Preservation of light signaling to the suprachiasmatic nucleus in vitamin A-deficient mice

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To investigate the role of retinal-based pigments (opsins) in circadian photoreception in mice, animals mutated in plasma retinol binding protein were placed on a vitamin A-free diet and tested for photic induction of gene expression in the suprachiasmatic nucleus. After 10 months on the vitamin A-free diet, the majority of mice contained no detectable retinal in their eyes. These mice demonstrated fully intact photic signaling to the suprachiasmatic nucleus as measured by acute *mPer* mRNA induction in the suprachiasmatic nucleus in response to bright or dim light. The data suggest that a non-opsin pigment is the primary circadian photoreceptor in the mouse.

circadian photoreceptor | retinol binding protein

In mammals, it appears that the eye is the sole photosensitive organ responsible for both vision and entrainment of the circadian clock to external light–dark cycles (1). Although it is well established that the opsin protein family comprises the visual photoreceptors, the classical opsins (rhodopsin and color opsin) are not required for circadian photoreception. Behavioral and *in vivo* biochemical analyses in retinal degenerate (*rd*) mice have shown that the outer retina (containing the rod and cone photoreceptors) is not necessary for circadian entrainment by light, suggesting that a pigment located in the inner retina acts as the circadian photoreceptor (2–5). Three candidate photoreceptive proteins, melanopsin (6) and cryptochromes 1 and 2 (7, 8), are primarily expressed in the inner retina. Melanopsin is a recently discovered mammalian opsin with greater sequence homology to the invertebrate opsins than vertebrate opsins, but with no known function in mammals (6). Cryptochrome is a FAD- and pterin-containing pigment (7) with high sequence homology to the light-activated DNA repair enzyme, photolyase (9–12), and one class of plant blue-light photoreceptors involved in plant growth and development (13, 14). Cryptochromes function as circadian photoreceptors in *Arabidopsis* and *Drosophila* (15–17) and are essential for the normal functioning of the circadian clock in mice (18–21) and in some *Drosophila* tissues (22, 23). The photoreceptive role of cryptochromes in mammals, however, has not been firmly established (24), although a recent study by using cryptochromeless, retinally degenerate mice has suggested that both cryptochromes and classical opsins may function as circadian photoreceptors in mice (25). However, this study could not categorically demonstrate that opsin-based pigments were dispensible for circadian photoreception.

A conceptually simple way to determine the requirement for a retinal-based pigment such as melanopsin for circadian photoreception would be to deplete this cofactor by maintaining animals on a vitamin A-free diet. Such a study was first performed on *Drosophila* 30 years ago. Zimmerman and Goldsmith raised *Drosophila melanogaster* on a synthetic vitamin A-free diet and found that visual sensitivity was reduced about 1,000-fold, but circadian photosensitivity was not affected, suggesting that a nonopsin pigment was the circadian photoreceptor in the fly (26). This prediction has recently been confirmed by genetic and nutritional analyses that show that eyelessness or vitamin A deprivation leaves the flies circadian photoreceptive but with possibly reduced sensitivity (ref. 16; see also ref. 27).

Until recently, this approach was not feasible in mice. Vitamin A is required for development; adult mice placed on a vitamin A-free diet have sufficient hepatic stores of vitamin A from dietary intake during the weaning period to supply retinol to the eye for the lifetime of the animal. However, the recent generation of a plasma retinol-binding protein (RBP)-deficient mouse (28) has created the opportunity to deplete ocular retinal in adult mice. RBP is the only known specific serum transport protein for retinol and mobilizes hepatic retinol stores to tissues, including the retina where retinol is converted to retinal for use as the opsin chromophore. In $RBP^{-/-}$ animals (mice homozygous for RBP null mutation) maintained on a vitamin A-free diet, there is no detectable retinol in the plasma after 1 week of vitamin A starvation. These animals progressively become visually blind; after 130 days on a vitamin A-deficient diet, no electroretinogram signal can be detected (28). In this study, we investigated the circadian photoresponse in vitamin A-depleted $RBP^{-/-}$ animals as measured by acute gene induction in the suprachiasmatic nucleus in response to light. Our data indicate that ocular retinal is not required for light signaling to the murine circadian pacemaker.

