Supplementary Methods:

Chicken iris preparation. White leghorn E15 embryos (maintained at 39^{o}C for 15 days) were sacrificed and enucleated. The posterior eyecup was dissected away from the anterior segment. The lens was removed and the iris ciliary dilator muscle incised and removed. The remaining internal iris sphincter muscle and cornea were mounted onto a Petri dish with cyanoacrylate glue. Irises were maintained in Tyrode's solution (134 mM NaCl, 3 mM KCl, 20 mM $NaHCO_3$, 1 mM $MgCl_2$, 3 mM $CaCl_2$, 12 mM glucose) at room temperature. High K^+ (100 mM) solution was made by replacing Na^+ with K^+. Removal of iris pigment layers was performed via manual debridement.

Pupillometry. Pupillary light responses were recorded from dark-adapted (≥30 min) irises on a hooded stage using a digital CCD video camera equipped with close-up and dark red infrared filter lenses (~650 nm cutoff). The irises were visualized under infrared illumination. A xenon arc lamp (Sutter Instrument Company) served as the light source; wavelength and intensity were manipulated via narrow bandwidth (half bandwidth 10 nm) interference filters and neutral density filters (Thorlabs, Inc). Irradiance measurements were made using a calibrated radiometer (Advanced Photonics International).

Irradiance-response curve and action spectra analysis. Percent constriction was calculated as 100·(1 - (pupil area / dark-adapted pupil area)). Percent of maximum constriction was then calculated as 100·(% of constriction / % of constriction to a saturating broad spectrum light, xenon 350 W/m^2). For each color of light tested, an irradiance-response curve was fitted via the Zettler modified Naka-Rushton equation previously utilized to describe photoreceptors: $y=a\cdot x^b/(c^b+x^b)$ (using SigmaPlot 2001)

(*1-3*). These curves were used to calculate the irradiance level required to obtain 50% of maximum constriction (IR50) for each color tested. Percent sensitivity was calculated as $100 \cdot (IR50_{350}/IR50)$, where $IR50_{350}$ = IR50 at 350 nm, the wavelength of maximum sensitivity.

Bleaching/Photopotentiation. Pupil area was normalized to set the response to the first 430 nm light pulse =1 and the area in response to saturating white light =0; normalized pupil area was calculated as (pupil area – minimum pupil area during white light exposure) / (pupil area after 45 sec during the 1st 430 nm light pulse – minimum pupil area during white light exposure). To calculate the half-life, the difference in normalized pupil areas between the 1st and 2nd 430 nm light pulses (45 sec subsequent to light onset) was calculated; these data were fit with a logarithmic function of the form $y = m \cdot Ln(x) + b$. Best fit was obtained with m=0.25 and b = 0.32.

UV light retinoid depletion. All-*trans*-retinol (Sigma Chemical, St. Louis, MO, USA) was dissolved in methanol at 20 μg/ml and exposed to 312 nm UV light from a transilluminator at maximum intensity (8000 $\mu W/cm^2$) (FB-TIV-88A, FisherBiotech). Samples were collected at various time points and tested via HPLC for retinoid content. Retinoids were separated by normal phase HPLC (Beckman, Ultrasphere-Si, 4.6 mm x 250 mm) with a flow rate of 1.4 ml/min using 0.5% ethyl acetate in hexane for 15 min, followed by 4% ethyl acetate in hexane for 65 min. Retinoids were detected at 325 nm using an Agilent 1100 HPLC with a diode-array detector and Agilent Chemstation A.10.01 software. Chicken irises were exposed (or mock-exposed) to the same 312 nm UV light for 20 min (n=57). Four irises in each group were tested for irradiance-response to 450 nm light. Percent of maximum constriction was calculated as the

percent constriction to a given fluence after UV light (or mock) exposure relative to the maximum PLR prior to UV light exposure.

Pharmacology. Cholera toxin, pertussis toxin, neomycin, thapsigargin, forskolin, U-73122, and U-73343 were obtained from Sigma Chemical (St. Louis, MO, USA). Staurosporine was obtained from AG Scientific (San Diego, CA, USA). Tetrodotoxin was obtained from Alomone Labs (Jerusalem, Israel). RO-31-8220 and H-89 were obtained from LC Laboratories (Woburn, MA, USA). Stock solutions were made according to supplier's protocols. Drugs dissolved in DMSO were tested against vehicle controls. Irises were tested at several time points following addition of pharmacologic agents; pupillary responses recorded after incubating irises for 1 hour in drug (or vehicle control) solutions were utilized for the comparisons in Table 1 and Supplemental figure 5.

