Regulation of the Mammalian Pineal by Non-rod, Non-cone, Ocular Photoreceptors

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In mammals, ocular photoreceptors mediate an acute inhibition of pineal melatonin by light. The effect of rod and cone loss on this response was assessed by combining the *rd* mutation with a transgenic ablation of cones (*cl*) to produce mice lacking both photoreceptor classes. Despite the loss of all known retinal photoreceptors, *rd/rd cl* mice showed normal suppression of pineal melatonin in response to monochromatic light of wavelength 509 nanometers. These data indicate that mammals have additional ocular photoreceptors that they use in the regulation of temporal physiology.

The possibility that mammals use uncharacterized ocular photoreceptors in the regulation of circadian physiology has been a topic of recent interest (*1*–*3*). This speculation is, however, at odds with one of the oldest beliefs of visual science: that the classical rod and cone photoreceptors account for all photoreceptive input to the mammalian central nervous system (*4*). We tested this assumption by examining the photic suppression of pineal melatonin in mice lacking both rod and cone photoreceptors.

Melatonin, the principal product of the mammalian pineal gland, acts as an internal representative of nighttime. Production is confined to the hours of darkness both by an appropriately phased circadian rhythm of pineal stimulation and by an extreme sensitivity of pineal melatonin synthesis to inhibition by light (*5*–*7*). The mammalian pineal, unlike that of other vertebrates, is not directly light-sensitive, and photic information reaches it via a multisynaptic pathway originating in the retina and passing through suprachiasmatic regions of the hypothalamus (*8*). In mammals, removal of the eyes abolishes this response, demonstrating that ocular photoreceptors are used (*5*, *9*).

Photic melatonin suppression survives the loss of rod photoreceptors (*9*), and because the murine retina contains two populations of cone photoreceptors [sensitive to green light, maximum wavelength (λ_{max}) 508 nm, and ultraviolet (UV) light, λ_{max} 359 nm] (*10*, *11*), it was previously thought that these cells might contribute to pineal responses. To test this hypothesis, we examined the effect of cone photoreceptor loss in mice bearing a specific transgenic (*cl*) ablation (*12*, *13*). In C3H/He mice, the *cl* transgene induced a profound degeneration of cone photoreceptors. Immunocytochemical (*14*) and mRNA analysis (*15*) (Fig. 1) showed that green cones were almost entirely lost in *cl* retinae. Nevertheless, the sensitivity of pineal melatonin to suppression by monochromatic 509-nm light was unattenuated (Fig. 2) (*16*). UV cones are insensitive to this wavelength over the range of irradiances used in this study (*11*). Consequently, although our analysis indicates that a substantial population of UV cones survives ablation by the *cl* transgene (Fig. 1), we conclude that neither green nor UV cone photoreceptors form an essential component of the photoreceptive input to the mammalian pineal.

To test whether the absence of either rods or cones is compensated for by the presence of the other, we generated mice completely lacking rod and cone photoreceptors by introduction of the *cl* transgene into C3H/He mice homozygous for the *retinal degeneration* (*rd*) allele. The *rd* allele inactivates rod phototransduction (*17*, *18*) and triggers a degeneration of rod and subsequently cone photoreceptors (*19*). Histological examination of 80-day-old *rd/rd cl* mice (*20*) revealed retinae completely lacking an outer nuclear layer and immunoreactivity for any of the three known photoreceptor types (Fig. 3), a result confirmed by mRNA analysis (Fig. 4). Despite this degenerate retina, *rd/rd cl* mice exhibited normal entrainment to a light:dark cycle (*21*) and complete suppression of pineal melatonin in response to a monochromatic 509-nm light pulse (2.6×10^{-2} $\mu W/cm^2$) (Fig. 5). Given the irradiance-dependent nature of this response, and the fact that light is the only environmental variable known to induce such an acute effect on the activity of the pineal, we conclude that *rd/rd cl* mice remain capable of light detection.

Thus, in mice, cells other than rods and cones can act as photoreceptors. Circumstantial evidence suggests that these photoreceptors reside in the retina because this is thought to be the source of all photoreceptive input to the mammalian pineal. A subset of retinal ganglion cells form a retinohypothalamic tract (RHT) innervating the suprachiasmatic nuclei (sites of a circadian clock), which drive the activity of the pineal. Removal of the eyes or sectioning of the RHT confirms that the retina and its efferents comprise essential components of the pathway by which light reaches the pineal (*5*, *22*). Although it has long been assumed that the rods and cones are the only directly photosensitive elements of the retina, photic responses of *rd/rd cl* mice suggest that a subset of those retinal cells currently thought not to be directly sensitive to light can act as photoreceptors. On the basis of recent tract tracing studies, retinal ganglion and amacrine cells appear to be strong candidates (*23*).