Experimental Procedures

Vitamin A-Free Diet. $RBP^{-/-}$ mice are true null mutants that have been described previously (28). We maintained our $RBP^{-/-}$ stock by crossing homozygous animals. For vitamin A deprivation, wild-type (The Jackson Laboratory) and $RBP^{-/-}$ mice from a mixed background (129xC57BL/6J) were placed on a vitamin A-deficient diet 19–25 days after birth. The purified vitamin A-deficient and control diets and the control chow diet were obtained from Purina Mills Test Diet (www.testdiet.com). The purified diet was based on Basal Diet 5755. Over the course of this study, the purified diets were stored at −20°C and protected from hydration in sealed plastic bags. Actual lot analysis of the vitamin A-deficient diet indicated that the vitamin A content of the diet was below the detection limit ($<0.066\ \mu g/g$ diet) used for this analysis. Aside from the vitamin A content of this diet, all other minerals and vitamins were present in the purified diet at control levels.

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Abbreviations: SCN, suprachiasmatic nucleus; RBP, retinol binding protein; ZT, Zeitgeber time.

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Table 1. Total retinal levels (in nanograms) in the two eyecups of mice on control diet or vitamin A-free diet

Genotype/diet	Retinal, ng	
	5-month diet	10-month diet
Wild-type/control	271, 293, 242, 226, 305, 313, 296, 266 277 ± 31 (n = 8)	327, 280, 83, 235, 361, 337, 298, 284, 244, 265 271 ± 77 (n = 10)
Wild-type/deficient	321, 275, 312, 218, 251, 211, 302, 305, 267, 277, 274 274 ± 36 (n = 11)	139, 289, 155, 257, 193, 379, 87, 570 259 ± 156 (n = 8)
$RBP^{-/-}$/control	70, 51, 91, 87, 77, 105, 57, 64 75 ± 18 (n = 8)	115, 170, 445, 365, 242, 231, 85, 289, 106, 507 256 ± 146 (n = 10)
$RBP^{-/-}$/deficient	17, 34, 27, 23, 8, 19, 27, 20, 27 22 ± 8 (n = 9)	0, 0, 20, 0, 25, 1, 0, 0, 0, 12 6 ± 10 (n = 10)

The numbers for the individual mice, the averages, and the SDs are given in nanograms per two eyecups.

Measurement of Retinal in Eyecups. Retinal measurements were obtained from eyecup homogenates by normal phase HPLC and were corrected for recovery by using all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetreen-1-ol(TMMP-ROH) as an internal standard (29).

Eye Histology. Eyes were fixed in formalin and embedded in paraffin. Six-micrometer sections were cut and stained with hematoxylin and eosin. Photoreceptor nuclei counts were obtained by counting nuclei within photoreceptor columns in sections containing optic nerve cross-section, at a location one high-powered field away from the optic nerve. Counts were averaged from at least five different sections and analyzed by ANOVA (for genotype and diet effects) and by pairwise Student's t test assuming equal variance.