RT-PCR. Total RNA was isolated from irises (RNeasy, Qiagen) and genomic DNA was removed from RNA samples via DNase I treatment (DNA-free, Ambion). MuLV reverse transcriptase was used to perform reverse transcription reactions primed with both random hexamers and oligo $d(T)_{16}$ (Applied Biosystems). AmpliTaq® DNA polymerase was then used to amplify ~300 bp regions of *β-actin*, *Opn4*, *Cry1*, *Cry2* with the following primer sets: *β-actin* (upstream 5'-GACTGTTACCAACACCCACAC C-3', downstream 5'-CTTCACAGAGGCGAGTAACTTCC-3'); *Cry1* (upstream 5'-AATGCCCCAGAGAGTGTCCAGAAG-3', downstream 5'-CACATGTCTGAACGCCAACTGTC-3'); *Cry2* (upstream 5'-GCCAAGTGCATCATTGGAGTGG-3', downstream 5'-CTTCAGTGCACAGCTCTTCTGCTC-3'); *Opn4* (upstream 5'-

TGCCTGAGGGCTTGATGATAT-3', downstream 5'-AATACGGCGTTAGAGTGTTTCCT-3').

siRNA and antisense knock-down experiments

siRNA. The Block-IT® siRNA system (Invitrogen, Carlsbad, CA) was used to generate heterogeneous diced siRNAs targeting LacZ, *Cry1*, *Cry2*, and *Opn4*. The following primers were used to amplify target regions from vectors containing *LacZ, Cry1, Cry2, or Opn4* cDNA: *LacZ* according to manufacturer's protocol; *Cry1* (upstream primer 5'-GGTAGCAAACTTTGAAAGACCAC-3', downstream primer 5'-CATTAGGCCACCATTTCCATTTC-3'); *Cry2* (upstream primer 5'-CCAGCAGATGGAGACATGTAAA-3', downstream primer 5'-TCCACTCCAATGATGCACTT-3'); *Opn4* (upstream primer 5'-GTGGATCCTTGGAGATATAGGTTG-3', downstream primer 5'-CTCTACATCACTCCAGTTAGCTTC-3'). Sequence lengths of *Cry1, Cry2, Opn4* siRNA targeted regions were 859, 879, and 853 nt respectively. RNA transcripts synthesized from PCR-derived templates were used to produce diced siRNA molecules that were subsequently purified, visualized via gel electrophoresis, and quantified according to manufacturer's protocols.

Fugene 6 transfection agent (Roche Applied Science) was mixed first with diced siRNAs before addition of chilled 25% pluronic F-127 and injection via Hamilton syringe into the anterior chamber of intact eyes isolated from E15 embryos. The total amount of diced siRNA injected into each eye (290 ng) was kept constant by adding *LacZ* siRNA to *Cry1* and *Cry2* reactions. Transfected eyes (intact globes) were incubated at 37°C for 1 hour and then at room temperature for 2 hours before dissection. Pupillary light responses were tested 26-29 hours after transfection. Percent of control

constriction was calculated as 100·(% constriction / avg % constriction of *LacZ* siRNA transfected irises).

30 hours after transfection, irises were collected; total RNA isolation, genomic DNA removal, and reverse transcription reactions were performed as described previously. Quantitative RT-PCR (using the RT-PCR primers described previously) was performed on reverse transcription products to quantify levels of *β-actin*, *Cry1*, *Cry2*, and *Opn4* (iQ™ SYBR® Green, Bio-Rad Laboratories). Serial dilutions of cDNA-containing plasmids were used to create standard curves for each gene. The amounts of *Cry1*, *Cry2*, and *Opn4* measured were normalized to corresponding *β-actin* levels. Percent of control transcript was calculated as 100*(*β-actin*-normalized copy number / average *β-actin*-normalized copy number of *LacZ* siRNA-transfected control irises).

Antisense. Phosphothiorate antisense or sense oligonucleotides corresponding to the 5' sequences of chicken *Cry1*, *Cry2*, *Opn4* were injected into anterior chambers of isolated E15 embryo eyes. Oligonucleotide sequences used were as follows, where * represents phosphothiorate bonds: *Cry1 sense* (5'-A*T*G*GGGGTGAACGCCGT*G*C*A-3'), *Cry1 antisense* (5'-T*G*C*ACGGCGTTCACCCC*C*A*T-3'), *Cry2* sense (5'-A*T*G*GCGGCGGCTGCGTC*C*C*C-3'), *Cry2 antisense* (5'-G*G*G*GACGCAGCCGCCGC*C*A*T-3'), *Opn4 sense* (5'-A*T*G*GATTTGAACATGGG*C*A*C-3'), *Opn4 antisense* (5'-G*T*G*CCCATGTTCAAATC*C*A*T-3'). The same basic transfection protocol was used as described previously; modifications include a longer post-transfection incubation (45-48 hours) and higher concentrations of injected oligonucleotide (0.7 µg per oligonucleotide per eye). Because less total RNA was used per RT-PCR reaction in antisense experiments than in siRNA experiments, *Opn4* mRNA levels were undetectable in the majority of samples from antisense transfected irises.

