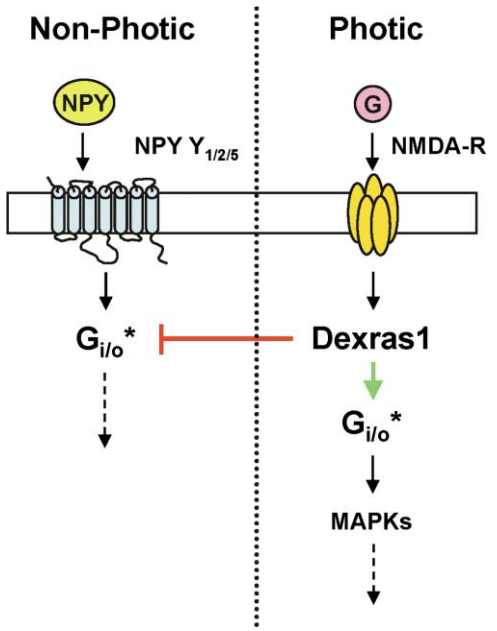


Figure 9. Proposed Model of Dexras1 Function in Modulating Photic and Nonphotic Effects of the Circadian Clock

Photic effects are mediated, in part, by light-dependent activation of NMDA receptors expressed in the SCN. NMDA receptor activation upregulates Dexras1 function, increasing the guanine nucleotide exchange activity of Dexras1. As a result, Dexras1 activates $G_{i/o}$ and MAPKs, leading to photic resetting. Nonphotic effects are mediated by neuropeptide Y (NPY) receptors in the SCN, which, in turn, activate $G_{i/o}$ proteins via enhanced guanine nucleotide exchange. Dexras1 inhibits NPY-mediated signal transduction and ultimately nonphotic effects, potentially by competing with NPY receptors for the common downstream substrate $G_{i/o}$, thereby reducing G protein-coupled receptor (GPCR)-mediated activation of $G_{i/o}$ signaling processes. G, glutamate. $G_{i/o}$*, $G_{i/o}$ in the active state. Green arrow, activation. Red arrow, inhibition. Dashed arrows, unidentified downstream processes.

clock are potentiated in the absence of Dexras1 and that this enhanced nonphotic response is also a $G_{i/o}$-mediated process. However, in this case, we believe that the loss of Dexras1 allows for more efficient coupling of $G_{i/o}$ proteins *directly* to activated NPY receptors (Figure 9). By imposing circadian rhythmicity upon the expression of Dexras1 (Panda et al., 2002; Ueda et al., 2002; Takahashi et al., 2003), the SCN clock is able to regulate simultaneously both photic and nonphotic inputs and, conceivably, maintain the proper temporal relationship between photic and nonphotic responsiveness. More generally, our results suggest a concrete mechanism by which various intracellular signaling pathways are coupled to produce an integrative response in a complex yet essential biological system.

The second issue involves the well-known differences among various species in the responsiveness of their circadian system to photic and nonphotic inputs. Unlike hamsters, mice typically do not respond to acute, nonphotic manipulations (Edgar et al., 1991). However, mutation of dexras1 has produced a mouse strain that shows strong nonphotic responses and, therefore, responds more like another species. One tempting speculation is that species-specific responsiveness of the circadian system may be brought about by natural selection of the function or expression of a few discrete genes, such as Dexras1, that play a key role in regulating inputs to the clock.

## Conclusions

Our results show that the lack of Dexras1 leads to reduced photic and enhanced nonphotic effects on the circadian timekeeping system. The attenuation in photic effects is attributed to a deficit in NMDA signaling to $G_{i/o}$ and MAPK activation within the SCN. Increased sensitivity of NPY signaling is likely to contribute to increased nonphotic responses. Notably, Dexras1 modulates the strengths of photic and nonphotic responses in opposite directions. Furthermore, the circadian system of *dexras1*$^{-/-}$ animals is highly unstable under conditions of constant illumination. In summary, our results identify a biological paradigm of regulation in the circadian system, in which one signaling molecule, Dexras1, serves as a differential modulator of photic and nonphotic responses of the mammalian central pacemaker.

## Experimental Procedures

More detailed description of Experimental Procedures is available in the Supplemental Data at http://www.neuron.org/cgi/content/full/43/5/715/DC1.