***In Situ* Hybridization.** Animals on light–dark 12/12-h schedule were exposed to a broad spectrum fluorescent light at a rate of either 45.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 1 h (total dose = 1.65×10^5 $\mu mol \cdot m^{-2}$) or 1.67 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 30 min (total dose = 3.0×10^3 $\mu mol \cdot m^{-2}$) between ZT18 and ZT20. Animals were killed 1.5 h after initiation of the light pulse. Eyes and brain were immediately frozen in liquid nitrogen. The antisense *mPer1* and *mPer2 in situ* hybridization probes were generated by *in vitro* transcription with T7 RNA polymerase (Promega) in the presence of ^{35}S-UTP from pBluescript SK+ containing 0.9-kb and 1.0-kb segments of the genes, respectively. Eighteen-micrometer coronal brain sections were fixed with formalin, treated with 10 μg/ml proteinase K (BMB), and acetylated with triethanolamine and acetic acid anhydride. After dehydration with ethanol, brain sections were hybridized overnight at 55°C with 6×10^5 cpm of probe in 50% formamide/20 mM Tris-HCl, pH 8.0/5 mM EDTA/0.3 M NaCl/10 mM phosphate buffer/1 × Denhardt's solution/10% dextransulfate/0.2% sarcosyl/0.2 mg/ml salmon sperm DNA. A high stringency wash was carried out at 65°C for 45 min with 50% formamide/4 × SSC/7.7 mg/ml DTT, and the sections were treated with 1 μg/ml RNase A for 30 min and washed again in high stringency wash buffer. The slides were dehydrated with ethanol and subjected to autoradiography. Suprachiasmatic nucleus (SCN) images were obtained by using a Leica M420 macroscope density-calibrated with a Kodak control scale T-14 by using optronics DE1750 camera. Quantitation was performed by using Scion Image 1.62a (version of National Institutes of Health Image). The background signal (optical density of a region of the brain with virtually no *Per* gene expression) was subtracted from the SCN signal. Induction was expressed as ratios of light-induced levels to basal levels in dark-kept animals.

Results

Depletion of Ocular Retinal by Vitamin A Deprivation. $RBP^{-/-}$ and wild-type mice were weaned 19–25 days after birth and were maintained on a vitamin A-deficient diet for 10 months. Control animals were maintained on either a chow diet or a nutritionally complete purified diet providing control levels of vitamin A (3 μg of retinol/g diet). Vitamin A-depleted mice were maintained on the same purified diet lacking any form of vitamin A. Animals were killed periodically over a 10-month period to measure the retinal levels in the eyecups. The ocular retinal levels of wild-type animals on either the control diet or the vitamin A-free diet did not significantly change over this period (Table 1), a striking demonstration of the unfeasibility of ocular retinal depletion in wild-type mice. In $RBP^{-/-}$ animals maintained on control diet, there was markedly less ocular retinal after 5 months because of the inability of the $RBP^{-/-}$ mice to transport liver retinol to the retina. Interestingly, in this group of animals, the ocular retinal levels after 10 months on vitamin A-containing diet recovered to normal levels because of activation of compensatory mechanisms for retinol transfer from the periphery to the eye (28). In $RBP^{-/-}$ animals on vitamin A-free diet for 5 months, the eyecup retinal level dropped to 8% of the control (Table 1); at this stage, no electroretinogram signal can be detected (28). After 10 months of vitamin A-deficient diet, 6 of 10 animals had no detectable retinal in the eyecup, whereas the remaining 4 animals contained eyecup retinal levels ranging from 0.4% to 10% of the wild type (Table 1). Our assay's retinal detection limit is 0.5 ng per pair of eyecups (Fig. 1), and thus most of these mice had less than 0.2% of the wild-type retinal level. This value is at least an order of magnitude lower than the retinal levels in the eyes of aged *rd/rd* mice (2), which have been used as a model system for non-rod, non-cone-mediated circadian photoreception (2–4).

Normal Retinal Histology in $RBP^{-/-}$ Mice. Because vitamin A deficiency might cause retinal degeneration (30, 31) and possibly atrophy the SCN (32), which may indirectly affect the function of a circadian photoreceptor or the response of the master circadian clock, we examined the retinas of $RBP^{-/-}$ mice on vitamin A-free diet for 10 months for degenerative changes. As shown in Fig. 2, the retinas of these animals are essentially indistinguishable from the control by light microscopy. Remarkably, there is minimal outer retinal degeneration in vitamin A-depleted mice. Cell counts in photoreceptive columns in the outer nuclear layer show a minimal decrement in the number of nuclei per column with genotype (wt on vitamin A+ diet: 10.75 + 0.55 nuclei/column; wt on vitamin A− diet: 10.47 + 0.43 nuclei/column; $RBP^{-/-}$ on vitamin A+ diet: 10.22 + 0.45 nuclei/column; $RBP^{-/-}$ on vitamin A− diet: 9.87 + 0.53 nuclei/column). By ANOVA, no significant ($P < 0.05$) differences could be detected between groups, based on either genotype or diet. By pairwise t test, there was a small but significant difference in number of nuclei per column between wild-type mice raised on a vitamin A+ diet and $RBP^{-/-}$ mice raised on vitamin A− diet. In contrast, mice with the rd/rd mutation