Supplemental Text

Non-opsin based photoreceptors have been described in many non-vertebrate organisms. Plants utilize at least three different photoreceptor families – cryptochromes, phytochromes, and phototropins – for mediating their growth, circadian, and phototropic responses to light (*4*). *Drosophila melanogaster* also utilize cryptochrome in mediating light information to the circadian clock. Flies carrying a loss-of-function mutation in their sole cryptochrome gene show markedly decreased circadian phase shifting responses (*5*), and *Drosophila* Cryptochrome is able to bind Timeless (*6*) and Period (*7*) in a light-dependent fashion in a yeast two-hybrid system. Non-opsin-based photoreceptors have also been suggested in vertebrates. The circadian rhythms of zebrafish embryonic cell lines can be entrained to external light-dark cycles, demonstrating the presence of a functioning photopigment in these cells (*8, 9*). The photopigment responsible may not be opsin-based as the action spectrum is not consistent with an opsin template, and these cells do not appear to have detectable retinaldehyde. Similarly, at least one of the photopigments in the chick pineal gland *in vitro* is resistant to severe vitamin A depletion, which partially blocks the acute decrease of melatonin synthesis and release by light, but does not appear to affect resetting of the circadian clock in this tissue (*10*). However, both zebrafish and chick pineal systems utilize gene expression as the measured endpoint for photic stimulation. As this occurs on a timescale of minutes, further characterization of the photopigment underlying these phenomena has been difficult. Using the embryonic chick pupillary light response, we are able to demonstrate an absence of bleaching with bright light exposure, and are also able to quantitatively assay the effect of specific inhibitors and gene-knockdown treatments. Together, these data support the hypothesis that a vertebrate eye employs a non-opsin-based photopigment for a non-visual function.

The utility and function of the intrinsically photosensitive iris during this phase of embryonic chick development is not clear. Retinal neurogenesis is occurring during the E14-E18 period, and intrinsic iris photosensitivity may allow a way to limit light exposure through the eggshell and embryonic membranes during this critical developmental period. Alternatively, this may represent simply "ontogeny recapitulating phylogeny", as irises of some teleost fish and amphibians continue to show intrinsic photosensitivity throughout life (*11-13*). However, the mechanisms of intrinsic iris photosensitivity may not be well conserved among vertebrates. The action spectrum for intrinsic iris photosensitivity in the frog, for example, has an opsin-like action spectrum with peak sensitivity ~500 nm (*12*), while that of the eel has significantly more blue-light sensitivity and does not fit a simple opsin template (*11*).

