Almost a century ago, Stiles and Crawford reported that the human eye is more sensitive to light entering through the pupil center than through its periphery (Stiles-Crawford effect). This psychophysical phenomenon, later found to correlate with photoreceptor orientation toward the pupil, was dynamically phototropic, adjustable within days to an eccentrically displaced pupil. For decades, this phototropism has been speculated to involve coordinated movements of the rectilinear photoreceptor outer and inner segments. We report here that, unexpectedly, the murine photoreceptor outer segment has a seemingly light-independent orientation, but the inner segment’s orientation undergoes light-dependent movement, giving rise to nonrectilinear outer and inner segments in adult mice born and reared in darkness. Light during an early critical period (−P0 to P8), however, largely sets the correct photoreceptor orientation permanently afterward. Unexpectedly, abolishing rod and cone phototransductions did not mimic darkness in early life, suggesting photosignaling extrinsic to rods and cones is involved.

RESULTS
Light-dependent photoreceptor orientation in mouse retina
Consistent with previous reports on a nonhuman primate and several other vertebrates (2, 3, 5, 7), we found that, in control adult wild-type (WT) mice kept in cyclic light (14-hour light/10-hour dark cycle) for 1 to 3 months since birth, the rectilinear outer and inner segments of mouse rods were oriented roughly toward the center of the pupil (Fig. 1A, top; note that the pupil is invariably dilated upon death of an animal; thus, the location of the center of the pupil is only approximate). For quantification (Materials and Methods), we graphically divided the midsagittal section of the retina into seven sectors (−3, −2, −1, 0, 1, 2, and 3) and plotted the outer- and inner-segment tilting angles against retinal sector [see collected data in Fig. 1A (bottom)]. Hence, the angles θ and φ formed between the radial direction (i.e., perpendicular to the retinal surface and pointing approximately toward the eyeball center), and the outer- and inner-segment axes, respectively, were roughly equal, and they co-increased progressively with photoreceptor position away from the posterior pole (retinal sector 0) toward the retinal periphery (retinal sectors +3 and −3) (Fig. 1A, bottom).

To test whether the photoreceptor orientation is light dependent, we studied adult WT mice born and reared in continuous darkness for 1 to 3 months after birth before euthanasia. Unexpectedly, the outer and inner segments under these conditions no longer showed the same coordinated orientation, thus becoming nonrectilinear. Although the outer segments remained aligned approximately toward the pupil center, the inner segments became approximately radially oriented, i.e., pointing toward the center of the eyeball (Fig. 1B, top). Collective data gave the same conclusion (Fig. 1B, bottom). The different alignments of the outer and inner segments in dark-reared mice were evident as early as postnatal days 18 to 21 (P18 to P21)
light and dark conditions for $P_A$, but there is a large difference for $A_cyclic$ light or continuous darkness.

Fig. 1. Retinal photoreceptor orientation in adult WT mice born and raised in cyclic light or continuous darkness. (A) Top: Representative micrographs showing the outer- and inner-segment orientations in an adult WT mouse born and raised in cyclic light for 1 to 3 months. The middle bottom panel shows the image of the midsagittal section of one eyeball in its entirety. Five locations along the same retina are zoomed in to show the orientations of the respective photoreceptors. In each panel, the solid red arrow indicates the orientation of outer segments, the solid blue arrow indicates the orientation of inner segments, and the dashed red or blue arrow is perpendicular to the retinal surface, pointing approximately radially toward the eyeball center. The rectilinear alignment of solid red and blue arrows indicates that the rods are straight overall, with the inner and outer segments pointing roughly toward the pupil center. Bottom: Collected data from nine animals. The whole retina is divided into seven sectors, as shown in the cartoon on the left. The angles, $\theta$ and $\psi$ (formed between the perpendicular direction and the outer- and inner-segment axes, respectively), were measured and plotted on the right against sector number. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer, INL, inner nuclear layer. (B) Same display format as in (A), but for an adult WT mouse born and raised in darkness for 1 to 3 months. Collected data are from nine mice. Data points are means ± SD. Significance of the statistical data was assessed by two-way analysis of variance (ANOVA). There is no significant difference between light and dark conditions for $\theta$, but there is a large difference for $\psi$ ($P < 0.001$).