In addition to lacking rod and cone cell bodies, the *rd/rd cl* retina also lacks the molecular machinery by which these cells act as photoreceptors: rod phototransduction is precluded by the *rd* gene defect (*17*), and cone phototransduction by the absence of green and UV cone opsins and cone-specific arres-

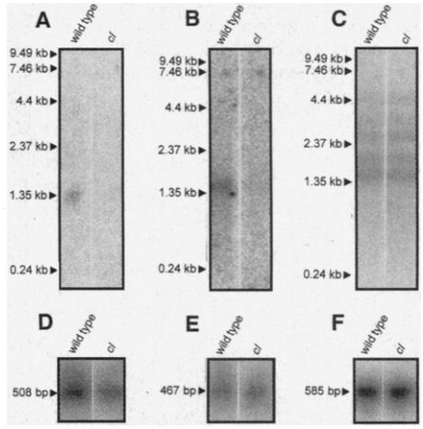

Fig. 1. Effects of the *cl* transgene on the expression of photoreceptor-specific genes in C3H/He mice. Northern blot (**A** to **C**) and RT-PCR (**D** to **F**) detection of mRNA encoding green cone opsin (A and D), UV cone opsin (B and E), and rod opsin (C and F) in wild-type and *cl* eyes (*15*). The *cl* transgene rendered green cone opsin mRNA undetectable by Northern blot (A) in the transgenic mice. RT-PCR in which green cone–specific primers were used also failed to amplify a band visible on an ethidium bromide–stained agarose gel. However, hybridization of a radiolabeled probe to the Southern (DNA) blot of this gel indicated a residual expression of this transcript (D). UV cone opsin mRNA was also reduced in the transgenic eye, although this transcript remained detectable by both Northern blot (B) and RT-PCR (E) techniques. Rod opsin expression (C and F) was unaffected by the transgene.

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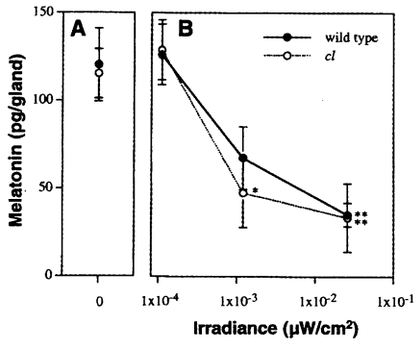

Fig. 2. Photic suppression of pineal melatonin in *cl* and wild-type mice. In comparison with sham-pulsed controls (**A**), both wild-type and *cl* mice exhibited an irradiance-dependent suppression of pineal melatonin (**B**) in response to 15-min exposure to monochromatic 509-nm light (*16*). Data represent mean ± SEM for six to eight animals per genotype at each irradiance; $*P < 0.05$, $**P < 0.001$ compared with unpulsed group, post hoc Bonferoni's test after one-way analysis of variance (ANOVA).

