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Nonvisual Photoreception in the Chick Iris

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The embryonic chicken iris constricts to light ex vivo, but with characteristics atypical of visual phototransduction. The chick iris was most sensitive to short-wavelength light, demonstrating an action spectrum consistent with cryptochrome rather than with opsin pigments. Pupillary responses did not attenuate after saturating light exposure, but showed paradoxical potentiation. Iris photosensitivity was not affected by retinoid depletion or inhibitors of visual phototransduction. Knockdown of cryptochrome expression, but not of melanopsin expression, decreased iris photosensitivity. These data characterize a non-opsin photoreception mechanism in a vertebrate eye and suggest a conserved photoreceptive role for cryptochromes in vertebrates.

The ability to sense ambient light without forming an image (nonvisual photoreception) is widespread among vertebrates. Nonvisual photosensitive tissues in vertebrates include brain and parietal eye photoreceptors in lizards (1, 2), pineal and iris photoreceptors in avians (3, 4), iris and dermal photoreceptors in amphibians (5, 6), and retinal ganglion cell photoreceptors in mammals (7). These photoreceptors regulate diverse nonvisual functions, including the entrainment of circadian rhythms to the light-dark cycle, pupillary constriction, and hormone secretion. The mechanisms underlying murine retinal ganglion cell photoreception have recently been investigated with targeted mutations in genes that encode potential photopigment. The opsin family member melanopsin has been found to be essential to inner retinal photoreception (8, 9), whereas the potential flavin photopigments cryptochrome 1 and 2 contribute to the amplitude of nonvisual light responses (10, 11).

Despite being recognized for more than 150 years (12), little is known about the mechanisms underlying the intrinsic pupillary light response (PLR) of some vertebrate irises. In the chick, intrinsic iris photosensitivity is present during a brief developmental window [embryonic days (E) 14 to 18] (4). Infrared pupillometry of E15 iris maintained ex vivo showed strong constriction and dilation to sequential light and dark exposure (Fig. 1A and movies S1 and S2). Light-induced constriction was preserved after removal of the pigmentary epithelium layer, indicating that nonpigmented tissue contains functional photopigment (fig. S1). The kinetics of response to subsaturating light exposure showed an initial peak constriction preceding a slightly lower steady state (Fig. 1B). Prolonged exposure to heat-filtered, bright white light induced sustained maximal constriction (Fig. 1C), which could be maintained for at least 3 hours under continuous illumination. Quantitatively identical PLRs were observed after subsequent stimulation for more than 10 hours, and the preparation maintained robust photoresponsiveness for at least 48 hours (13).

To characterize the photopigment(s) mediating this light response, action spectra were constructed from irradiance-response relations with 10-nm half-bandwidth filtered light from 350 to 590 nm (fig. S1). The action spectrum showed preferential sensitivity to ultraviolet-blue light, with a local secondary peak at 430 nm (Fig. 1D and fig. S2), qualitatively similar to the absorption spectra of cryptochrome purified from *Vibrio cholera* (14) (Fig.1E) and of heterologously expressed human cryptochrome (15) (Fig. 1F), but not to that of any opsin photopigment.

Opsin-based photopigments bleach after saturating white light exposure because of *cis/trans*-retinaldehyde photoisomerization, leading to pigment desensitization. However, rather than desensitizing after saturating stimulation, the isolated chick iris PLR demonstrated increased sensitivity to subsequent dim light pulses (Fig. 2). We have termed this phenomenon photopotentiation. Photopotentiation was wavelength-dependent: It could be elicited after exposure to bright monochromatic blue light, but did not result from exposure to bright green light of equivalent irradiance (13). Because the same color of light can both maximally stimulate the PLR and induce its photopotentiation, this is unlikely to represent behavior of a bistable opsin (16). Bright light–induced photopotentiation showed logarithmic decay with a half-life of ~2.8 min (Fig. 2 and fig. S3).