Supplemental references

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Fig. S1

● **Peak PLR**

○ **Steady-state PLR**

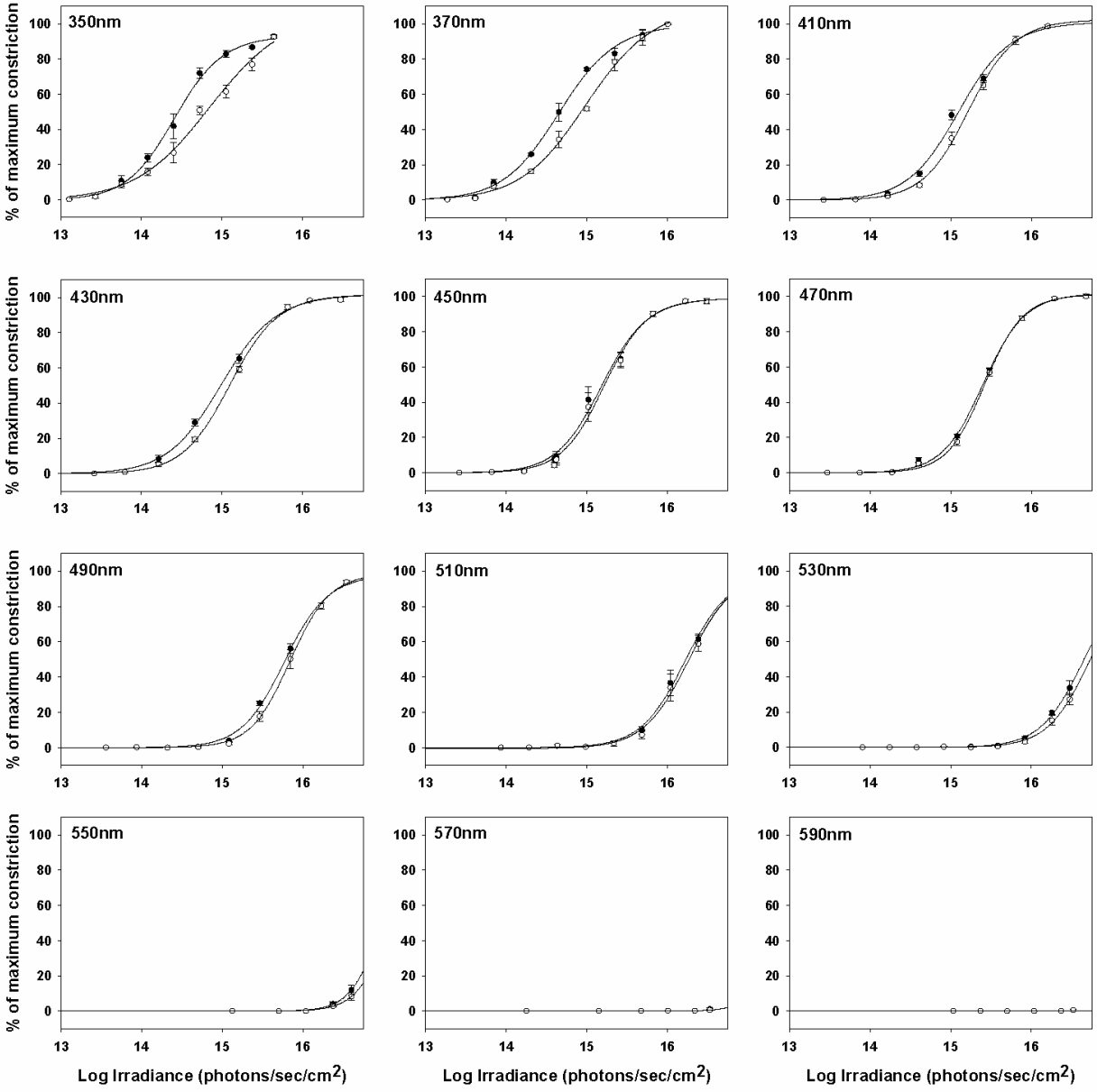


Fig. S2

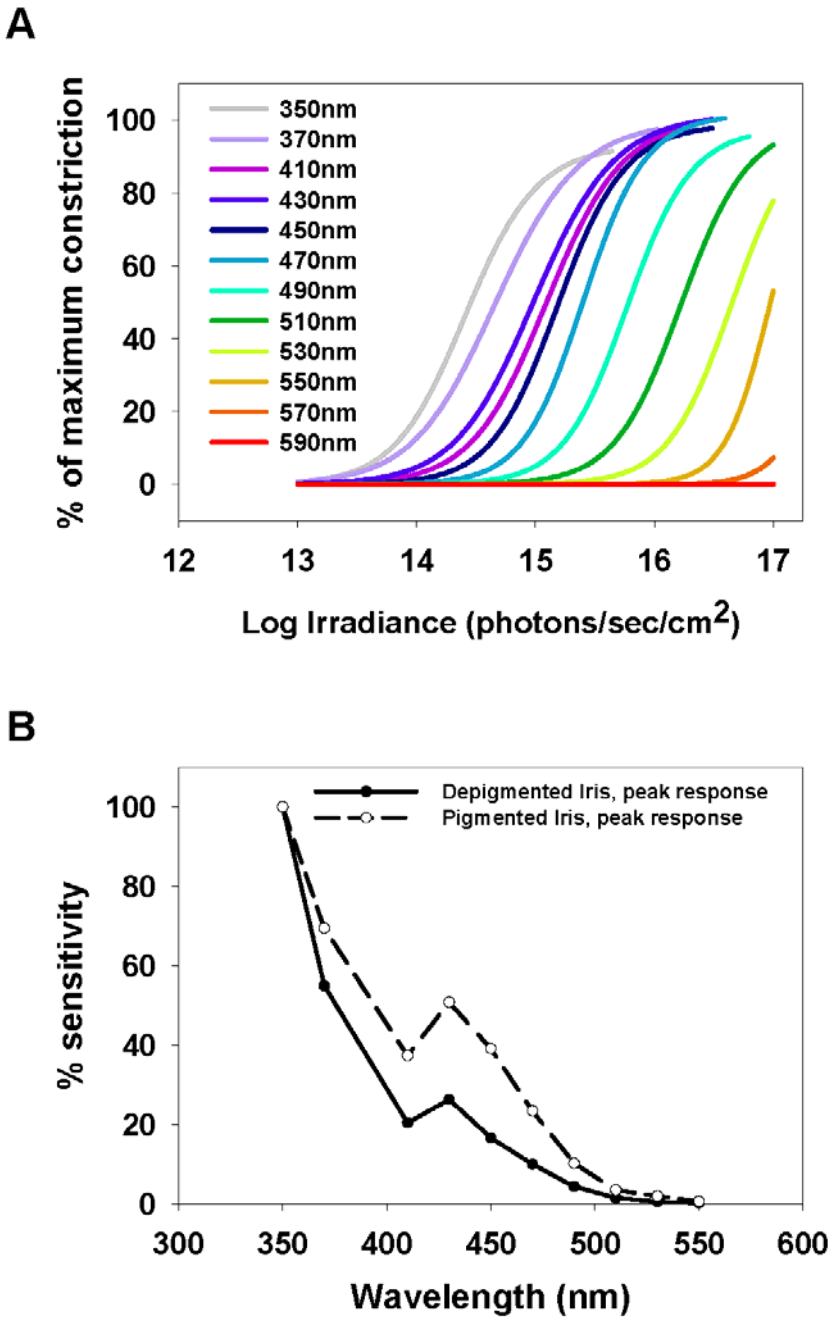

A
% of maximum constriction
100
80
60
40
20
0
350nm
370nm
410nm
430nm
450nm
470nm
490nm
510nm
530nm
550nm
570nm
590nm
12
13
14
15
16
17
Log Irradiance (photons/sec/cm2)
B
% sensitivity
100
80
60
40
20
0
Depigmented Iris, peak response
Pigmented Iris, peak response
300
350
400
450
500
550
600
Wavelength (nm)