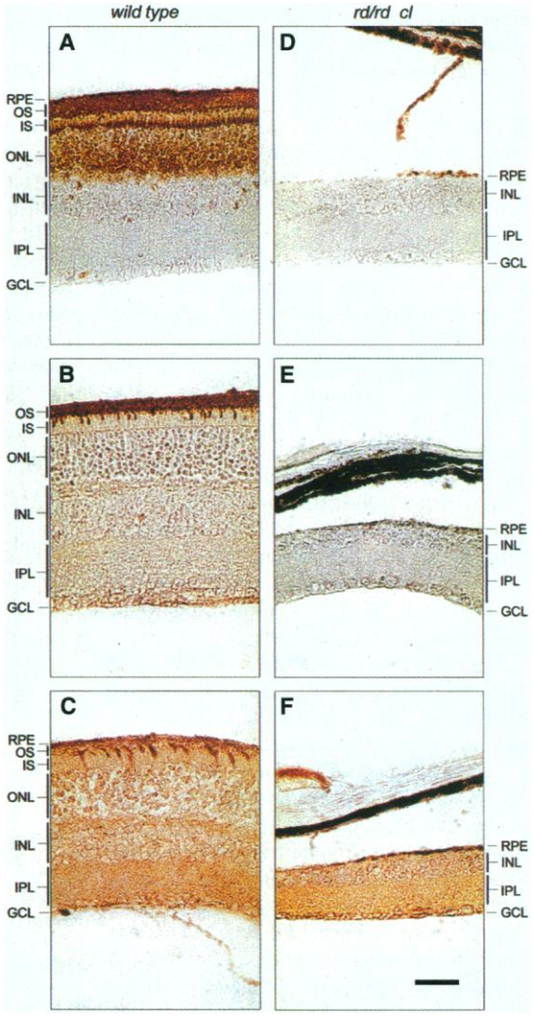

Fig. 3. Immunocytochemical analysis of retinae from *rd/rd cl* mice. Introduction of the *cl* transgene into mice homozygous for the *rd* mutation induced a retinal phenotype in 80-day-old mice that lacked both rod and cone cells. Tissue from wild-type (**A** to **C**) and *rd/rd cl* (**D** to **F**) mice was fixed with Bouins (75% picric acid, 25% formalin, 5% acetic acid) for 24 hours and embedded in paraffin, and 8-μm-thick sections were treated with antisera recognizing rod (A and D), rod and green cone (B and E), and UV cone (C and F) photoreceptors (*20*). Visualization was accomplished with ABC methods (Vectastain Elite, Vector Labs). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments; RPE, retinal pigment epithelium. Scale bar, 40 μm.

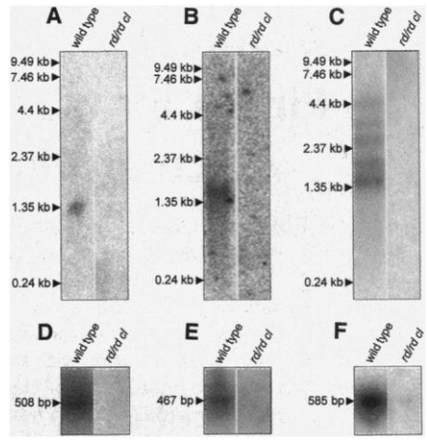

Fig. 4. *rd/rd cl* mice lack mRNA encoding cellular components of the known ocular photoreceptors. Northern blot (**A** to **C**) and RT-PCR (**D** to **F**) analysis of retinae from 80-day-old *rd/rd cl* mice confirms the lack of mRNA encoding green (A and D) and UV cone opsins (B and E) and cone arrestin (*21*). The expression of rod opsin mRNA was sufficiently impaired in this genotype to render it undetectable by Northern blot (C). After RT-PCR with rod opsin–specific primers, a product of the appropriate size was observed for wild-type but not *rd/rd cl* tissue on an ethidium bromide–stained agarose gel. Southern blot analysis of this gel and hybridization with a radiolabeled probe revealed a barely detectable band in the *rd/rd cl* lane (F), indicative of an extremely small amount of rod opsin mRNA. This message was not translated into measurable rod opsin protein (Fig. 2) and is precluded from driving photoreception by the nature of the *rd* mutation (*17*, *18*). Northern blot for GAPDH and RT-PCR specific for tubulin mRNA (*21*) was used to confirm the integrity of the *rd/rd cl* RNA used for this analysis.

tin (Fig. 4). Thus, *rd/rd cl* mice do not retain photosensitivity through ectopic expression of rod or cone cell components in other retinal cell types. Therefore, a satisfactory explanation of photic responses in this genotype awaits the description of a non-rod, non-cone photopigment that acts in a cell type previously thought not to be directly photosensitive.

A variety of candidate non-rod, non-cone photopigments have been suggested, the most recent being the mammalian cryptochromes (CRY1 and CRY2), vitamin B2–based putative photopigments (*3*, *24*). Mouse *cry1* and *cry2* genes are expressed within the inner retina and retinal ganglion cells (among many other sites in the body) (*25*). Other candidates include two non-rod, non-cone photopigments of the classical opsin:vitamin A family that have been identified in nonmammalian vertebrates (*2*, *26*). Both genes are expressed in cells of the retinal inner nuclear layer outside of the classical photoreceptors. Presently, there is limited direct evidence linking any of these putative photopigments with circadian photoreception. We anticipate that studies of *rd/rd cl* mice will prove successful in addressing this deficit. For example, our demonstration that these mice are highly sensitive to monochromatic 509-nm light already excludes those photopigments whose absorbance spectrum does not encompass this wavelength. *rd/rd cl* mice also provide an ideal retinal phenotype in which to determine the effects of ablating candidate photopigments.

