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26. ERN amplitude increases when subjects favor accuracy over speed, whereas ERN amplitude is reduced as error rates increase (*13, 16*). If we would accept higher error rates after alcohol, then ERN amplitude might vary with alcohol dosage because of the effects of alcohol not on error detection (expressed in ERN amplitude) but on error rate (which in turn covaries with ERN amplitude). Should our procedure incur a speed-accuracy tradeoff to obtain comparable error percentages across alcohol conditions, then the relative emphasis on accuracy after alcohol should result in larger ERNs, which would oppose the alcohol-induced reduction in ERN amplitude anticipated here, thus rendering our approach a conservative one.
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Tables S1 and S2

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Role of Melanopsin in Circadian Responses to Light

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Melanopsin has been proposed as an important photoreceptive molecule for the mammalian circadian system. Its importance in this role was tested in melanopsin knockout mice. These mice entrained to a light/dark cycle, phase-shifted after a light pulse, and increased circadian period when light intensity increased. Induction of the immediate-early gene c-*fos* was observed after a nighttime light pulse in both wild-type and knockout mice. However, the magnitude of these behavioral responses in knockout mice was 40% lower than in wild-type mice. Although melanopsin is not essential for the circadian clock to receive photic input, it contributes significantly to the magnitude of photic responses.

Several lines of evidence have recently indicated that melanopsin is a component of the photoreceptive system for circadian rhythms of mammals. Rods and cones are not necessary for circadian responses to light, which suggests that other photoreceptors exist (*1–3*). Melanopsin is found exclusively in the retina (*4–7*). Retinal ganglion cells of the inner retina that contain melanopsin mRNA and protein form dendritic plexuses in a network that allows these cells to capture photic stimuli across broad spatial domains (*7*). In these same cells, melanopsin is colocalized with pituitary adenylate cyclase activating polypeptide (PACAP); PACAP-containing ganglion cells form the retinohypothalamic tract that directly innervates the suprachiasmatic nucleus (SCN) (*8*), site of the mammalian circadian pacemaker. Furthermore, melanopsin-containing cells that innervate the SCN are intrinsically photosensitive in a manner consistent with their being irradiance detectors, but they are not suited for fine visual discrimination tasks (*5*). Cryptochrome photopigments are also found in the inner retina as well as in the SCN (*9, 10*), but there has been disagreement about their role in circadian photoreception (*9–12*).

Despite the data in support of melanopsin, there are no data to confirm a functional role in transducing photic input to the circadian pacemaker. Because input to the circadian pacemaker has several effects on the phase and period of circadian rhythms, one can test for melanopsin's involvement in these variables by investigating circadian photoresponsiveness in mice that lack melanopsin. We examined the capacity of mice with a targeted disruption of the melanopsin gene (fig. S1) to (i) entrain to a light/dark cycle, (ii) phase shift to brief light pulses, (iii) comply with Aschoff's rule [rhythm period (tau) increases with light intensity in nocturnal animals (*13, 14*)], and (iv) retain light-induced gene expression in the SCN (*15, 16*). We assessed circadian function in these mice by monitoring locomotor activity with infrared motion detectors (*17–19*). Melanopsin knockout (n = 7) and wild-type (n = 7) mice were housed with 12 hours of light per day for 13 to 16 days followed by constant darkness (DD). All animals were exposed to a 30-min bright-light pulse 16 days after DD began (*17, 18*). Mice remained in their home cages but were moved to a different room for the light pulse. The light pulse occurred four circadian hours [circadian time (CT) 16] after the onset of daily activity, at the time when light produces the maximal phase shift in most inbred mouse strains (*20, 21*). Because nonspecific factors associated with moving cages to novel locations sometimes produce phase shifts, all animals were given a control dark pulse 10 days after the light pulse at CT 16; animals were treated exactly as for the light pulse but remained in darkness during the procedure. Two weeks later, all mice were exposed to constant light (LL) for 14 days to test for conformity with Aschoff's rule. A separate group of wild-type (n = 3) and knockout (n = 3) mice was exposed to a 30-min light pulse, along with two mice exposed to a control dark pulse, on the second day of DD; c-*fos* expression was measured in the SCN of these animals by in situ hybridization (*17, 18*) as a cellular marker of SCN photosensitivity (*15, 16*).

