Rhodopsin and cone opsins are essential for light detection in vertebrate rods and cones, respectively. It is well established that rhodopsin is required for rod phototransduction, outer segment disk morphogenesis, and rod viability. However, the roles of cone opsins are less well understood. In this study, we adopted a loss-of-function approach to investigate the physiological roles of cone opsins in mice. We showed that cones lacking cone opsins do not form normal outer segments due to the lack of disk morphogenesis. Surprisingly, cone opsin-deficient cones survive for at least 12 mo, which is in stark contrast to the rapid rod degeneration observed in rhodopsin-deficient mice, suggesting that cone opsins are dispensable for cone viability. Although the mutant cones do not respond to light directly, they maintain a normal dark current and continue to mediate visual signaling by relaying the rod signal through rod–cone gap junctions. Our work reveals a striking difference between the role of rhodopsin and cone opsins in photoreceptor viability.

In two types of photoreceptors are involved in image-forming vision in the vertebrate retina: rods and cones. Rods are responsible for dim light vision, whereas cones mediate bright light and color vision. Both rods and cones rely on visual pigments that are embedded in their outer segment discs for light detection. Rods express one type of visual pigment, rhodopsin, whereas cones express different types of cone opsins for detecting light with different wavelengths (1, 2). The fundamental function of rhodopsin and cone opsins in phototransduction has been well established (3, 4). In addition to phototransduction, rhodopsin also has a structural role in disk membrane formation (5–7). In particular, rhodopsin-null mice (Rho−/−) did not form outer segments and underwent rod degeneration within ~90 d, suggesting rhodopsin is important for rod cell survival (6, 7).

In contrast to the wealth of information available on rhodopsin, much less is known about cone opsins despite their important role in mediating daylight vision and high-acuity vision. To better understand the function of cone opsins, we took a loss-of-function approach to study the role of cone opsins on the structure and function of cone photoreceptors. Because some mouse cones coexpress the medium-wavelength sensitive opsins (M-opsin encoded by Opn1sw) and short-wavelength sensitive opsin (S-opsin encoded by Opn1sw), we first generated the Opn1sw−/− mice and subsequently crossed them with the Opn1sw−/− mice (8) to generate the Opn1sw−/−Opn1sw−/− mice. We confirmed the absence of both M-opsin and S-opsin in the Opn1sw−/−Opn1sw−/− retina, while rhodopsin was located normally in the rod outer segment (ROS) (Fig. 1A). Opn1sw−/−Opn1sw−/− cones showed normal morphology except with much shorter length of inner plus outer segment as determined by labeling with the cone cell marker, cone arrestin (Fig. 1B). Quantitative assessment showed that the overall length of inner plus outer segment was reduced by 41, 56, and 54% in 1-, 6-, and 12-mo-old Opn1sw−/−Opn1sw−/− mice, respectively, in comparison with WT (Fig. 1C). This was primarily due to the lack of an outer segment of Opn1sw−/−Opn1sw−/− cones because the inner segment length of Opn1sw−/−Opn1sw−/− cones was only slightly reduced however, they maintain a normal dark current and continue to mediate visual signaling by relaying the rod signal through rod–cone gap junctions.