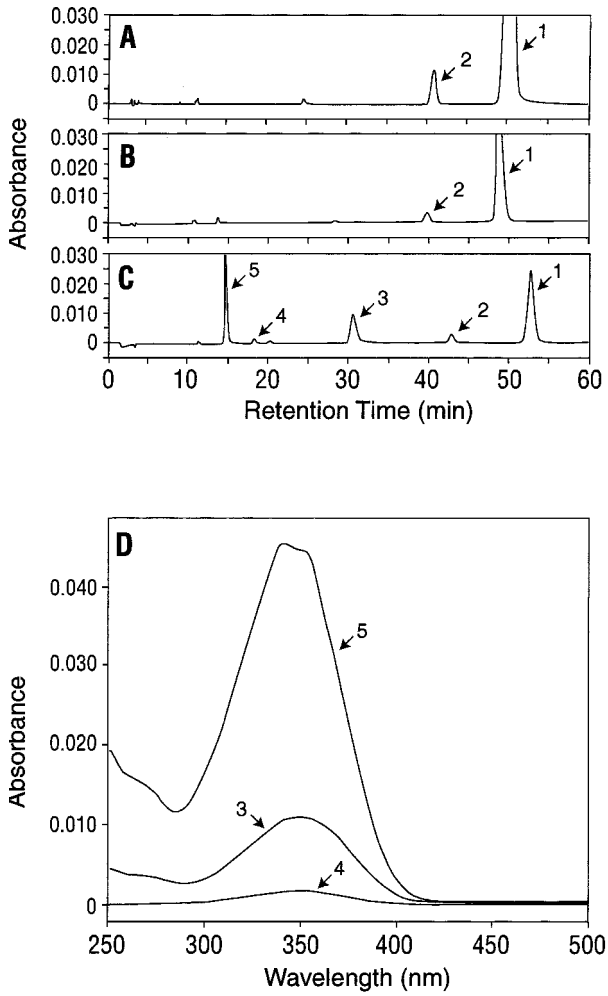

Fig. 1. Effect of 10 months of maintenance on the vitamin A-deficient diet on retinal levels in eyecups of RBP$^{-/-}$ mice. Retinal levels in dark adapted mouse eyecups were determined by normal phase HPLC. To assure quantitative extractions of the retinal present in the mouse eyecups, all extractions were carried out in the presence of hydroxylamine; thus, the HPLC profiles are for the oxime derivatives of retinal. (*A*–*C*) HPLC profiles of the internal standard all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetreen-1-ol (TMMP-ROH) alone (*A*), and extracts from eyecups of RBP$^{-/-}$ mice maintained on either the totally vitamin A-deficient diet (mouse no. 24, *B*), or a vitamin A-sufficient purified control diet (mouse no. 32, *C*). The recoveries of internal standard TMMP-ROH for the eyecup extracts shown in *B* and *C* were 68.7% and 46.7%, respectively. Absorbance was measured at 365 mm by using a Waters 996 photodiode array detector (*A*–*C*). (*D*) UV-Vis spectra for the oximes of retinal identified in the HPLC profile in *C*. Peak identification: 1, the all-*trans*-isomer of TMMP-ROH; 2, cis-TMMP-ROH; 3, anti-11-cis-retinal oxime; 4, syn all-*trans*-retinal oxime; and 5, syn 11-cis-retinal oxime.

typically show one nucleus per column in the outer nuclear layer, when that layer can be identified. The absence of outer retinal degeneration in these animals may be attributable to dissociation of retinol depletion from retinoic acid depletion (28). The inner retina, where the circadian photoreceptor is thought to reside, appears structurally normal in RBP$^{-/-}$ mice, both on and off vitamin A diet.