We next asked whether the inner-segment misalignment that developed in dark rearing could be reversed by light. We took adult WT mice born and reared in darkness for 1 to 2 months and exposed them to cyclic light for 1, 2, or 5 days (representative photomicrographs in different retinal sectors are shown in Fig. 2A; averaged data from each group are shown in Fig. 2B; data from individual animals in each group are shown in fig. S5A). After 1 day of light exposure, the situation was no different from continuous darkness, namely, with outer segments aligned toward the pupil and inner segments misaligned toward the eyeball center. After 2 days in the light, however, the correct inner-segment orientation was already restored in most (eight of nine) mice, with outer and inner segments both aligned toward the pupil. After 5 days of light exposure, all eight of eight prior-dark-reared mice showed a correct rectilinear rod outer-/inner-segment configuration oriented toward the pupil. The judgment of alignment or misalignment was qualitative, but the data predominantly showed that it was clearly either one or the other, instead of being a continuum (see fig. S5A). In short, the misaligned inner segments in continuous darkness were able to restore their tilt after several days of light exposure.

**P0 to P8 is the critical period for setting the correct and permanent photoreceptor orientation**

We wondered whether, conversely, after a time period of light exposure and then switching to continuous darkness, inner-segment misalignment would develop. Thus, we exposed adult WT mice, born and raised in cyclic light for 1 to 2 months, to different time periods of continuous darkness. We found that steady cyclic light followed by 10 days of darkness did not (zero of seven mice) make the inner segments misalign (Fig. 3A and fig. S5B). After 30 days of darkness, one of nine mice developed misaligned inner segments, and two others showed some signs of misalignment (Fig. 3A and fig. S5B). Even after 90 days of darkness, only two of nine mice developed some misaligned inner segments (Fig. 3A and fig. S5B). Again, as in fig. S5A, the indication of alignment or misalignment in fig. S5B was predominantly quite clear. The exceptional cases with misaligned inner segments caused some skewing of the averaged data in Fig. 3A (right), with fig. S4 showing one example each of these exceptions after 30 and 90 days of darkness. We have not looked beyond 90 days of darkness.

To possibly examine any age dependence early in life, we raised mice in cyclic light and then exposed them to continuous darkness for 20 days beginning at P0, P6 to P8, or P12 to P14 (Fig. 3B). When switched to darkness at P0, the inner segments showed misalignment (fig. S1), before which age they were structurally too short for quantification (14–16). The different orientations of the outer and inner segments created a bend on the rods, not at the ciliary neck but typically a short distance into the basal part of the rod outer segment [as examples, see yellow arrowheads in Fig. 1B (two top left panels)]. Electron microscopy showed some structural disorganization of the membrane disks at the bend (fig. S2). This dark-reared change in orientation apparently exclusive to the inner segment was never expected according to the longstanding notion inferred by psychophysicists from the human SCE, in which the outer and inner segments were implicitly conceived to always form a rectilinear structure together, even when adjusting to an eccentric pupil (9, 11, 13). Suction pipette recordings (Materials and Methods) from single rods of these dark-reared WT mice showed that their dark currents and flash responses are nonetheless normal. Thus, the cells apparently are functionally not adversely affected by the bend (fig. S3).
upon checking at P20 (we were unable to check earlier because the rods were still maturing before this time point; see earlier), which is expected on the basis of the experiments in Figs. 1B and 2 because the animal had never seen light since birth. When switched to darkness for 20 days at P6 to P8 or P12 to P14, however, the inner segments did not become misaligned. Hence, although not truly absolute given the exceptions alluded to above in connection with Fig. 3A, there is strong indication of a “critical period” predominantly between P0 and P6 to P8 during which light exposure has a high probability of permanently setting the correct alignment of the inner segment, regardless of darkness or light afterward.