Significance

The study of visual pigments (rhodopsin in rods and cone opsins in cones) in vision has a long and illustrious history. It is well established that rhodopsin is required for rod phototransduction, outer segment formation, and rod viability. It is not known if the same is true for the cones. By using the cone opsin-deficient mice, we made the surprising observation that cones survive for at least 12 mo without cone opsins, suggesting that cone opsins are dispensable for cone viability. Furthermore, cone opsin-deficient cones continue to mediate visual signaling by relaying the rod signal through rod–cone gap junctions. Our finding represents a major difference between the two types of photoreceptors in vertebrates: rods and cones.
compared with WT cones (SI Appendix, Fig. S1). In other words, the mean length differences of 10.0, 13.2, and 12.4 μm largely reflected the length of WT cone outer segment (COS) at 1-, 6-, and 12-mo, respectively. WT cone arrestin showed strong expression in the COS and synaptic terminal (Fig. 1D and SI Appendix, Fig. S2, arrows indicating strong colocalization signal between cone arrestin in green and peanut agglutinin [PNA] in red in the outer segment in WT). This pattern was shifted to the cone inner segment (CIS) and synaptic terminal due to the lack of normal COS in Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice (Fig. 1D and SI Appendix, Fig. S2, white arrowheads indicating localization of cone arrestin in the inner segment). Labeling of PNA (in red) showed long cone matrix sheaths in Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice that were comparable to WT. Cone cell densities were similar between Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> and WT mice in all regions at 1 mo of age (Fig. 1E, Left). Moreover, Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> cones survived an extended time. There was no significant difference in cone density between Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> and WT mice in all retinal regions at 6 and 12 mo of age, suggesting that cone viability is unaffected in the absence of cone opsins at least until 1 y of age (Fig. 1D and E and SI Appendix, Fig. S2). These observations are in sharp contrast to the rapid rod degeneration observed in Rho<sup>−/−</sup> mice (6, 7).

**Ultrastructure of COS in Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice.** To further examine the morphology of Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> cones, especially the COS in high resolution, we performed electron microscopy using biotinylated PNA to unequivocally identify cones in the rod-dominant retina of 6-wk-old WT and Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice (Fig. 2A and B; arrows show silver-gold particles surrounding cones). The specificity of this technique in labeling cones was demonstrated in SI Appendix, Fig. S3 (no silver-gold particles in either the ROS in SI Appendix, Fig. S3A, or rod inner segment [RIS] in SI Appendix, Fig. S3B). As expected, the COS of WT contained stacked discs (Fig. 2A). In sharp contrast, the PNA-identified cones did not form typical COS in Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice (Fig. 2B). The residual COS contained disorganized internal membranes. These observations are consistent with COS formation being initiated by the growth of opsin-containing membrane with retinal degeneration slow (RDS/peripherin-2)-mediated rim formation as a secondary step (10).

**Trafficking of Cone Phototransduction Proteins.** To determine whether the absence of opsins and normal disk membranes in the Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> cones affect the trafficking of cone phototransduction proteins, we examined the subcellular localization of guanylate cyclase 1 (GC1), G protein coupled receptor kinase 1 (GRK1), cone transducin alpha subunit (G<sub>α</sub>t2), and cone cyclic nucleotide-gated (CNG) channel A3 subunit in WT and Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> cones. In WT retinas, GC1 and GRK1 were markedly reduced (Fig. 3A, white arrows). Cone CNGA3 subunit was reduced in cones, although their expression in rods was normal (Fig. 3A, white arrows). Thus, cone opsins at least until 1 y of age (Fig. 1D and E and SI Appendix, Fig. S2). These observations are in sharp contrast to the rapid rod degeneration observed in Rho<sup>−/−</sup> mice (6, 7).