In addition to circadian physiology, many other aspects of mammalian biology are influenced by gross changes in environmental light, including pupil size, blood pressure, mood, and attention (*27*). Our results and those of an associated report in this issue (*28*) show that diverse aspects of temporal biology, including both photoentrainment and pineal melatonin suppression, respond to non-rod, non-cone photoreceptors. These unchar-

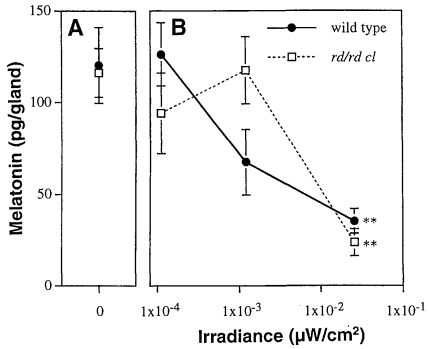

Fig. 5. The effect of 15-min exposure to monochromatic light (509 nm) on pineal melatonin content in *rd/rd cl* and wild-type mice. Compared with unpulsed animals (**A**), *rd/rd cl* mice showed irradiance-dependent suppression of pineal melatonin content after exposure for 15 min to 509-nm light (**B**). Data represent mean ± SEM for six to eight animals per genotype at each irradiance; $^{**}P < 0.01$ compared with unpulsed controls; post hoc Bonferoni's test after one-way ANOVA. Although the data suggest that the production of melatonin in *rd/rd cl* mice may be less sensitive to inhibition by light, this is not supported by statistical analysis (two-way ANOVA, $P > 0.05$).

acterized ocular photoreceptors might form the basis of a general non–image forming photoreceptive pathway mediating many, if not all, nonvisual responses to light.

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15. Total RNA was extracted from (n = 4 to 6) eyes collected from 80-day-old wild-type, *cl*, and *rd/rd cl* mice with the Promega (Southampton, UK) SV RNA isolation kit. For Northern (RNA) blot analysis, 5 μg of total RNA from each genotype was size-separated on a 1.2% agarose-formaldehyde electrophoresis gel and blotted overnight onto Hybond N+ membrane (Amersham). Three blots were hybridized with ^{32}P-labeled probes specific for mouse rod opsin [cDNA probe after W. Baehr *et al.*, *FEBS Lett.* **238**, 253 (1988)] and green and UV cone opsins [cRNA probes after M. von Schantz *et al.*, *Mol. Brain Res.* **770**, 131 (1997)], and subsequently with a ^{32}P-labeled cDNA probe for glyceraldehyde phosphate dehydrogenase (GAPDH) (positive control). For reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, cDNA was synthesized from 2 μg of total RNA from each genotype. PCR amplification was then undertaken over 35 cycles under optimized conditions with primers designed against the published sequences for mouse rod opsin, green cone opsin, UV cone opsin, cone arrestin, and tubulin (positive control). Appropriate negative controls (total RNA without reverse transcriptase step) were included for all genotypes. PCR reactions were separated on a 1.5% agarose gel and blotted overnight onto Hybond N+ membrane. Blots were hybridized with appropriate ^{32}P-labeled cDNA probes.
16. Mice stably entrained to a 12 hour light:12 hour dark cycle were individually either sham-pulsed (no light exposure) or exposed to 15 min of defined irradiance (1.2×10^{-4}, 1.2×10^{-3}, or 2.6×10^{-2} μW/ cm^2) with monochromatic light (λ_{max} = 509 nm, half band width 10 nm) at zeitgeber time 20-21. The melatonin content of pineal homogenates was assessed by radioimmunoassay as described (*9*).
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Vancomycin Derivatives That Inhibit Peptidoglycan Biosynthesis Without Binding D-Ala-D-Ala

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Vancomycin is an important drug for the treatment of Gram-positive bacterial infections. Resistance to vancomycin has begun to appear, posing a serious public health threat. Vancomycin analogs containing modified carbohydrates are very active against resistant microorganisms. Results presented here show that these carbohydrate derivatives operate by a different mechanism than vancomycin; moreover, peptide binding is not required for activity. It is proposed that carbohydrate-modified vancomycin compounds are effective against resistant bacteria because they interact directly with bacterial proteins involved in the transglycosylation step of cell wall biosynthesis. These results suggest new strategies for designing glycopeptide antibiotics that overcome bacterial resistance.

Vancomycin is a glycopeptide antibiotic that kills bacterial cells by inhibiting peptidoglycan biosynthesis (*1*). It is the most important drug in current use for the treatment of Gram-positive bacterial infections, representing the final option for curing infections that are resistant to other antibiotics. The emergence of vancomycin-resistant bacterial strains is a very serious public health problem. Recently, a set of carbohydrate derivatives of vancomycin that are active against resistant bacterial strains was discovered (*2*). We now show that the modified carbohydrates alone are specific inhibitors of the transglycosylation step of peptidoglycan biosynthesis. This finding changes the picture for how modified glycopeptides kill resistant bacteria.

Vancomycin functions by binding to the terminal D-Ala-D-Ala dipeptide of bacterial cell wall precursors (Fig. 1), thereby impeding further processing of these intermediates into peptidoglycan (*3*, *4*). The vancomycin complex involves a set of complementary hydrogen bonds between the peptide portion

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