All known opsin-based photopigments use specific retinoids as their chromophore. To test whether the chick iris photopigment(s) require retinoids, irises were depleted of endogenous retinoids by exposure to 312-nm ultraviolet (UV) light, a treatment that irreversibly degrades all retinoids to nonfunctional products (17). A 20-min exposure to 312-nm UV light (8 mW/cm^2) fully depleted control solutions of all-*trans*-retinol (Fig. 3A). From 57 embryos, irises from one eye received 20 min of 312-nm light, while irises from the other eye underwent mock exposure. A subset of both groups was tested for PLR to 450-nm light of varying irradiance. Endogenous iris retinoids were extracted and subjected to high-pressure liquid chromatography (HPLC) separation and quantitation. In the mock-exposed group of irises, very low amounts of retinoids were detected, mostly in the form of all-*trans*-retinyl esters (~0.2 pmol per iris) and all-*trans*-retinol (~0.1 pmol per iris) (Fig. 3B). No 11-*cis*-retinoids were detected. After UV light exposure, no retinoids could be detected in the irises (Fig. 3C), demonstrating complete depletion to the limit of HPLC detection (1.53 pmol for all-*trans*-retinyl ester, or 0.027 pmol per iris, and 1.8 pmol for all-*trans*-retinol, or 0.03 pmol per iris). Sensitivity of the PLR to blue light was not different between UV-depleted and mock-treated irises (Fig. 3D). The same result was obtained in irises lacking their pigment layer, demonstrating that the UV resistance of the light response is not due to shielding by iris pigment (fig. S4). These data suggest that the chick iris PLR uses a photopigment that does not require a retinoid-based chromophore.

Specific pharmacologic agents were used to dissect the signal transduction pathway of the iris-based photoreceptor (table S1). All known visual phototransduction cascades use specific G proteins in signaling. Neither cholera toxin (G$_s$ inhibitor) nor pertussis toxin (G$_i$ inhibitor) affected the PLR sensitivity.

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Invertebrate photoreception relies on the G protein G_q, which signals through inositol triphosphate and phospholipase C (PLC). Administration of two different PLC inhibitors (U-73122 and neomycin) had no effect on PLR sensitivity. Drugs that decreased PLR sensitivity included thapsigargin, forskolin, and staurosporine (fig. S5). Thapsigargin inhibits the PLR by depleting intracellular Ca^{2+} stores (18); thapsigargin also blocked acetylcholine (ACh)–induced pupillary constriction. Staurosporine, a pan-kinase inhibitor, equivalently blocked light-, ACh-, and K^+-mediated constriction (19). Staurosporine has been shown to block myosin light-chain phosphorylation, which may account for its effects on iris muscle (20). Forskolin, an adenylyl cyclase activator, increases cyclic adenosine monophosphate levels and is a smooth muscle relaxant (21); however, forskolin was a more potent blocker of light-induced than of ACh-induced constriction.

We performed reverse transcription–polymerase chain reaction (RT-PCR) to determine the expression of candidate photopigments in the embryonic iris (Fig. 4A). Melanopsin and cryptochromes 1 and 2 are three candidate blue-light photopigment proteins currently implicated in mammalian inner retinal nonvisual photoreception. mRNA for each was identified in embryonic chick iris, although (as assayed by quantitative RT-PCR) melanopsin mRNA was expressed at much lower levels (<0.5%) than either cryptochrome. A third cryptochrome (gCry4) has been cloned from chickens (GenBank accession no. AY102068), but we could not detect this transcript in chick iris by RT-PCR.

To determine whether melanopsin or cryptochromes contribute to the intrinsic PLR, irises were transfected with heterogeneous small interfering RNA (siRNA) to knock down melanopsin, cryptochrome 1, cryptochrome 2, or β-galactosidase (LacZ) mRNA (Fig. 4B). Thirty hours after transfection, expression of melanopsin, cryptochrome 1, and cryptochrome 2 mRNAs (as measured by quantitative RT-PCR) was reduced by 44%, 65%, and 50%, respectively, relative to transcript levels found in LacZ siRNA-transfected controls. LacZ siRNA had no effect on transcript levels of either cryptochrome or melanopsin. Although there was partial cross-suppression of Cry2 siRNA on Cry1 transcript levels (chicken cryptochromes 1 and 2 showed 76% nucleotide identity in their coding sequences), cryptochrome siRNA did not affect melanopsin mRNA expression, nor did melanopsin siRNA suppress cryptochrome mRNA expression. The PLR sensitivity of irises transfected with either cryptochrome 1 or 2 siRNAs was reduced by ~40% relative to LacZ siRNA-transfected control irises (Fig. 4C). When irises were transfected with siRNAs targeting both cryptochromes 1 and 2, the PLR sensitivity was