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**Fig. 1.** Long-term survival of Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> cones. (A) Retinal sections from 1-mo-old WT and Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice were immunostained with antibodies against rhodopsin, M-opsin, and S-opsin. (B) Cones of 1-mo-old WT and Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice were visualized by labeling cone arrestin in retinal sections. (Right) Magnified views of two boxed regions from Left. (C) Quantitative comparison of IS plus OS length between WT and Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> (dKO) mice. Data represent mean ± SD. ***P < 0.001. N = 80 cones from 1- and 6-mo-old mice for both genotypes (4 WT, 4 dKO, 20 cones per mouse). n = 60 cones from 12-mo-old mice for both genotypes (3 WT, 3 dKO, 20 cones per mouse). Statistics were performed with unpaired two-sample t test. (D) Retinal sections from 12-mo-old WT and Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice were labeled with anti-mouse cone arrestin antibody (in green) and rhodamine PNA (in red). Arrows indicate colocalization between cone arrestin and PNA in the outer segment of WT, while white arrowheads indicate cone arrestin in the inner segment of Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice. (E) Quantitative results of relative cone numbers at the ventral, central, and dorsal retina from 1-, 6-, and 12-mo-old WT and Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice. Data (mean ± SEM) were normalized to WT ventral region. N = 3 to 4 (1 mo), 4 (6 mo), and 4 to 6 (12 mo) for each genotype. The difference between WT and Opn1sw<sup>−/−</sup>/Opn1mw<sup>−/−</sup> mice was not significant (NS) for all comparisons by one-way ANOVA with Tukey post hoc analysis. IS, inner segment. (Scale bar, 10 μm in A and B, Left; 5 μm in B, Right; and 20 μm in D.)
Indeed, the photopic ERG responses of increase of the other through transcriptional compensation (8, and S-opsin for green and UV light detection, respectively (Fig. opsin) light stimuli, confirming the essential role of M-opsin and rod input at high intensities. Both the scotopic a-wave (504 nm) were slightly increased (Fig. 4), which was consistent was likely due to the increased S-opsin level (9). Similarly, the B at 360 nm were enhanced at high intensities (Fig. 4), associated with cone telodendria (Fig. 6), yellow arrows). Prph2 was normally expressed throughout the outer segments of both rods and cones (Fig. 3 F and H, white arrows indicating signal in cones). In Opn1sw−/−Opn1mw−/− mice, while Prph2 expression remained in the ROS, it appeared as dots at the base of residual COSs (Fig. 3 F and H, white arrowheads). In this experiment, we used cone arrestin and PNA double labeling (Fig. 3f) as a reference to help identify the junction of COS and CIS in the PNA/Prm1 and PNA/Prph2 labeled cones in Fig. 3 G and H (cone arrestin showed stronger signal in COS than in CIS, with red arrows indicating the junction of COS and CIS; see legend for details).

Functional Evaluation of Cone Photoreceptors by Electoretinography.

To evaluate the photoreceptor function of Opn1sw−/−Opn1mw−/− and control mouse lines (Opn1sw+/−, Opn1mw−/−, and WT), we performed photopic and scotopic electoretinography (ERG) recordings. Photopic ERG responses were not detectable in Opn1sw−/−Opn1mw−/− mice to either 504-nm (λmax for M-opsin) or 360-nm (λmax for S-opsin) light stimuli, confirming the essential role of M-opsin and S-opsin for green and UV light detection, respectively (Fig. 4 A and B). As some mouse cone photoreceptors coexpress both S- and M-opsins, loss of either one would lead to the increase of the other through transcriptional compensation (8, 18). Indeed, the photopic ERG responses of Opn1mw−/− mice at 360 nm were enhanced at high intensities (Fig. 4B), which was likely due to the increased S-opsin level (9). Similarly, the photopic ERG responses of Opn1sw−/− mice to green light (504 nm) were slightly increased (Fig. 4A), which was consistent with increased M-opsin level (8).

Scotopic ERG reflects rod input at low intensities and mixed rod and cone input at high intensities. Both the scotopic a-wave and b-wave amplitudes were reduced in Opn1sw−/−Opn1mw−/− mice compared with the other three genotypes (Fig. 4 C and D).

Because the cone sheaths are disordered (SI Appendix, Fig. S5; arrows) and the normal traction system for transporting shed cone discs across the gap between the COS tip and the retinal pigment epithelium is likely compromised in Opn1sw−/−Opn1mw−/− mice, this might lead to an accumulation of extracellular debris in the subretinal space that is deleterious to rods. Indeed, we found that the length of outer nuclear layer (ONL) was reduced by 3.3, 4.7, and 8.2% in 1-, 6-, and 12-mo-old Opn1sw−/−Opn1mw−/− mice, respectively, compared with those in age-matched WT (SI Appendix, Fig. S4), although no significant changes were observed for the length of ROS at 1 mo of age when ERG was recorded (SI Appendix, Fig. S5; see more in Discussion).