Photosignaling extrinsic to rods and cones may be involved in photoreceptor orientation

We wanted to know whether the above inner-segment misalignment in darkness was due to the absence of photosignaling in rods and cones, by examining Rho−/− (17, 18) and Opn1SW−/−; Opn1MW−/− mice (i.e., double knockout of S- and M-cone-pigment genes; mouse does not have OPN1LW pigment) (19, 20) so as to eliminate rod or cone phototransduction. Rho−/− rods do not form outer segments,
and they gradually lose their inner segments, eventually their cell bodies as well (17, 18). Upon examining these mice born and raised in cyclic light until P30 to P60, at which time even the inner segments had become quite short, we found nonetheless that the inner segments appeared to be aligned toward the pupil, although not as clearly as in WT (Fig. 4). The same normalcy was found for Opn1SW−/−; Opn1MW−/− mice. Last, we observed no misalignment in Gnat1−/−; Gnat2−/− double-knockout mice, where the genes for rod and cone transducin α subunits mediating the respective phototransductions are both deleted, thus abolishing rod/cone photosignalings, although normal photoreceptor morphologies are retained (21, 22). The results therefore suggest that intrinsic photosensitivities of rods and cones are not required for inducing the correct light-dependent tilt of the inner segments. Instead, some other photosignaling extrinsic to rods and cones may be involved.

DISCUSSION
In summary, we have found a dynamic, light-dependent orientation of the photoreceptor inner segment, which, so far, is the only morphological correlate of the phototropism in photoreceptor tilting implicated in human psychophysics (5, 8, 9). On the other hand, our finding that the outer segment shows no apparent dynamic change in orientation regardless of light or darkness tends to reject the long-held concept by psychophysicists (5, 9, 11, 13) that the phototropism implicated in SCE involves coordinated outer- and inner-segment movements to retain rectilinearity. Because no validating human ocular histology was ever carried out or could be done, the above decades-old concept may well be just an interesting possibility but turns out to be untrue. Given the invariant orientation of the outer segment independent of light or darkness during rearing, the question nonetheless remains: What signal besides light could ensure the outer segment’s accurate and consistent tilting toward the pupil for optimal photon capture? As for the darkness-induced misalignment of the inner segment toward the eyeball center, this phenomenon undoubtedly represents a malfunction brought on by darkness and is ill-fit for light capture, highlighting the importance of light in keeping the inner segment properly aligned to serve as a light guide. Future studies to examine whether the same phenomenon exists in other animal species will be of interest. Mouse has the distinct advantage of allowing genetic manipulations, but its small eyes do make it difficult to examine, on the basis of morphology, the previously reported SCE displacement associated with an eccentric pupil in humans (10–13). The existence in mouse of a critical period before P8 is also unexpected because one might have expected the photoreceptor orientation to be permanently set only upon maturation of the outer and inner segments at eye opening (~P12). Whether a related critical period of any kind exists in other vertebrates is unknown at present. At least in the adult mouse with light exposure since birth, however, the lack of plasticity is in agreement with the decades-old psychophysical observation that monocular light deprivation with an eye patch on an adult human subject failed to change the SCE (23). The mechanism for setting photoreceptor orientation during the critical period in mouse can be complex because of the many developmental events, including cell differentiation, happening during this period. Last, the mechanism underlying the dynamic changes in inner-segment orientation triggered by light versus darkness is currently also unknown. The lack of effect of disrupting rod/cone phototransductions suggests that another retinal light signal likely participates. The inner-segment misalignment involves major cellular motility and thus may require mechanical forces extrinsic to the photoreceptors, such as from Müller glial cell processes. Hence, our discoveries have opened up an entirely new set of fundamental questions regarding photoreceptor cellular mechanics serving to optimize visual optics in the eye.