The results of the three behavioral measures and the cellular index of circadian photoresponsiveness were consistent and show that melanopsin is not essential for transduction of photic stimuli to the circadian pacemaker. The light pulse at CT 16 produced robust phase delays in activity rhythms in both wild-type and knockout mice, although phase-shift magnitude was significantly lower (P = 0.02) in knockout mice than in wild-type mice (Fig. 1). Mean phase shifts for the dark pulse were <10 min for both groups. A second behavioral measure showed that diurnal rhythms under the LD cycle represent true entrainment rather than masking by the LD cycle because activity onsets in DD can

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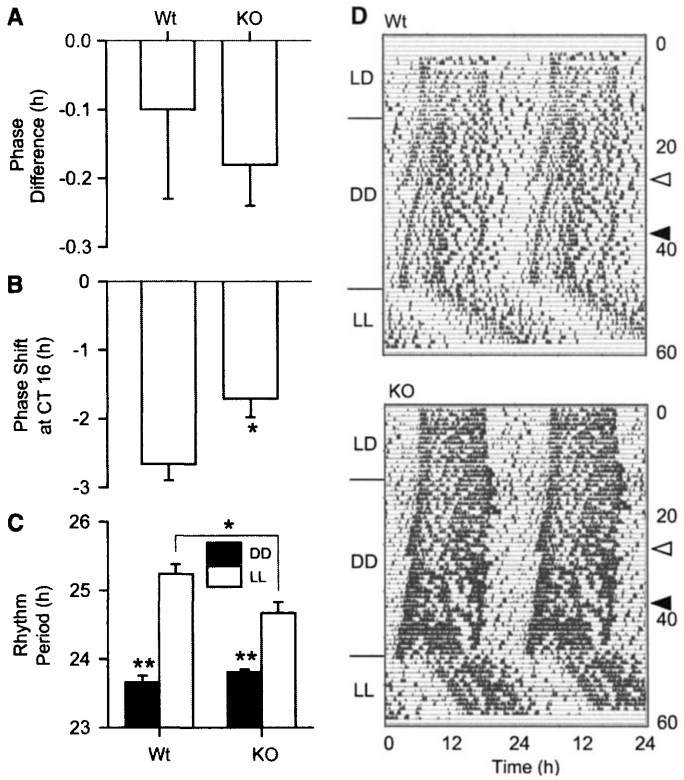

Fig. 1. Behavioral responses to light treatments in wild-type (n = 7) and melanopsin knockout (n = 7) mice. (**A**) Mean difference between the phase of activity onsets in LD and on the first day of DD. These values were determined by a linear regression through activity onsets in DD that were extrapolated back to the first day in DD. Activity onsets on that day, as determined by the regression, were within 15 min of the mean time of activity onset under the LD cycle (negative values indicate a delayed activity onset). Rhythms under LD were entrained and not the result of masking by the LD cycle. If knockouts were not entrained, activity onsets would have been distributed randomly throughout the day and night, which would have been reflected by high variability around the mean phase. (**B**) Phase shift after a light pulse at CT16. All knockout and wild-type mice phase-shifted in response to a light pulse at CT16 in DD, but this response was 36% less in knockout mice. Mean phase shifts for the dark pulse were <10 min for both groups. (**C**) Rhythm period (tau) in DD and LL. The effects of LL on tau depended on genotype (two-way analysis of variance, genotype and lighting condition interaction, $F_{1,12} = 7.0$; $P = 0.02$). There were no differences in tau among these groups in DD and all animals increased tau in LL; however, tau of knockout mice increased 46% less than it did in wild-type mice in LL (t test with Tukey's post hoc correction; $P = 0.02$). *$P < 0.05$, **$P < 0.01$. (**D**) Actograms from representative wild-type and knockout mice: open triangle, light pulse; filled triangle, dark pulse control. Days are indicated on the right.