Opn1sw−/−Opn1mw−/− Cones Mediate Rod Signaling via Rod–Cone Coupling. In addition to mediating COS–originated intrinsic cone photoreponses, WT cones receive extrinsic rod inputs through rod–cone coupling, which are mediated by gap junctions (19–22). We performed electrophysiological recordings to investigate whether Opn1sw−/−Opn1mw−/− cones receive rod-originated signals via rod–cone coupling despite the lack of intrinsic photoreponses. The cone photovoltages to 500- or 350-nm light were recorded at cone pedicles from retinal slices of different mouse lines to selectively stimulate the M-opsin or S-opsin, respectively (Fig. 5, green traces for 500 nm and blue traces for 350 nm). At 500 nm, WT cones exhibited an initial transient and fast component reflecting the contribution of the cone intrinsic response and a slower and long-lasting component reflecting the contribution of coupled rods (Fig. 5, Top Left, and SI Appendix, Fig. S6 A and E). In contrast, the fast component was absent in Opn1sw−/−Opn1mw−/− cones, and they only showed the slow rod-mediated response (Fig. 5, Bottom Left, and SI Appendix, Fig. S6 D and H). The threshold intensity T350 (the light intensity required to elicit 0.5 mV response to 350 nm light), which was mainly driven by the rod input, was similar in Opn1sw−/−Opn1mw−/− and WT cones (0.74 photons/μm2/20-ms versus 0.64 photons/μm2/20-ms; Table 1). Although the threshold intensity T350 at 350 nm light was approximately two times higher in Opn1sw−/−Opn1mw−/− cones than in WT cones, this difference was not statistically significant (P > 0.05 between four genotypes in Table 1 among all cone recordings with rod input by two-way ANOVA). Application of the rod–cone gap junction blocker meclofenamic acid (MFA) (23, 24) eliminated the slow rod component in WT cones while abolishing all light responses in Opn1sw−/−Opn1mw−/− cones. This indicates that Opn1sw−/−Opn1mw−/− cones mediate rod signaling via rod–cone coupling (Fig. 5, Top Right and Bottom Right, and SI Appendix, Fig. S6 D and H). As a control, deletion of S-opsin in Opn1sw−/− or M-opsin in Opn1mw−/− cones eliminated the fast cone response to UV or green light, respectively, while the remaining slow rod response persisted. This rod-mediated component was eliminated upon gap junction blocking (Fig. 5, Middle; SI Appendix, Fig. S6 B, C, F, and G; and Table 1). Finally, the rod photoreponses (recorded from the rod soma) to 500-nm light were not significantly different across all four genotypes (Table 1).

Since rod–cone coupling was mediated by connexin36 (Cx36), we compared the expression and distribution of Cx36 between WT and Opn1sw−/−Opn1mw−/− retinas. Similar to the distribution of Cx36 in the WT retina (Fig. 6 A–C) (21), Cx36 was expressed in the outer plexiform layer (OPL) and inner plexiform layer (IPL) on Opn1sw−/−Opn1mw−/− retinal sections (Fig. 6D), associated with cone telodendria (Fig. 6E), and at contact sites between rod spherules and cone telodendria (Fig. 6F, white arrows). Quantitative analysis showed that the number of Cx36 puncta/cone pedicle and the median volume of Cx36 puncta were similar between both genotypes (Fig. 6G), suggesting intact rod–cone coupling in Opn1sw−/−Opn1mw−/−.
retina that is consistent with the same threshold of rod-mediated responses in both cone types.

**Dark Current in Opn1sw<sup>−/−</sup> Opn1mw<sup>−/−</sup> Cones.** In WT rods and cones, the circulating dark current (mainly carried by Na<sup>+</sup>) through the cGMP-gated channel keeps photoreceptors in a depolarizing state (25, 26). Activation of the phototransduction cascade by light results in a reduction of the circulating current, which leads to photoreceptor hyperpolarization. Given that Opn1sw<sup>−/−</sup> Opn1mw<sup>−/−</sup> cones lack normal COSs and do not respond to light, we asked whether a dark current was still present. We first measured the resting membrane potential of dark-adapted WT and Opn1sw<sup>−/−</sup> Opn1mw<sup>−/−</sup> cones without rod input and found it to be similar in both genotypes at
approximately −35 mV (Fig. 7 A and D). This indicates the presence of an active conductance in the absence of rod–cone coupling and of an outer segment in Opn1sw−/− Opn1mw−/− cones. Next, we tested whether this conductance reflects the presence of the dark current. Because Na+ is the major carrier of the dark current, reducing extracellular Na+ would be expected to decrease the current, hyperpolarize the cones, and decrease the amplitude of their photovoltage. Indeed, replacement of Na+ by choline in WT cones produced a hyperpolarization from −34.20 to −48.20 mV (n = 5, P < 0.01) (Fig. 7A). In addition, we observed a reduction in the cone photoresponse amplitude during Na+ replacement by up to 80% at the brightest intensities (Fig. 7 B and C). This effect was reversible as the resting membrane potential returned to about −35 mV after washing out the cells in normal Ringer solution.