Fig. S3

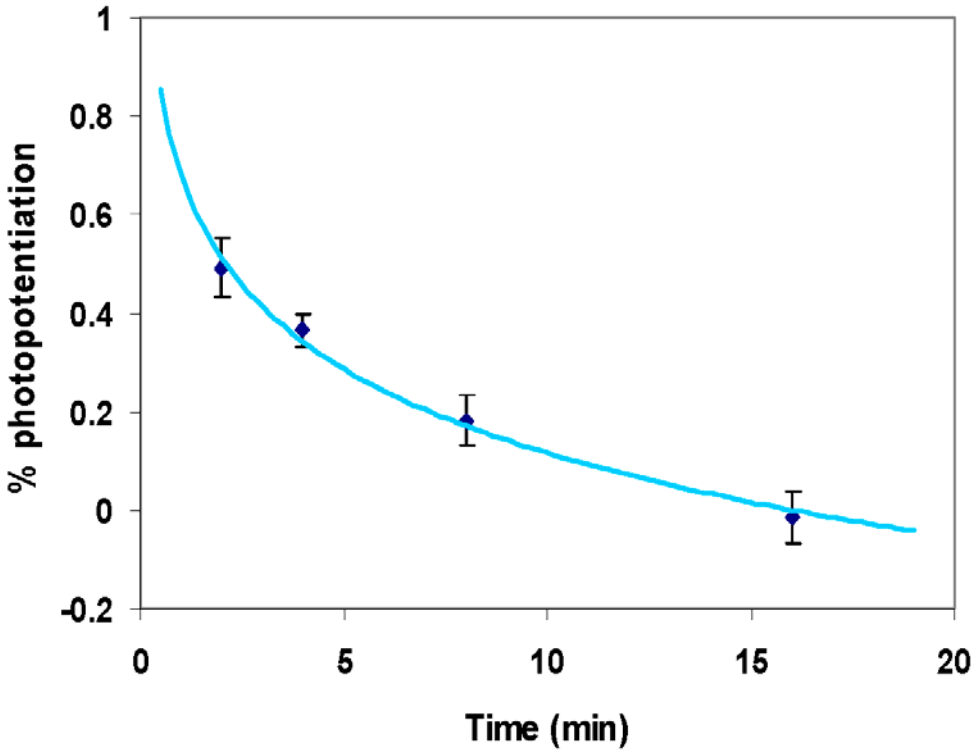


Fig. S4

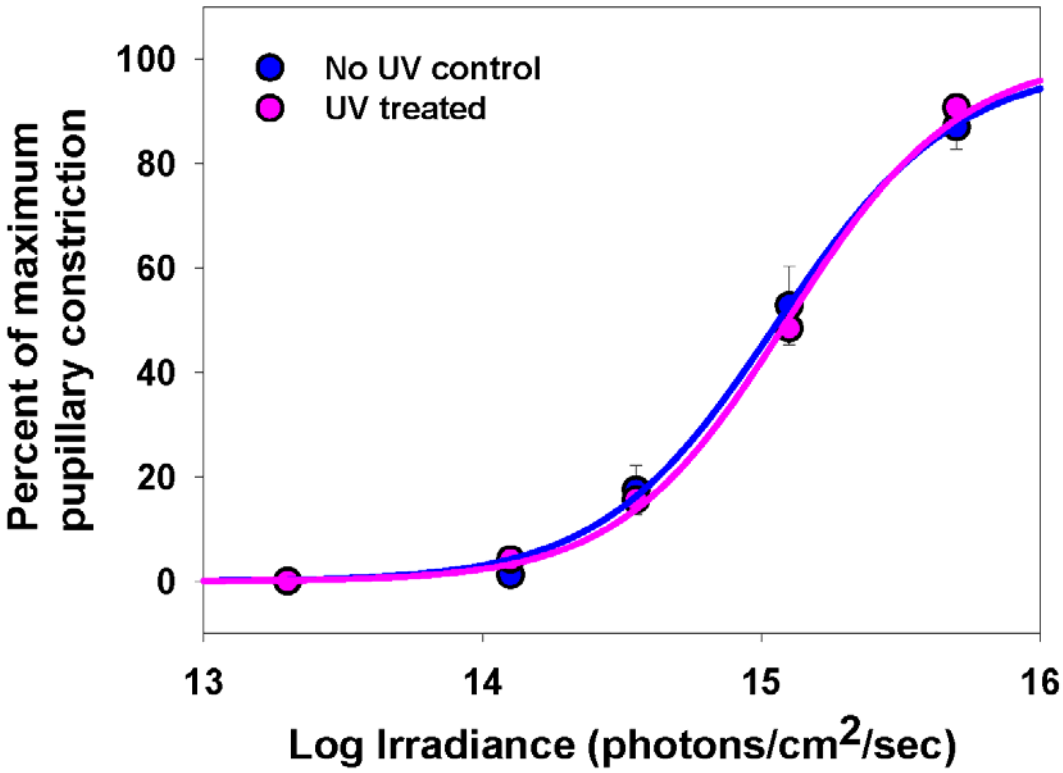