reduced by 58%. Melanopsin siRNA-transfected irises showed PLR sensitivity indistinguishable from paired LacZ siRNA-transfected controls. Qualitatively identical results were also obtained 45 to 48 hours after transfection with phosphothiorate antisense oligonucleotides to mediate knockdown of cryptochromes or melanopsin (fig. S6). These data suggest that cryptochromes 1 and 2 are additive in their contributions to the sensitivity of the intrinsic light response, whereas melanopsin may not be required.

The isolated embryonic chicken iris is a very robust and readily available photosensi-

tive ocular tissue for the study of nonvisual photoreception in a vertebrate. Five lines of evidence support the hypothesis that this nonvisual photoreceptive phenomenon is not opsin-based: (i) The action spectrum is not consistent with an opsin template (but shows univariance consistent with a single pigment); (ii) the photoreceptor is not bleached by bright light exposure (as are most opsins), but rather shows paradoxical photopotentiation to supersaturating stimulus; (iii) substantial retinoid depletion does not alter the sensitivity of the photoreceptor; (iv) pharmacologic blockade of vertebrate and inverte-

Fig. 1. Isolated embryonic chicken iris sphincter muscle constricts in response to light. (**A**) Two sequential light-induced pupillary constrictions. Images were captured at the time points indicated above and depict the same iris muscle in the following states: (i) dark-adapted, (ii) after a 1-min white light exposure (350 W/m²), (iii) after 30 min in the dark, and (iv) after a second 1-min white light exposure (350 W/m²). (**B**) PLR to subsaturating blue light (430 nm, 11.44 W/m², n = 5 irises). (**C**) PLR to bright broad-spectrum light (180 s of white light, 350 W/m²). Normalized pupil area was calculated as pupil area/dark-adapted pupil area (mean ± SEM; n = 5 irises). (**D**) Action spectrum of depigmented embryonic chick iris PLR (steady-state PLR measurements). (**E**) V. cholerae cryptochrome absorption