In Opn1sw−/− Opn1mw−/− cones, replacement of Na+ by choline produced a hyperpolarization from −34.35 to −46.86 mV (P < 0.01) (Fig. 7D), which was similar to the amplitude change observed in WT cones (Fig. 7A). As expected, Opn1sw−/− Opn1mw−/− cones remained insensitive to light before, during, and after Na+ replacement (Fig. 7 E and F). The most parsimonious explanation is that the current in Opn1sw−/− Opn1mw−/− cones is carried by residual cGMP-gated channels (Fig. 3D) that are insensitive to light because key elements of the phototransduction cascade (e.g., cone opsins and Gβγ12) are missing (Discussion).

We tested a specific blocker of cGMP-gated channels, L-cis-diltiazem (20 to 150 μM), on the resting and light-evoked changes in membrane potential of rods and cones. Although we observed the expected effects of the drug on the membrane potential of rods (i.e., hyperpolarization of the membrane and decrease in the light-evoked response amplitude), we were not able to detect a clear effect of the drug on the cone membrane potential, either in the WT or in the mutant mice (SI Appendix, Table S1). We attribute the failure of this experiment to the fact that the drug, which is water-soluble and was dissolved in the recording pipette, had to diffuse from the site of the clamp (pedicle) all the way up to the CIS that is about 50 μm away. Nonetheless, despite the technical limitations, we recorded a small but significant decrease in resting membrane potential (about −5 mV) in all three recorded Opn1sw−/− Opn1mw−/− cones (SI Appendix, Table S1). This result is consistent with the presence of active cGMP channels in these cells.

Discussion

By taking a loss-of-function approach, here we show that cone opsins are important for cone disk morphogenesis and outer segment formation. However, the most surprising finding of this work is that cones can survive for an extended time (at least 12 mo) without cone opsins. This finding revises the established dogma based on the absolute requirement of rhodopsin for rod viability. It represents the starkest difference between rod and cone photoreceptors. The reason why cones but not rods can survive without their respective opsins is not clear yet, but we present several possibilities below.

One possibility is the different volume of outer segments between mouse rods and cones. For example, the volume of a mouse COS is ∼1/3 of ROS (36 μm³ in rods versus 14 μm³ in cones) (25, 27). Thus, Opn1sw−/− Opn1mw−/− cones only need to degrade ∼1/3 of the proteins destined for the outer segment compared to Rhodopsin−/− rods. Assuming the protein densities are the same and similar gene regulation mechanisms, this translates into a greater reduction in metabolic stress. For primate photoreceptors, this difference is much smaller (40 μm³ in rods versus 30 μm³ in cones) (28, 29). This would indicate that primate cones may be more susceptible to cone opsin loss than their mouse counterparts. This is indeed the case in human X-linked blue cone monochromacy patients carrying deletions in the red/green opsin gene array can survive in reduced numbers with abnormally shortened outer segments (30). Nevertheless, these patients possess a sufficient number of red/green cones that may warrant future gene augmentation therapy. Thus, the susceptibility of primate cones to cone opsin loss seems to lie between mouse cones and rods and could be related to their relative size. If this hypothesis holds true, we can predict that amphibian rods and cones will exhibit the biggest difference in terms of susceptibility to opsin loss (2,000 μm³ in rods versus 70 μm³ in cones) (29, 31). Photoreceptors are the only neurons of central nervous system (CNS) that possess a highly specialized structure of outer segment, which is dedicated to light detection. Our results indicate that an intact outer segment is not essential for cone survival; the degeneration of rods without an outer segment may be caused by a secondary effect (e.g., metabolic stress caused by degradation of large amounts of phototransduction proteins).