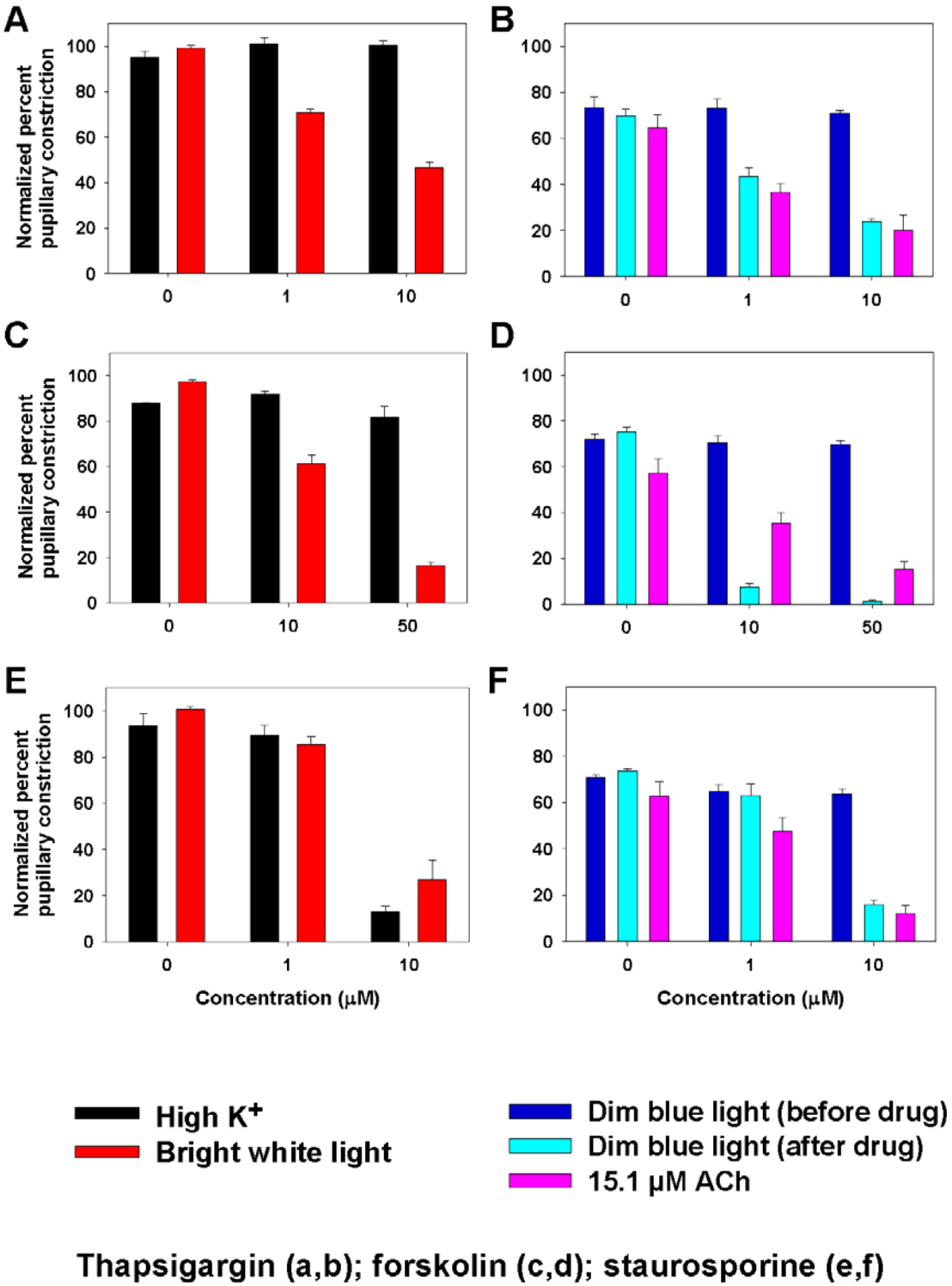
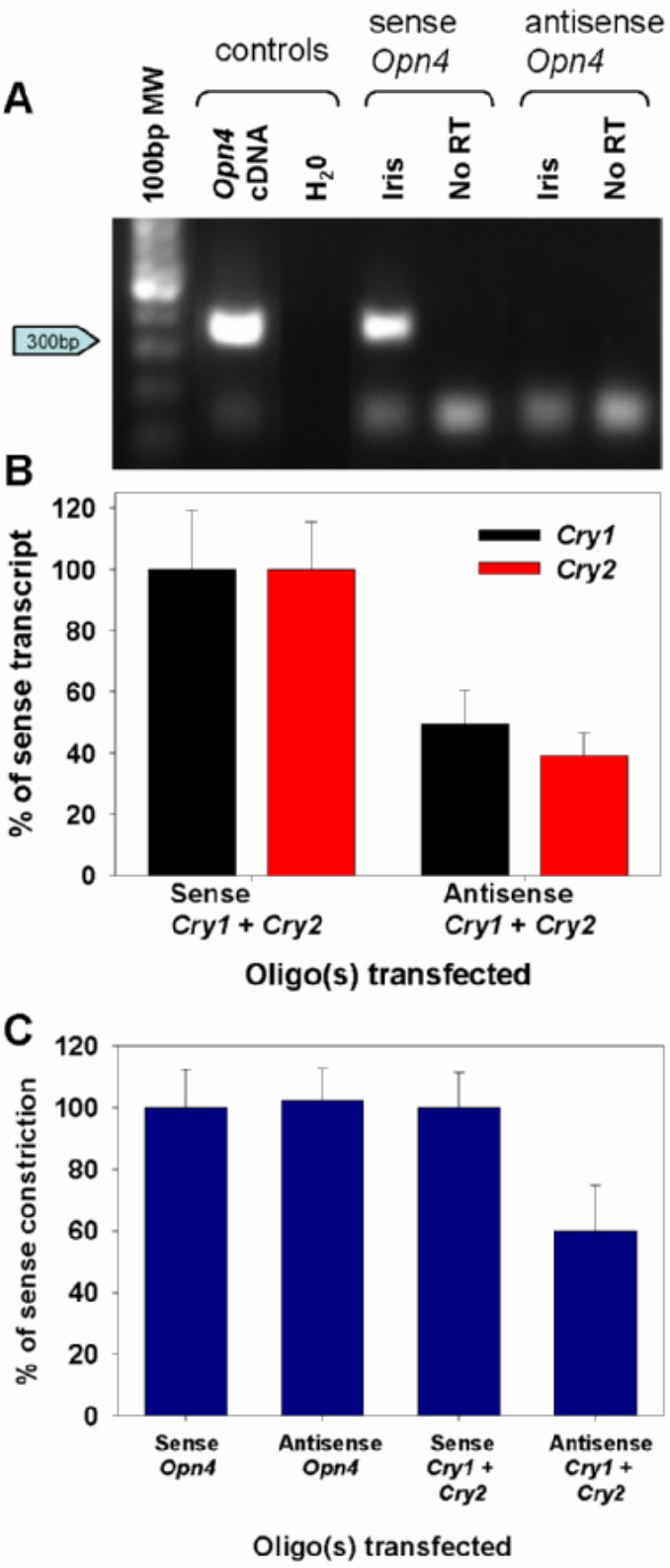