spectrum (14). [Modified and reprinted with permission from (14), © 2003 American Society for Biochemistry and Molecular Biology]. (**F**) Absorption spectrum for heterologously expressed human Cry1 (solid line) and Cry2 (dashed line) (15). [Modified and reprinted with permission from (15), © 1996 American Chemical Society].
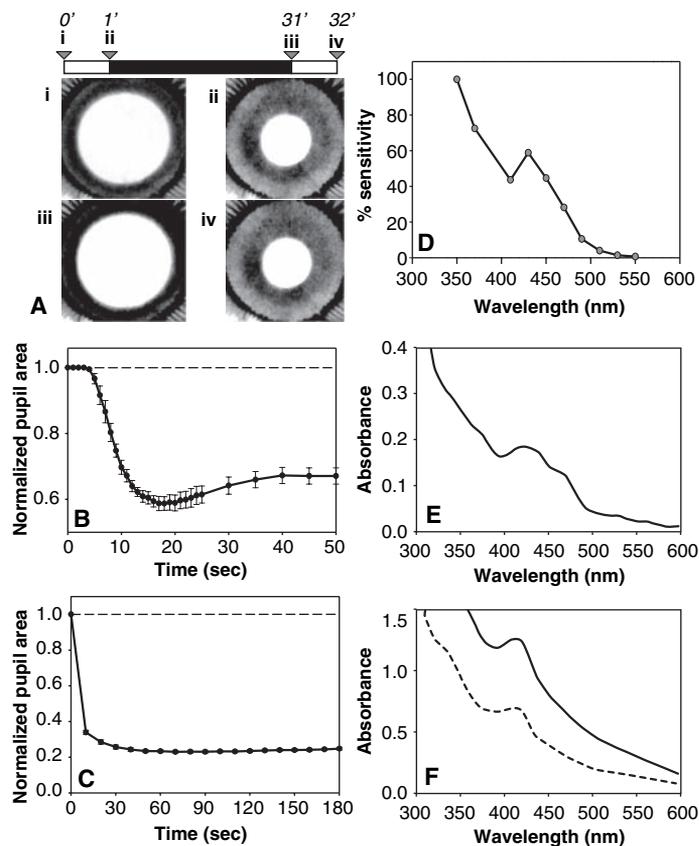
Fig. 2. Bright white light exposure potentiates subsequent pupillary responses. Dark-adapted irises were exposed to four sequential periods of illumination or darkness in the following order: 45 s of blue light (430 nm, 11.44 W/m²), 1 min of bright white light (400 W/m²), 2 to 16 min of complete darkness, then 45 s of blue light (430 nm, 11.44 W/m²). (**A**) Time series of pupillary constriction to the first 45-s pulse of blue light (mean ± SEM; n = 6 to 8 irises). (**B**) Time series of pupillary constriction to the second 45-s pulse of blue light (mean ± SEM; n = 6 to 8 irises).
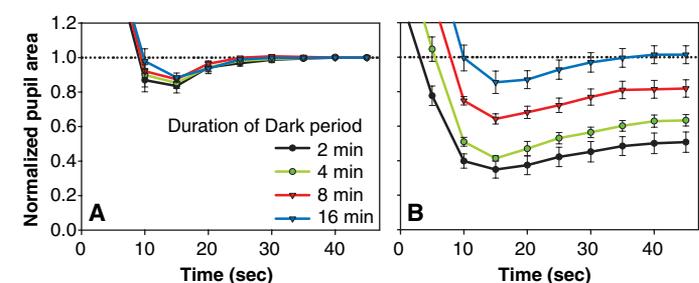
brate opsin-mediated signal transduction pathways does not affect the PLR sensitivity; and (v) genetic knock-down of mRNA for melanopsin does not decrease the PLR sensitivity. This last experiment must be qualified: Because antibodies for detection of melanopsin are not presently available, it is not known to what extent mRNA knockdown is reflected in decreased protein levels. However, the action spectrum is qualitatively similar to the absorption spectrum of purified *V. cholerae* and human cryptochrome, and knockdown of cryptochrome 1 and 2 mRNA decreases PLR sensitivity. These lines of evidence also suggest the involvement of cryptochrome-based photopigments in the chick PLR. Invertebrate and plant cryptochromes are believed to be bona fide photopigments (*22*), whereas in vertebrates it has been unclear if cryptochromes serve a photoreceptive function separate from their role in circadian rhythms (*10*). The ultimate demonstration of vertebrate cryptochrome photopigment function will require elucidation of its photocycle (*23*).

