



Connexin 36 in photoreceptor cells: studies on transgenic rod-less and cone-less mouse retinas

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Purpose: Rod-cone gap junctions permit transmittal of rod visual information to the cone pathway. A recent report has shown that this transfer does not occur in mice in which the gap junction protein connexin 36 is knocked out indicating that rod-cone gap junctions are assembled from this protein. It remains unresolved, however, whether rods, cones or both express connexin 36. We have tried to address this question with the use of transgenic rod-less and cone-less mice.

Methods: Deletion of *Nrl*, a transcription factor, results in a complete loss of rods with a concomitant increase in S-cones. We used this as the rod-less (cone-only) model. Cone-less (rod-only) retinas were from mice expressing an attenuated diphtheria toxin gene under the control of a promoter selective for cones. Nearly all long wavelength cones and 95% of short wavelength cones are missing in this model. Fixed retinal sections from these two models and age matched controls were used to detect connexin 36 gap junctions by immunofluorescence.

Results: Punctate immunofluorescence, indicating the presence of gap junctions, was observed in the inner and outer plexiform layers of both wild type and cone-less and rod-less retinas. Our assumption was that immunofluorescence due to photoreceptor gap junctions would be observed in the outer plexiform layer. In all the animals, most of the immunofluorescence was in the inner plexiform layer, with only a marginal reaction in the outer plexiform layer. In cone-only (rod-less) retina, immunofluorescence in the outer plexiform layer increased by more than 20 fold compared to wild type. In rod-only (cone-less) retina, the outer plexiform layer showed about a 30% decrease in immunofluorescence. In both rod-less and cone-less retinas, immunofluorescence in the inner plexiform layer was higher than in the wild type by 25-50%.

Conclusions: Cones constitute only about 3% of photoreceptors in the wild type retina while they make up 100% of the photoreceptors in cone-only retina. This increase in their numbers coincided with a 20 fold increase in immunofluorescence in the outer plexiform layer, strongly suggesting that cones express connexin 36. Conversely, when the cone numbers went down from 3% to near zero in cone-less retina, immunofluorescence decreased by about 30% in the outer plexiform layer, suggesting again that cones express the connexin and that they contribute to its presence disproportionately more than their numbers indicate. The results from both rod-less and cone-less animals are strongly indicative of cones expressing connexin 36, but are not sufficient to conclude whether rods express the protein. An unexpected observation from our experiments is that immunofluorescence increases slightly in the inner plexiform layer in both rod-less and cone-less retina for reasons that need further investigation.

Gap junctions are clusters of intercellular channels between neighboring cells that permit passage of small molecules of up to 1,000 kDa [1,2]. Each channel is formed by the coupling of two half channels, one in each of the apposed cell membranes. The half channels are made up of six units of a class of proteins called connexins; a half channel may be made up of one or more types of connexins of which about twenty are known, and the connexin composition of the two half channels may also be different. Knowledge of the types of connexin proteins forming the channel is important in understanding the possible mechanisms that might be involved in regulating the flow of information through the gap junction.

Connexin 36 and its orthologs were discovered recently and shown to be preferentially expressed in brain and retina [3-8]. In retina, antibodies to connexin 36 react with both outer plexiform layer (OPL) and inner plexiform layer (IPL) [7],

though most of the antibody reaction is in the latter where it is shown to be due to processes of AII amacrine cells making contacts with processes of other AII amacrine cells and with those of cone bipolar cells [7,8]. Since AII amacrine cells couple the rod and cone pathways, gap junctions made by connexin 36 may play an important role in transmitting rod visual information to the cone pathway [9-13]. In a recent report, Deans et al. [14] have shown that in connexin 36 knockout mouse, on-center responses mediated by rods, but not cones, do not reach ganglion cells, suggesting a complete absence of information transfer from AII amacrine cells to on-cone bipolar cells under scotopic conditions and from rods to cones under mesopic illumination [10,15,16]. Defective rod-cone transfer in the connexin 36 knockout mouse suggests that rod-cone gap junctions in the wild type mouse are made up of connexin 36 and that rods, cones or both express this protein. From their studies on these knockout mice in which the connexin 36 coding sequence was replaced by sequences for histological reporters, Deans et al. noted that connexin 36