Normal Retinohypothalamic Phototransduction in RBP$^{-/-}$ Mice. Signaling of luminance levels to the circadian pacemaker occurs through the retinohypothalamic tract (RHT), which connects a subset of retinal ganglion cells to the circadian pacemaker in the SCN. Perception of light by the circadian system may be assayed by measuring acute induction of immediate early or light-responsive circadian clock genes in the SCN following a brief pulse of light (33, 34). Under conditions of 12-h light:12-h dark, the expression of clock genes *mPer1* and *mPer2* in the SCN reaches a maximum at ZT6–8 [ZT (Zeitgeber time) = 0 corresponds to lights on] and declines to a minimum at ZT16–20. When the expression is low (night phase), a brief light pulse can induce transcription of the *Per* genes that is thought to mediate the corresponding shift in the phase of the circadian clock (35, 36). Hence, the *Per* gene induction may be used as a reasonable substitute for behavioral assays such as measurement of locomotor activity. Thus, to assess the requirement of an opsin/retinal-based pigment for circadian photoreception, we measured acute *mPer1* and *mPer2* gene induction in the SCN of RBP$^{-/-}$ mice on a vitamin A-free diet for 10 months in response to a light pulse delivered between ZT18 and ZT20.

Fig. 3 shows the *Per* gene induction measured by *in situ* hybridization of two mice with no detectable ocular retinal and exposed to two different light doses. Qualitatively, there is no difference between the level of *Per* gene induction in the control and retinal-depleted animals. Quantitative analysis of data from these and other animals are summarized in Fig. 4. At the two light doses used in our study, there was no significant difference in *Per* induction between animals maintained on vitamin A-free and control diets. Statistical analysis by using a two-tailed *t* test yielded *P* values for *mPer1* induction of $P = 0.9$ and $P = 0.2$ and for *mPer2* induction of $P = 0.6$ and $P = 0.2$ at saturating and subsaturating light doses, respectively. At the lower luminance, there is a trend for reduced *Per* induction in the animals on vitamin A-free diet, which is not statistically significant. Although it is possible that with a larger sample size some reduced circadian photosensitivity may be evident in animals with no detectable retinal in the eye, these data demonstrate that retinal-free mice exhibit robust circadian gene induction by light. Significantly, however, at the low irradiance the level of *mPer1* induction was lower ($P < 0.01$) in both groups of animals than the induction level at the higher irradiance (Fig. 4). Because at a limiting light dose the amount of the photopigment becomes rate limiting, any reduction in the predominant photoreceptor

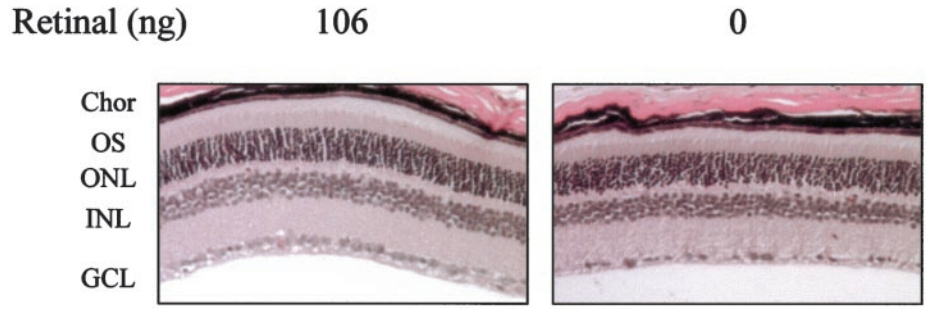

Fig. 2. Histology of the retinas of RBP$^{-/-}$ mice on (*Left*) control diet and (*Right*) vitamin A-free diet for 10 months. Chor, choroid; OS, outer segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