Fig. 3. UV light depletion of endogenous retinoids does not alter photic sensitivity of the pupillary light response. (**A**) UV light (312 nm) depletion of all-*trans*-retinol in methanol (mean ± SEM). (**B**) HPLC separation of retinoids from a pooled sample of 57 chick irises mock-exposed to UV light. Very low levels of all-*trans*-retinyl ester (peak 1, ~0.2 pmol per iris) and all-*trans*-retinol (peak 2, ~0.1 pmol per iris) were detected. (**C**) HPLC from a matching set of 57 irises after a 20-min exposure to 312-nm UV light (8 mW/cm²) with loss of all-*trans*-retinyl ester and all-*trans*-retinol peaks. (**D**) Irradiance-response relationship of PLR to 450-nm light with and without UV light pretreatment (mean ± SEM; *n* = 4 irises).
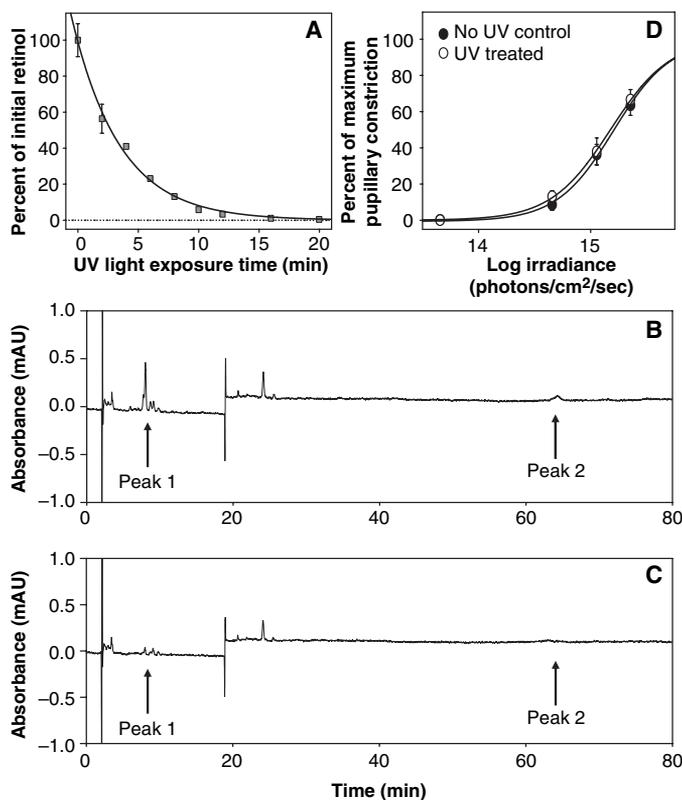
Fig. 4. Knockdown of candidate photopigment genes expressed in embryonic chicken iris. (**A**) RT-PCR of chicken β-*actin*, *Cry1*, *Cry2*, and *Opn4* (melanopsin) in E15 embryonic chick iris tissue. PCR reactions used 35 cycles of amplification. "No RT" lanes represent RT-PCR reactions performed without reverse transcriptase. "H₂O" lanes contained no template mRNA. bp, base pairs. (**B**) Quantitative RT-PCR of total RNA collected from the irises tested in (C), showing the amount of *Opn4*, *Cry1*, or *Cry2* transcript (normalized to β-*actin* levels) relative to that measured in the *LacZ* siRNA-transfected control irises (mean ± SEM; *Cry1* and *Cry2*, *n* = 16 to 18 irises; *Opn4*, *n* = 9 irises). Asterisks indicate statistically significant differences (*P* < 0.05, unpaired two-tailed *t* test) between experimental and control groups. (**C**) Percent pupillary constriction for irises transfected with siRNAs targeting regions of *LacZ*, *Opn4*, *Cry1*, *Cry2*, or *Cry1* and *Cry2*, relative to the control irises transfected with *LacZ* siRNAs (mean ± SEM; *n* = 7 to 8 irises). Asterisks indicate statistically significant differences (*P* < 0.05, unpaired two-tailed *t* test) between experimental and control groups.
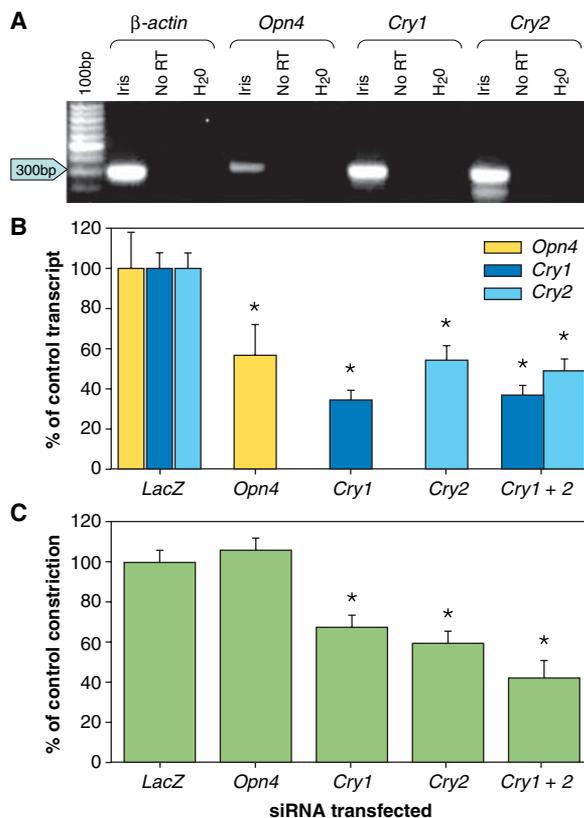
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