Another possibility is that cones are less vulnerable to outer segment disruption compared with rods. Goldfish COSs become shortened and even disappear when living in lower temperature, but they regenerate within approximately a week at room temperature (32). Similarly, the outer segments of ground squirrel cones are much shortened during hibernation, when body temperature can be as low as 4°C, yet they regrow rapidly after hibernation (33, 34). These data point out the potential to regenerate the COS of patients with red/green opsin deficiency by gene therapy.

Alternatively, cones without opsins may survive because rods can provide them with trophic support. Several viability factors have been identified, including the well-characterized rod-derived cone viability factor (RdCVF), a thioredoxin-like protein secreted by rods that promotes cone survival by increasing glucose intake.
in cones and stimulating aerobic glycolysis (35). Because rods outnumber cones by about 30 to 1 (27), rods may provide both metabolic and structural support to the cones (36). In addition, rods and cones may interact metabolically and/or electrically through rod–cone gap junctions, although the exact nature and/or benefit of these interactions remains uncertain. On one hand, rod–cone gap junctions have been proposed to support the diffusion of apoptotic signals from the dying rods to the cones. In degenerative diseases that primarily affect rods such as retinitis pigmentosa, the diffusion of these apoptotic signals may cause the secondary death of overwise healthy cones—a form of “bystander killing” effect (37). Although it has been demonstrated that gap junctions mediate bystander cell death in the retina (38), eliminating Cx36 in the retinal degeneration-1 (rd1) mouse model did not slow down secondary cone loss (39), indicating that the rod–cone coupling role in the secondary death of cones may not be significant or required. On the other hand, rod–cone gap junctions may play a role in supporting cone survival when cones are functionally impaired. Clearly, Cx36 does not appear required for the normal development, function, and maintenance of cones (or of rods), as

| Table 1. The threshold intensity of cones and rods from WT, Opn1sw−/−, Opn1mw−/−, and Opn1sw−/− Opn1mw−/− mice |
|-----------------------------------------------|
| Wavelength (nm) | Cone (+ rod input) (photons/μm²/20 ms) | Cone (- rod input) (photons/μm²/20 ms) | Rod (photons/μm²/20 ms) |
|-----------------|---------------------------------|---------------------------------|----------------------|
| WT              |                                 |                                 |                      |
| 500             | 0.64 ± 0.35 (n = 5)             | 12.1 ± 3.62 (n = 6)             | 0.21 ± 0.07 (n = 7)  |
| 350             | 0.49 ± 0.34 (n = 4)             | 5.33 ± 2.10 (n = 6)             | 0.21 ± 0.10 (n = 4)  |
| Opn1sw−/−       |                                 |                                 |                      |
| 500             | 1.17 ± 0.49 (n = 4)             | 15.8 ± 9.28 (n = 6)             | 0.07 ± 0.03 (n = 5)  |
| 350             | 0.15 ± 0.00 (n = 4)             | 16.7 ± 9.33 (n = 6)             | 1.08 ± 0.98 (n = 5)  |
| Opn1mw−/−       |                                 |                                 |                      |
| 500             | 2.04 ± 1.08 (n = 5)             | ≥ 50 (n = 2)*                   | 0.23 ± 0.07 (n = 11) |
| 350             |                                 | ≥ 2,000 (n = 2)^†              |                      |
| Opn1sw−/− Opn1mw−/− |                      |                                 |                      |
| 500             | 0.74 ± 0.28 (n = 10)            | > 2,000 (n = 5)                 | 0.11 ± 0.04 (n = 5)  |
| 350             | 1.03 ± 0.51 (n = 9)             | > 50 (n = 5)                    | 0.49 ± 0.26 (n = 5)  |

Values are mean ± SEM, with the number of cells studied indicated in parentheses. Threshold is the intensity at which the response is ≥ 0.5 mV.