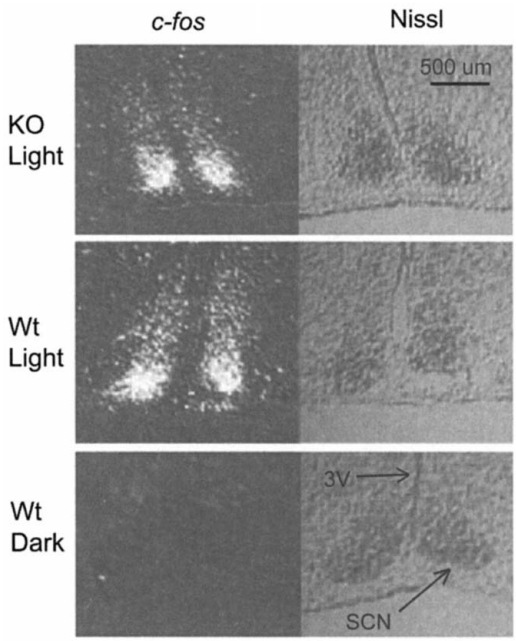

Fig. 2. Photic induction of c-*fos* mRNA in the SCN in representative coronal brain sections of wild-type (Wt) and melanopsin knockout (KO) mice. KO and Wt mice were exposed to light pulses that began at CT16 and lasted 30 min; a section from a wild-type mouse sacrificed at the same time, but held in darkness during the 30 min, is also shown (**bottom**). Note that both Wt and KO mice respond with typical c-*fos* induction (*15*, *16*) after light exposure. (**Left**) Dark-field image of silver grains representing c-*fos* mRNA levels. (**Right**) The same section in bright-field image showing Nissl staining. 3V, third ventricle.

be extrapolated back to the mean time of activity onsets under the LD cycle in each animal (Fig. 1). If locomotor activity of knockout mice were masked by the LD cycle, the extrapolation would reveal high variability among individual animals in the phase at which the free run started. Instead, the extrapolation revealed that the phase of activity onsets on the first day of DD was within 15 min of the phase in LD for both groups of animals. Thus, there were no significant differences ($P = 0.61$) (Fig. 1) between wild-type and knockout mice in the phase at which the free run began in DD. A third behavioral measure revealed that melanopsin knockout mice conform to Aschoff's rule, as do wild-type mice. There was no significant difference in tau among knockout and wild-type mice in DD ($P = 0.19$), and both genotypes had significantly longer tau values in LL ($P < 0.001$) (Fig. 1). However, the effect of increased light intensity on tau was significantly smaller in knockout than in wild-type mice (Fig. 1). In situ hybridization revealed clear induction of c-*fos* in the SCN of wild-type and knockout mice that were exposed to the light pulse (Fig. 2).

Multiple measures of circadian function in these knockout mice show that melanopsin is not essential for entrainment. However, phase and period responses of melanopsin-deficient mice were about 40% less than in wild-type animals; thus, melanopsin appears to be a significant contributor to circadian function. Melanopsin may function as a photopigment, but it is also possible that it is critical for some other photopigment to perform normally. For example, it might serve as a retinaldehyde isomerase, like some other members of the opsin family (*22*). As with all knockout studies, the absence of a functional protein may trigger compensation during development or it may alter functioning of the adult system. We believe our results are more likely attributable to the lack of functional melanopsin in mature retinal ganglion cells; however, conditional knockout studies are needed to address this developmental question.