MATERIALS AND METHODS
Animals
All animal experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee at Johns Hopkins University. For control WT mice, we used 129-Elite Mouse (strain code: 476) purchased from Charles River Laboratories. The Rho−/− mouse line (17, 18) was originally made by P. Humphries (Trinity College, Dublin, Ireland), and the Gnat1−/− mice (21) was originally made by J. Lem (Tufts University School of Medicine). Opn1 SW−/− mice (19) were made in the laboratory of E. Pugh Jr. (University of California, Davis), and Opn1 MW−/− mice (20) were made by W. Baehr (University of Utah), who also gave us the Opn1 SW−/−; Opn1 MW−/− double-knockout line. The Gnat2−/− mouse line (22) was from M. Burns (University of California, Davis). We generated Gnat1−/−; Gnat2−/− double-knockout mice by crossing. All...
mice were raised under either 14-hour/10-hour cyclic light or continuous darkness, unless indicated otherwise for specific experiments. For experiments requiring mice born and kept in continuous darkness, the mothers were also kept in continuous darkness from pregnancy on. Both male and female mice were used in all experiments. Light exposure was with room light, 200 to 300 lux throughout.

**Suction pipette recording**

For suction pipette recordings (24–26), all mice exposed to cyclic light were dark-adapted overnight before experiments, whereas the dark-reared mice were in darkness throughout before recordings. The eyes were removed after euthanasia under dim red light, and the retinae were isolated from the retinal pigment epithelium under infrared illumination in Locke’s solution, containing 112.5 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, 3 mM Na₂-succinate, 0.5 mM Na-glutamate, 0.02 mM EDTTA, 10 mM glucose, 0.1% MEM (Eagle’s minimum essential medium) vitamins (M6895, Sigma-Aldrich), 0.1% MEM amino acid supplement (M5550, Sigma-Aldrich), 10 mM Hepes (pH 7.4), and 20 mM NaHCO₃. Retinae were stored at room temperature in Locke’s solution bubbled with 95% O₂/5% CO₂, to be used for experiments within 6 hours of isolation. Before recording, a piece of retina was chopped into small fragments with a razorblade in Locke’s solution on a SYLGARD-coated petri dish (24236-10; Electron Microscope Sciences). The recording chamber was perfused with Locke’s solution at 37°C. Temperature was monitored by a thermistor held within several millimeters of the recorded cell. Suction pipette recording was carried out from individual rods under infrared light using a tight-fitting glass pipette containing 140 mM NaCl, 3.6 mM KCl, 2.4 mM MgCl₂, 1.2 mM CaCl₂, 0.02 mM EDTTA, 10 mM glucose, and 3 mM Hepes (pH 7.4). The pipette resistance was ideally ~7 megohms in recording solution without a cell drawn in. Light pulses (500 nm) were delivered in 10-ms duration with different intensities. Responses were primarily digitized at 2 kHz through an Axopatch 200B amplifier and low-pass filtered at 1 kHz. An additional channel was low-pass filtered at 20 Hz (RC filter, Krohn-Hite 3343).

The 2-kHz and 20-Hz channels were compared to correct for the time delay caused by filtering.

**Histology**

For histology, the mice were perfused with 1/2 Karnovsky buffer [2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2)]. After animal perfusion, the two eyeballs of each mouse were marked at the superior pole for the purpose of orientation, followed by enucleation. A window with dimension of ~1 mm by 2 mm was opened on the lateral side of each eyeball for better infiltration of solutions and then placed in 1/2 Karnovsky buffer for at least 24 hours at 4°C for further fixation. Afterward, the fixed eyeballs were washed in 0.1 M cacodylate buffer (pH 7.2) for 3 × 20 min and then immersed in 1% osmium tetroxide in 0.1 M cacodylate buffer overnight at 4°C. The eyeballs were then washed by ice-cold distilled water for 3 × 20 min and then dehydrated at room temperature by successive immersion for 20 min each in 30, 50, 70, and 90% ethanol and then for 3 × 10 min in 100% ethanol. After dehydration, the eyeballs were treated for 2 × 10 min with 100% propylene oxide, followed by infiltration. Because of the difficulty in infiltration presented by the whole eyeball, we adopted an extended infiltration protocol with a mixture of Epon and Spurr’s resin at room temperature. Briefly, the eyeballs were successively incubated with a 1:3 ratio of resin:propylene oxide for 12 hours, 1:1 ratio of resin:propylene oxide for 12 hours, and lastly fresh 100% resin for 3 × 12 hours. The eyeballs with 100% resin were placed in a vacuum for 2 to 3 hours to get rid of the bubbles inside. It was made sure that 100% resin was not used for over 24 hours for better infiltration. After infiltration, the eyeballs were placed in molds filled with fresh 100% resin and baked in an oven for 1 to 2 days at 60°C. Once hardened, the resin-embedded eyeballs were trimmed and cut into 1-μm sections on an ultramicrotome (Leica UC7) with a Histo Jumbo Diamond Knife (8 mm; DiATOME), followed by staining with toluidine blue (1% toluidine blue and 1% sodium borax in distilled H₂O). The sections were photographed and stitched automatically on a light microscope (BZ-X700, KEYENCE).