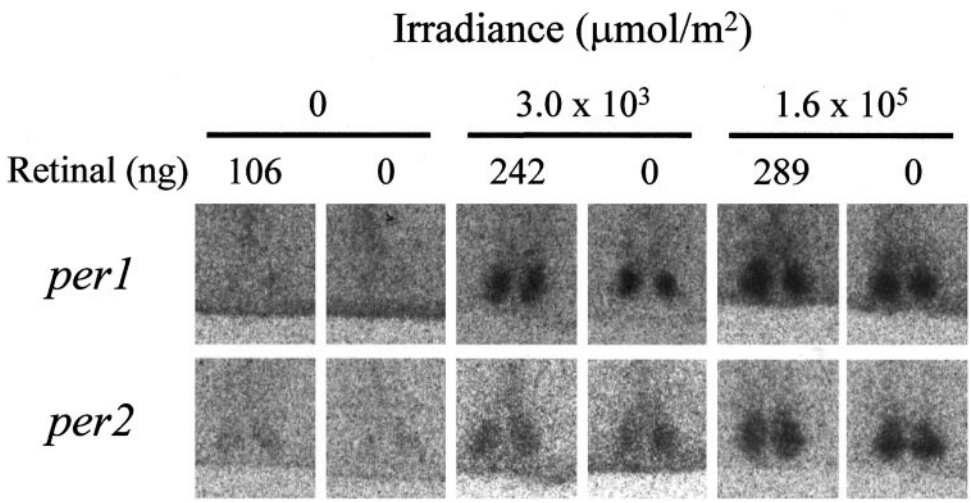

Fig. 3. *mPer* gene induction by light in the SCN of mice with no detectable ocular retinal. Each column represents SCN slices from one animal. Control animals (columns 1, 3, and 5) and animals on vitamin A-free diet for 10 months were exposed to the indicated doses of light at ZT 18–20, killed 90 min after the beginning of light exposure, and the induction of the *mPer1* and *mPer2* genes in the SCN was measured by *in situ* hybridization. The panels show slices exhibiting the peak signal at each light dose in the SCN for each animal. The levels of ocular retinal determined from both eyecups of each animal are listed.

for this response would be expected to cause a comparable reduction in photoresponse. As noted above, under limiting irradiance, there is no statistically significant difference in the reduction level of *mPer1* gene induction, indicating that retinal-based pigments are not necessary for photoinduction of this gene, which is a component of the molecular clock.

Discussion

Circadian Photoreceptors in Mammals. Extensive analyses have demonstrated that the classical photoreceptors (rods and cones) are not necessary for transmission of light information to the circadian clock in the suprachiasmatic nucleus (3, 5). Two families of candidate photoreceptors have been proposed to function in the absence of classical ocular photoreceptors to mediate signal transduction of light to the SCN: novel opsins and cryptochromes. Several novel opsin family members have been identified in mammals in recent years (37); the recent completion of the human genome has not revealed any additional obvious candidates. Of the novel opsins, melanopsin is the most likely to function in circadian photoreception. Melanopsin is a vertebrate opsin originally isolated from amphibian melanocytes. The mammalian homologue is expressed selectively in the inner retina, primarily in a subset of retinal ganglion cells (6). On the basis of its localization, it has been proposed to function in circadian photoreception; however, at present, there is no evidence for such a function. In contrast, the cryptochrome family of flavin-based blue-light photoreceptors has been shown to function as a circadian photoreceptor in plants such as *Arabidopsis* (38) and animals such as *Drosophila* (27, 39, 40). Mammals contain two cryptochrome family homologues, Cry 1 and Cry 2. Genetic analysis of mouse cryptochrome 1 and 2 knockouts has demonstrated that these proteins are necessary for normal circadian clock function (18, 20, 21). In contrast, in light–dark cycles, mice lacking both cryptochromes retain behavioral rhythmicity (20, 21), which has led some investigators to conclude that cryptochromes do not function as photoreceptors in mammals and to propose that a non-rod, non-cone opsin, such as melanopsin, is the circadian photoreceptor (41, 42). However, recent studies with triple mutant mice lacking rods and cones and both cryptochromes have revealed that these animals lack behavioral rhythmicity under light–dark cycles, strongly suggesting that cryptochromes do function as photoreceptors in mammals (25).