*Two Opn1sw−/− cones did not respond to 350 nm light without rod input.
†Two Opn1mw−/− cones did not respond to 500 nm light without rod input.
demonstrated in Cx36−/− animals (21, 40). However, a “bystander trophic” effect has been proposed in an experimental mouse model in which loss of Cx36 resulted in increased vulnerability to secondary photoreceptor cell loss following focal laser-induced lesions (41). Thus, Cx36 may contribute to the survival of photoreceptor cells after trauma. Although the nature of the trophic agent exchanged via gap junction coupling remains unknown, it is possible that Opn1sw−/−/Opn1mw−/− cones survive because of electrical coupling between rods and cones. In support of this, mutations that either constitutively activate or constitutively inactivate the phototransduction cascade—and therefore keep the membrane constantly hyperpolarized or constantly depolarized—invariably lead to photoreceptor cell death (42). The intriguing possibility that rods may support the phototransduction cascade—and therefore keep the membrane constantly hyperpolarized or constantly depolarized—is supported by our findings. This possibility warrants further investigation.

Although Opn1sw−/−/Opn1mw−/− cones survive for an extended time, we found evidence that cones without outer segments affect rod viability and function as reflected by a reduction of ONL length and scotopic ERG a- and b-wave amplitudes of Opn1sw−/−/Opn1mw−/− mice compared with WT mice (Fig. 4C and SI Appendix, Fig. S5). Why does a 3.3% reduction in ONL thickness lead to twofold to threefold reductions in scotopic a-wave of Opn1sw−/−/Opn1mw−/− mice? One possibility is that an accumulation of extracellular debris in the subretinal space of Opn1sw−/−/Opn1mw−/− mice might affect chromophore supply from RPE to rods.

Previously, Opn1sw−/− mouse was shown to respond to 360-nm light albeit with substantially reduced sensitivity by ERG (8). However, we did not observe photopic ERG responses to 360-nm light of Opn1sw−/−/Opn1mw−/− mouse even at high light intensities (Fig. 4B). Mouse M-opsin in individual cones is about 10-fold less sensitive to light at 360 nm than it is at 510 nm (8). In the Ganzfeld ERG, WT mouse absolute b-wave sensitivity at 510 nm (due to M-opsin) is about 0.2× that at 360 nm (due to S-opsin) (43). The net result is that measured with the ERG b-wave, in absolute units, the M-opsin in the WT mouse is at

**Fig. 6.** Cx36 distribution in (A–C) WT and (D–E) Opn1sw−/−/Opn1mw−/− (dKO) retinas. (A and D) Cx36 (red) was expressed in the OPL and IPL in both genotypes on retinal sections. Note the OPL labeling was weaker compared to IPL. Nonspecific labeling of some blood vessels by Cy3 anti-mouse secondary antibody is indicated by white asterisks. (B and E) Cx36 immuno-puncta (red) were associated with cone telodendria in OPL on whole-mount retinas in both genotypes (green). (C and F) Representative images showing Cx36 puncta (red) at points of contact (white arrows) between rod spherules (blue) and cone telodendria (green). (G) Quantification of the number of Cx36 puncta per cone pedicle and of the median volume of Cx36 puncta at photoreceptor terminals. Data represent mean ± SEM. n = 3 mice per genotype. For each animal, a volume of 32 to 41 cone pedicles including between 1,745 and 1,987 Cx36 puncta was surveyed. The mean value of the number of Cx36 puncta per cone pedicle and the median volume of Cx36 puncta were calculated for each animal to generate a single value. NS, P > 0.05. Statistical analysis was performed by unpaired two-sample t test. Mice were about 2 mo old. Cones were labeled for cone arrestin (cARR; green), rod spherules were labeled for the vesicular glutamate transporter 1 (VGLUT1; blue), and nuclei were stained with DAPI (cyan). INL, inner nuclear layer; GCL, ganglion cell layer. (Scale bar, 50 μm in A and D and 5 μm in B, C, E, and F.)
in our experiments: 1) M-opsin is primarily expressed in the dorsal retina (44–46), so that depending on how homogeneously the retina is stimulated, we could be delivering less 360 nm light to the dorsal retina. 2) Because we did our ERG experiments from low intensities to high intensities, the Opn1sw<sup>–/–</sup> mouse might have lost some overall responsivity as the experiment progressed at higher intensities.