### Generation of *dexras1*$^{-/-}$ Mice

A targeting vector (521 bp short arm and 7.6 kb long arm) was constructed using the pGK-neo vector. A portion of the *dexras1* genomic DNA containing *dexras1* cDNA nucleotides +179 to +822 was replaced with a neomycin (*neo*) resistance cassette. The targeting construct was electroporated into E14K ES cells to obtain homologously recombined clones. Two independent *dexras1*$^{+/-}$ ES cell lines were injected into C57BL/6-derived blastocysts to generate chimeric mice, which were crossed to C57BL/6 mice to produce *dexras1*$^{+/-}$ mice. These *dexras1*$^{+/-}$ mice were subsequently backcrossed into the C57BL/6 background for five generations to analyze the consequence of the null mutation in a more homogeneous genetic background. Wild-type and *dexras1*$^{-/-}$ littermate male mice generated from heterozygous intercrosses were used for all studies. Mice were maintained at the animal facilities of the Ontario Cancer Institute and the University of Toronto in accordance with institutional guidelines.

### Behavioral Analyses

Mice were singly housed in cages equipped with a running wheel in light-tight, ventilated boxes with controlled lighting. Wheel running activity was monitored and analyzed with VitalView and Actiview (MiniMitter Co., Inc., Sunriver, OR). For the photic phase shift experiment, mice were entrained to a 12:12 LD cycle (400 lux) for at least 1 week. Two hours after light-off on the final LD cycle (ZT14), mice received a 15 min light pulse of 10 lux and were subsequently released into DD for 7–10 days. Animals were reentrained, and the procedure was repeated for a 40 lux light pulse.

To assess the effect of spontaneous activity on period length, mice were entrained to a 12:12 LD cycle (400 lux) for 2 weeks with free access to a running wheel and then released into DD for an additional 3 weeks (stage I). Subsequently, mice were transferred to individual cages equipped with a running wheel and a raised metal platform positioned beneath the spout of the drinking bottle. Immediately upon transfer of the animal, wheels were locked by inserting a metal rod through the bars of the cage top and the running wheel. During this stage of the experiment, drinking activity was continuously monitored using VitalView (stage II). After 6 weeks, animals were returned to original housing conditions with free access to the running wheel, and wheel running activity was monitored for an additional 4–5 weeks (stage III). For the novel wheel exposure experiment, mice were entrained to a 12:12 LD cycle (400 lux) for

1 week. Animals were confined to novel running wheels from ZT6 to ZT9. Once the transfer was complete, the lights were turned off. After the 3 hr pulse, animals were transferred back to their home cages under dim red light (1 lux). The animals remained in DD for at least 1 week after the pulse.

All other behavioral tests that were performed have been described previously (Cheng et al., 2002).

### Electrophysiological Studies

Recording of extracellular single-unit activity of SCN cells was performed as described (Yannielli and Harrington, 2001). Unless otherwise stated, all chemicals were purchased from Sigma, resuspended in artificial cerebrospinal fluid (ACSF), and applied to the SCN as a 200 nl microdrop. NMDA (1–100 μM) was applied at ZT15. NPY (0.2–20 ng) alone was applied at ZT15 or 5 min after application of NMDA at ZT15. For MAPK inhibition experiments, U0126 (20 μM) or vehicle (0.08% [v/v] DMSO) was applied to the SCN tissue by continuous perfusion between ZT14.333 and ZT15.667. For $G_{i/o}$ inhibition experiments, SCN tissue slices were maintained for 6 hr (ZT9–ZT15) in a static bath of warmed, oxygenated ACSF with or without PTX (1 μg/ml), before being transferred to the slice chamber. All drug treatments occurred on the first day in vitro, and extracellular single-unit activity in the SCN was measured on the second day in vitro.

### Light-Induced ERK Phosphorylation

Mice were exposed to 10 min of light of different intensities at ZT15 (3 hr after lights off) and immediately sacrificed. Control animals not exposed to light were sacrificed at ZT15.167. Tissue processing, immunohistochemistry, and image analysis were performed exactly as described (Butcher et al., 2003). The following antibodies were used: phospho-p44/42 MAP kinase (Thr202/Tyr204) (1:500 dilution; Cell Signaling) and Alexa Fluor 488 goat anti-rabbit IgG (1:500; Molecular Probes). Data are presented as absolute pERK intensity values using a 0–255 intensity scale.

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