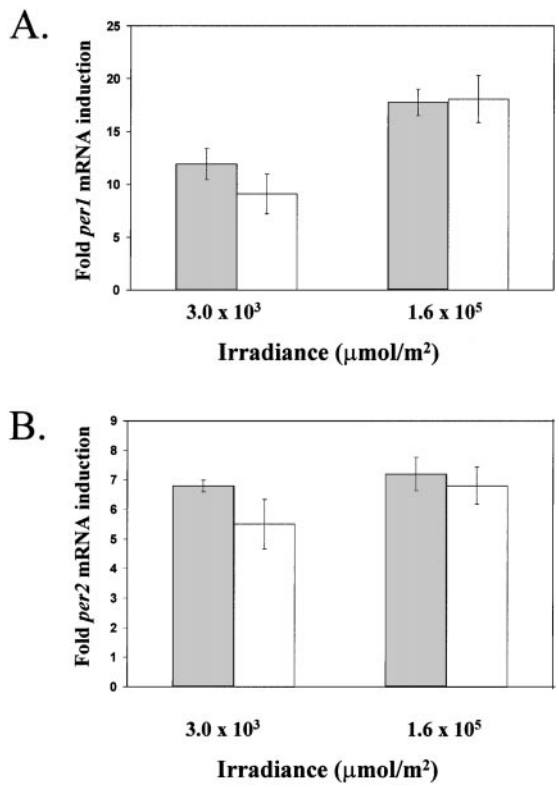

Fig. 4. Quantitative analyses of *mPer* gene photoinduction in the SCN in RBP$^{-/-}$ mice on control (shaded boxes) and vitamin A-free (open boxes) diets. The bars indicate standard deviations; $n = 4$ for all classes of animals.

Circadian Photoreception in the Absence of Opsins. The present experiments were designed to test the hypothesis that a retinal-based photoreceptor is necessary for circadian photoreception. The results demonstrate that mice without detectable levels of ocular retinal retain normal *Per* induction in the SCN in response to brief light pulses. The most parsimonious explanation of these results is that retinal-based photoreception is not required for retinohypothalamic signaling. This conclusion is based on the assumption that vitamin A depletion disables all opsin-based phototransduction, including phototransduction by rhodopsin,

cone opsins, and the putative photoreceptor melanopsin. However, because melanopsin's photochemical mechanism and function are unknown, we cannot formally conclude that 500-fold depletion of ocular retinal is sufficient to disrupt its function. The photoreceptive function of melanopsin is based at present on its localization in the inner retina and its sequence homology to invertebrate opsin. The photochemistry of retinal regeneration differs between vertebrate and invertebrate opsins (43); whereas reisomerization of all-trans retinal to 11-cis retinal for vertebrate rhodopsin occurs enzymatically in the retinal pigment epithelium, reisomerization of invertebrate all-trans to 11-cis retinal occurs within the apoprotein, mediated by a second photochemical event. If melanopsin is an unusually long-lived holoprotein, it could be argued that the *in situ* regeneration of cofactor may protect it from systemic retinal depletion. Such a model is at odds, however, with the behavior of invertebrate opsins following vitamin A starvation; *Drosophila* subjected to vitamin A depletion rapidly lose visual photosensitivity, both in terms of standard responsiveness to light and in terms of marked decrement in sensitivity to entrainability of adult behavior in light–dark cycling conditions (ref. 26; see also ref. 27). Additionally, such a model is strained by the necessity of simultaneously proposing a highly stable holoprotein and continual two-photon absorption for activation and regeneration of the protein, which would be likely to induce additional photochemical damage to the protein. Thus, we suspect that in RBP $^{-/-}$ mice with less than 0.2% of total ocular retinal, the melanopsin holoenzyme is also reduced to at least this level without affecting retinohypothalamic phototransduction.