Our data clearly show that although Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones do not respond to light directly, their membrane potential still senses rod-originated changes in membrane voltage via gap junction. Thus, we have generated an interesting mouse that separates the two functions mediated by cones, the residual cone CNGA3 subunits likely responsible for the observed dark current. Since the amount of cone CNGA3 subunits was substantially reduced in Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones (Fig. 3D), how can they mediate a normal dark current? This might occur because under normal conditions, in the WT, only about 5% or less of the channels are activated in the dark to maintain the dark current (48), suggesting that very few channels are needed to maintain a normal dark current. Thus, our data support the view that residual expression of CNGA3 channels in Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones likely supports a dark current that allows the cells to maintain a quasi-normal resting membrane potential, and this current is not sensitive to changes in light. Since we were unable to detect GC1 in Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones by immunostaining (Fig. 3A), it raised the question of the origin of cGMP in Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones. An intriguing possibility is that cGMP may flow into Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones, which are heavily coupled to neighboring rods via gap junctions (Fig. 6) (21). Gap junctions are permeable to molecules up to 1 kDa in molecular size (49), and cGMP has a molecular size of 345.21 Da. Although the lack of effect of the gap junction blocker MFA on the resting membrane potential of Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones would suggest that rods are not the source of cGMP for the mutant cones, although it is not regulated by light. The residual cone cGMP-gated channels are likely responsible for the observed dark current.

**Fig. 7.** Effects of Na<sup>+</sup> substitution by choline on photovoltage and resting membrane potential of WT and Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones. Resting membrane potential of (A) WT and (D) Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones before, during, and after (i.e., washout) Na<sup>+</sup> replacement. n = 5 (WT) and 4 (Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup>) cones (one to two cells per animal). Representative recordings of the cone light responses to 20-ms light flashes of increasing intensities (0.2, 2, 20, 200, and 2,000 R<sup>+</sup>) for (B) WT and (E) Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones. Averaged photovoltage amplitudes from (C) five WT and (F) four Opn1sw<sup>–/–</sup>/Opn1mw<sup>–/–</sup> cones (one to two cells per animal) at different intensities before, during, and after Na<sup>+</sup> replacement. Data represent mean ± SEM in A, C, D, and F. ***P < 0.001 by one-way analysis of variance (ANOVA) with Tukey post hoc analysis in A and D.
definitive demonstration will require genetic elimination of rod-cone coupling as well as the determination of the turnover of cGMP and other important biological complexes in the electrically isolated mutant cones. Future research will answer the fundamental question of the origin of cGMP.

Finally, although the results with L-diltiazem point toward the involvement of cGMP-gated channels in maintaining depolarized dKO cones, we cannot exclude the contribution of ionic exchangers to the dark current. Indeed, several Na+/Ca2+ and K+ exchangers (e.g., NCKX2 and NCKX4) have been found in the cones (50, 51). Their relative contribution will require further investigation.

Materials and Methods

Opn1sw+/− and Opn1sw−/− mice were generated and genotyped as described previously (8, 9). Opn1sw+/− mice were bred with Opn1sw−/− mice to obtain Opn1sw+/Opn1sw−/− mice. Mice were reared under cyclic light (12 h light/12 h dark). Samples were collected during light cycle unless otherwise noted. All animal experiments were approved by the Institutional Animal Care Use Committee at the University of Utah. The University of Texas Health Science Center, Houston, and the Baylor College of Medicine. All the experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Immunodetection and confocal microscopy, ERG, electro-physiological recordings, and transmission electron microscopy were done as previously described (21, 23, 24, 52–55). Detailed methods are described in SI Appendix, Materials and Methods.

Data Availability. There is no shared data (e.g., code analyses, sequences, dataset, structural data, genomic and proteomic data, etc.) in this study. All study data are included in the article and/or SI Appendix.

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