The most parsimonious explanation for these data is that multiple types of retinal photoreceptors and photopigments contribute to the transduction of photic information to the circadian system. Cryptochromes, for example, appear to be capable of transmitting light information to the SCN in mice depleted of retinal and therefore lack functional rods, cones, and melanopsin (*23*). Light pulses are also able to induce c-*fos* in mice that lack rods and most cones as well as both cryptochrome proteins (*24*). These data suggest that there are redundant photic inputs to the clock, each of which is sufficient to sustain photic entrainment. Similar redundancy of function has been reported for plants (*25*), flies (*26*), and several classes of vertebrates (*27*). Un-

like the latter two groups, mammals lack functional extraocular photoreceptors (*28*); thus, redundancy in photoreception is confined to the retina. One challenge is to determine the relative contributions of melanopsin, rod/cone opsins, cryptochromes, and other currently uncharacterized photopigments in communicating photic information to the circadian system.

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Supporting Online Material

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Materials and Methods
Fig. S1
Reference

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Melanopsin (*Opn4*) Requirement for Normal Light-Induced Circadian Phase Shifting

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The master circadian oscillator in the hypothalamic suprachiasmatic nucleus is entrained to the day/night cycle by retinal photoreceptors. Melanopsin (*Opn4*), an opsin-based photopigment, is a primary candidate for photoreceptor-mediated entrainment. To investigate the functional role of melanopsin in light resetting of the oscillator, we generated melanopsin-null mice ($Opn4^{-/-}$). These mice entrain to a light/dark cycle and do not exhibit any overt defect in circadian activity rhythms under constant darkness. However, they display severely attenuated phase resetting in response to brief pulses of monochromatic light, highlighting the critical role of melanopsin in circadian photoentrainment in mammals.

Photoentrainment of circadian rhythms can occur in the absence of classical visual photoreceptors (rods and cones) (*1*, *2*) but not in animals without eyes (*3*, *4*). Therefore, nonvisual ocular photoreceptor(s) must mediate light entrainment. Recently, melanopsin has been suggested as a candidate circadian photopigment in mammals on the basis of several lines of evidence (*5*–*10*). First, it is expressed in retinal ganglion cells (RGCs), which directly project to the suprachiasmatic nucleus (SCN) and express the neuropeptide pituitary adenylyl cyclase activating peptide (PACAP) (*11*). PACAP has also been implicated in circadian photoreception (*12*). Furthermore, physically isolated melanopsin-containing RGCs depolarize in response to direct illumination with a spectral sensitivity that closely matches the behavioral action spectrum of circadian photoentrainment in rodents (*8*, *10*). To formally investigate the role of melanopsin in light resetting of the circadian clock in mammals, we have generated melanopsin-null mice ($Opn4^{-/-}$) by replacing exon 1 of the melanopsin gene with a neomycin-resistance gene by homologous recombination in embryonic stem cells (fig. S1). We verified interruption of the *Opn4* gene immunohistochemically with an antiserum to melanopsin (figs. S1 and S2). The targeted locus exhibits normal autosomal Mendelian inheritance, and the $Opn4^{-/-}$ mice are apparently healthy with anatomically normal eyes and no obvious developmental defects.

Photoreceptors can contribute to circadian oscillation in three ways: (i) as oscillator components (*13*, *14*), (ii) in acute light suppression of activity (masking) (*15*), and (iii) in light entrainment of the clock (*16*). To determine whether melanopsin is required for normal functioning of the circadian oscillator, we characterized locomotor activity rhythms in driven and free-running conditions in $Opn4^{-/-}$ mice and littermate controls (Fig. 1, A and B) (*17*). $Opn4^{-/-}$ mice entrained to a 12-hour white light (800 lux)/12-hour dark (LD) cycle (*18*) and exhibited no detectable defect in locomotor activity rhythms in constant darkness (DD). During entrainment, the phase angle of activity onset in relation to the LD cycle was similar in both wild-type and $Opn4^{-/-}$ mice. In DD, the free-running period length (τ) of the locomotor activity rhythm in the knockout mice was not significantly different from that of wild-type littermates (Fig. 2D). Total activity and the length of the activity phase during a

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