Identity of the Non-Opsin Circadian Photoreceptor. Concluding that opsin-based photoreception is not necessary for functional light signaling to the SCN requires positing the existence of a non-opsin-based ocular photoreceptor. Although definitive identification of such a photoreceptor has not been achieved, substantial evidence suggests that cryptochromes may serve this function in mammals. Cryptochromes are evolutionarily highly conserved proteins related to the photolyase family of light-dependent DNA repair enzymes (10–12). Direct photoreceptive function of these proteins has been demonstrated in *Arabidopsis* (14), and *Drosophila* cryptochrome has been shown genetically to function in the circadian photoreceptive pathway (16). *Drosophila* cryptochrome seems to function as a photoreceptor when expressed in the heterologous yeast two-hybrid system (44, 45). The discovery that mammalian cryptochromes are essential components of the circadian clock (18, 20, 21), and the failure to detect light-mediated functions for human cryptochromes expressed heterologously in yeast or mouse fibroblasts (46), led to the suspicion that mammalian cryptochromes lack photoreceptive function (41, 47). More recent data, however, have suggested a role for cryptochromes in circadian photoreception in mammals. Mice lacking cryptochromes have attenuated immediate-early gene induction in the SCN in response to light (25), whereas mice lacking classical photoreceptors have exaggerated SCN photoresponsiveness (48, 49). Concomitant loss of both cryptochromes and classical photoreceptors nearly eliminates SCN photoresponsiveness (25). Such triply mutant animals also show substantially reduced light-responsive behavior, indicative of certain functional redundancy between cryptochromes and classical photoreceptors. Significantly, a recent study in *Drosophila* has also revealed that circadian blindness occurs after elimination of all known photoreceptors (50).

Redundant Circadian Photoreceptors. Because neither cryptochrome-dependent nor retinal-dependent pathways seem necessary for retinohypothalamic phototransduction, it is important to ask which system is primarily responsible for daily entrainment to light–dark cycles. If light induction of immediate-early and circadian clock gene expression in the SCN is taken as a measure of the strength of circadian photoreception, it would seem that the cryptochrome-dependent phototransduction system has greater sensitivity than the opsin-based system. Mice lacking cryptochromes show a 10–20-fold reduction in SCN *c-fos* induction in response to a brief subsaturating light pulse (25), whereas mice lacking classical photoreceptors in fact show enhanced induction of immediate-early gene expression (25). In the present study, we find that total depletion of retinal, which in all likelihood affects not only classical photoreceptors but also all retinal-based pigments, has no effect on the magnitude of induction of *mPer* genes by light in the SCN. This finding suggests that nonclassical opsins, such as melanopsin, are not major contributors to circadian photoreception and that at least in dim-light conditions circadian photoreception via the cryptochrome-dependent pathway has greater light sensitivity than the classical opsin pathway. However, the ability to assimilate environmental information through different photoreceptive pathways that enable the organism to assess the quality and quantity of light would be advantageous in organizing its circadian behavior. Thus, in mice, as has recently been shown in *Drosophila* (16, 50), in addition to cryptochromes, classical opsins may be considered important circadian photoreceptors as well, as evidenced by the phenotype of *mCry1*$^{-}$*/mCry1*$^{-}$*;mCry2*$^{-}$*/mCry2*$^{-}$*;rd/rd* mice (25). In conclusion, it seems that in both organisms, cryptochromes function as dedicated circadian photoreceptors that are intimately integrated into the clock mechanism itself (22, 23, 25), whereas classical opsins are the essential visual pigments with additional photoreceptive function in circadian regulation.

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