No UV control
UV treated
Percent of maximum pupillary constriction
100
80
60
40
20
0
13
14
15
16
Log Irradiance (photons/cm2/sec)

Fig. S5



Thapsigargin (a,b); forskolin (c,d); staurosporine (e,f)

Fig. S6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100bp MW
controls
Opn4 cDNA
H20
sense Opn4
Iris
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antisense Opn4
Iris
No RT
300bp
B
% of sense transcript
120
100
80
60
40
20
0
Cry1
Cry2
Sense Cry1 + Cry2
Antisense Cry1 + Cry2
Oligo(s) transfected
C
% of sense constriction
120
100
80
60
40
20
0
Sense Opn4
Antisense Opn4
Sense Cry1 + Cry2
Antisense Cry1 + Cry2
Oligo(s) transfected

Table S1

Table S1. Pharmacologic influences on the iris light response. Results represent comparison of vehicle control and drug-exposed irises (n ≥4) at multiple incubation periods (30 min to 4 hours) and at multiple irradiance levels of 430 nm and broad spectrum light. Decreases in pupillary light response were calculated as the difference in peak pupillary constriction to 430 nm light between vehicle and drug treated samples (logIR= 14.6, after a 1 hour drug incubation). See Fig. S5 for details regarding thapsigargin, forskolin and staurosporine effects.