**Quantitative analysis of photoreceptor orientation**

Photomicrographs were taken using a 20× objective on a light microscope (BZ-X700, KEYENCE) throughout an entire section of the eye, with images being stitched together using KEYENCE software. Image analysis was carried out using a custom MATLAB program, available at https://github.com/dsilve33/PhotoreceptorOrientation. Example images and example analysis results are available at https://osf.io/4kuzj/. Briefly, whole-eye images were loaded into MATLAB, and photoreceptor orientation angles (θ and φ) were determined as follows. First, the perimeter of the eye was defined by converting the image to a binary format and extending neighboring pixels to trace the outer edge surrounding the eye. To identify a reference point for measuring θ and φ, the approximate center of the eye was located using the regionprops (“Centroid”) function in MATLAB. This function determines the approximate center of the eye by calculating the averages of all x and y coordinates of pixels within the perimeter of the eye. The retina was divided into seven equal sectors (−3, −2, −1, 0, +1, +2, and +3), with sector 0 being at the posterior pole. θ and φ were measured in each retinal sector by manually identifying three clearly resolved outer segments and three clearly resolved inner segments. Typically, most or all the outer segments within a given sector are parallel to each other, as are the inner segments, so the step of choosing which outer/inner segments as representatives of the sector was not critical. Radial lines were drawn automatically from the outer-segment distal tip to the center of the eye and from the inner-segment distal tip to the center of the eye, as shown in one panel marked with “θ” and “φ” in each text figure, giving the red and blue dashed arrows, respectively. A straight line connecting the outer-segment distal tip and the inner-segment distal tip (same as proximal tip of outer segment) defines the vector orientation of the outer segment. Likewise, a straight line connecting the inner segment distal and proximal tips defines the vector orientation of the inner segment. θ and φ were then computed from the dot product of the outer- or inner-segment vector and the corresponding radial lines.

The approximate center of the pupil was used only as a qualitative reference point. We identified the approximate pupil center by drawing a straight line connecting the two sides of the iris. This straight line was then manually dragged to the anterior surface of the lens, and the midpoint of the line was calculated automatically, yielding a defined point that was reliably near the center of the pupil.

**Transmission electron microscopy**

After getting the 1-μm sections for histology, the eyeballs showing clear morphology of outer and inner segments of photoreceptors were chosen for transmission electron microscopy (TEM). Briefly, the blocks were further trimmed to an area of ~0.5 mm by 0.5 mm,
and 80-nm sections were cut. The sections were picked up on formvar-coated copper slot grids with dimensions of 1 mm by 2 mm and stained with uranyl acetate followed by lead citrate. Grids were viewed on a Hitachi 7600 TEM operating at 80 kV. Digital images were captured with a 5-megapixel XR50 charge-coupled device camera by AMT.

**Statistics analysis**

All experiments were performed side by side with controls in a random order and replicated at least three times. All values presented are means ± SD. The number of mice used for each set of experiment was dictated by the measurement variations and is given in the text and figure legends. For quantitative conclusions, P values from two-way analysis of variance (ANOVA) or Student’s t test are reported after tests of normality. All tests were conducted using the Statistical Package for the Social Sciences version 20.0. Significant differences were accepted at P < 0.05.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/5/eabe2782/DC1

View/request a protocol for this paper from Bio-protocol.

**REFERENCES AND NOTES**


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