Drug	*Mechanism of action*	*Effect on PLR sensitivity*	*Conc. range examined; Effective dose*	*Effect on ACh-mediated pupillary constriction*	*Effect on High K+ pupillary constriction*
Cholera Toxin	ADP-ribosylation of G_s; Inhibits transducin	-	10-1000 ng/ml; -	-	-
Pertussis Toxin	ADP-ribosylation of G_i; Inhibits transducin	-	10-1000 ng/ml; -	-	-
U-73122 (and U-73343)	Phospholipase C inhibitor (and inactive analog)	-	10-20 µM; -	-	-
Neomycin	Phospholipase C inhibitor	-	1-100 µM; -	-	-
RO-31-8220	Protein kinase C inhibitor	-	2-24 µM; -	-	-
H-89	Protein kinase A inhibitor	-	10-30 µM; -	-	-
Tetrodotoxin	Na^+ channel blocker	-	0.1-1 µM; -	-	-
Thapsigargin	Inhibits intracellular Ca^{2+} pump resulting in depletion of intracellular Ca^{2+} stores	↓ 47%	0.1-10 µM; 1 µM	Inhibited to same degree as light response	-
Forskolin	cAMP elevation via adenylyl cyclase activation; smooth muscle relaxation	↓ 68%	1-50 µM; 1 µM	Inhibited, but less affected than light response	-
Staurosporine	Non-specific kinase inhibitor	↓ 48%	0.1-10 µM; 10 µM	Inhibited to same degree as light response	Inhibition at highest drug concentration

Supporting figure legends:

Fig. S1. Irradiance-response relations for irises after manual debridement of pigment layers. Curves represent fitting of data points to a modified Naka-Rushton equation. The maximum pupillary constriction during a 45 sec light pulse is represented by the "peak PLR," while the level of constriction at the end of the 45 sec is represented by the "steady-state PLR." (mean ± S.E.M.; n=7-12).

Fig. S2. (**A**) Irradiance-response curves for peak pupillary responses compiled from data in Supplementary Figure 1. Curves represent fitting with a modified Naka-Rushton equation of the form $y=a \cdot x^b/(c^b+x^b)$. The average coefficients for curves (350 nm – 530 nm) are "a"= 99.35 ±0.86, "b"=52.47 ±2.42 (mean ± S.E.M.) where "a" represents the maximum, "b" represents the slope, and "c" equals the log irradiance yielding 50% of the maximal response. (**B**) Action spectra derived from peak PLR measurements (both irises with and without pigment layers) show the same basic waveform as those derived from steady-state measurements (Fig. 1D).

Fig. S3. Photopotentiation decays following a logarithmic function (mean ± S.E.M.; n=6-8); with a half-life of ~2.8 min. The logarithmic function used was of the form: $y = m \cdot Ln(x) + b$. Best fit was obtained with m=0.25 and b = 0.32.

Fig. S4. UV light depletion of retinoids from manually depigmented irises. After manual debridement of pigment layers, irises were exposed (or mock-exposed) to the

same 20 min, 312 nm light schedule used in (Fig. 3). Following UV light exposure, pupillary light sensitivity was tested to 430 nm light (mean ± S.E.M.; n=3).

Fig. S5. Effects of thapsigargin (**A**,**B**), forskolin (**C**,**D**), and staurosporine (**E**,**F**) on pupillary responses. Pupillary constrictions were normalized to the response elicited by a saturating broad-spectrum light (350 W/m^2) prior to drug exposure. Constriction in response to high K^+ bath solution or to a bright white light (350 W/m^2) after incubation with thapsigargin, forskolin, or staurosporine for 1 hour is shown in (**A**,**C**,**E**) respectively. PLRs elicited by a dim blue light (430 nm, 11.6 W/m^2) before and after drug exposure are compared with pupillary constrictions from addition of ACh (15.1 μM) subsequent to drug exposure in (**B**,**D**,**F**) for thapsigargin, forskolin, or staurosporine respectively. Plotted here are peak pupillary responses as a percent of the pupillary constriction to a bright white light (350 W/m^2) prior to drug treatment; inhibition of light responses by these pharmacologic agents affected peak and steady-state levels of pupillary constriction equally.

Fig. S6. Antisense-mediated gene knockdown in chick iris. Irises (n =7-8) were transfected with phosphothiorate antisense oligonucleotide targeting either *Opn4* or a combination of *Cry1* and *Cry2*, while irises from contralateral eyes were transfected with the respective sense oligonucleotide(s). 45-48 hours after transfection, irises were isolated and total RNA extracted. Levels of *Opn4*, *Cry1, Cry2,* and *β-actin* as assayed by qRT-PCR (40 cycles of amplification). (**A**) Antisense oligo transfection decreased the *Opn4* transcript level below the limit of detection by quantitative PCR and (**B**) decreased the levels of *Cry1* (50% decrease) and *Cry2* (60% decrease) relative to sense-transfected control irises. (**C**) Just prior to tissue collection (45-48 hours after

transfection), irises were tested for pupillary sensitivity to blue light (430 nm, 11.6 W/m^2). These results were replicated on two independent trials.

Movie S1. Pupillary light response. Pupillary constriction in response to a bright 45 sec pulse of broad spectrum light (350 W/m^2). Light onset is marked as t= 0 sec. Shown at 2x speed.

Movie S2. Pupillary constriction and relaxation. Pupillary constriction and relaxation in response to a 5 sec pulse of bright 430 nm light (logIR 15.75, 20 W/m^2) followed by 1 min of dim background lighting (20 mW/m^2). The end of the 430 nm light pulse is marked as t= 0 sec. Shown at 3x speed.