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is expressed by rods, but its presence in cones, which make up only 3% of photoreceptor cells in the mouse retina [17,18], could not be determined [14]. Using transgenic mice with *lacZ* reporter expressed under the control of connexin 36 promoter, Feigenspan et al. suggested that connexin 36 is present in cones, but not rods [19]. In other species, connexin 36 plaques were reported in salamander retina between rods and between rods and cones [20], while only cones appeared to express the protein in the guinea pig retina [21].

To address the question of expression of connexin 36 by cones and rods in mouse retina, we took advantage of the availability of rod-less (cone-only) and cone-less (rod-only) transgenic animals. Deletion of *Nrl*, encoding a transcription factor [22,23], in a knockout mouse model (*Nrl*^{-/-}) resulted in complete loss of rods with a concomitant increase in S-cones [24]. We used this as the cone-only model. Cone-less retinas were from mice expressing an attenuated diphtheria toxin gene under the control of a promoter selective for cones. Nearly all long wavelength cones and 95% of short wavelength cones are missing in these animals, lost during the first postnatal week [25,26]. Immunofluorescence analysis of connexin 36 in fixed retinas using a connexin 36 specific antibody showed that the expression of the protein is greatly increased in the OPL of cone-only retina in comparison to the control and that it is marginally lower in the absence of cones. These results suggest that cones express connexin 36.

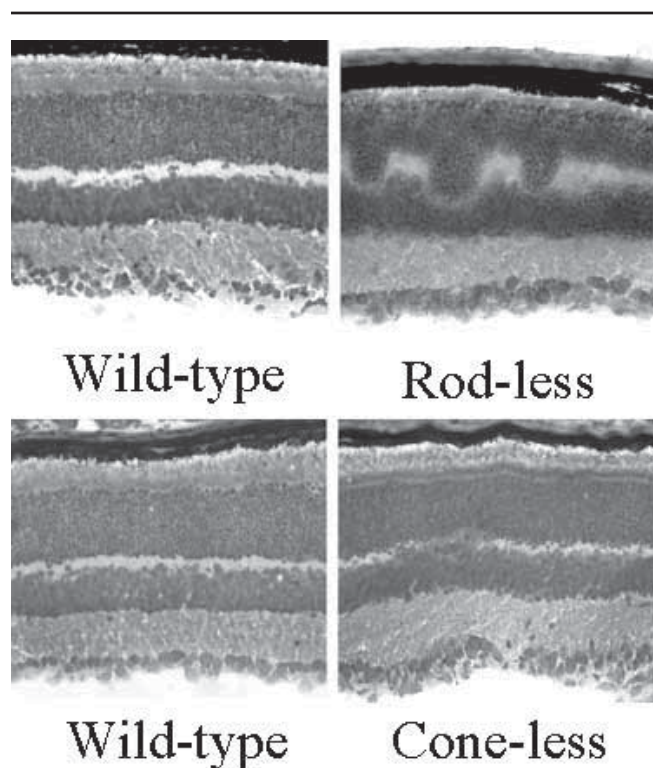


Figure 1. Comparable outer nuclear layer thickness in the wild type and transgenic mouse retinas. Fixed sections of rod-less and cone-less retinas and their age matched wild type retinas were stained with toluidine blue. The thickness of outer nuclear layer in rod-less, cone-less and wild type retinas was comparable.

METHODS

The use of animals conformed to the guidelines established by the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Institutional Animal Care and Use Committees of Oakland University, the University of Michigan, and the University of California at Santa Barbara. Eyes were removed from euthanized wild type and transgenic mice and embedded in OCT compound and frozen at -80 °C. Rod-less mice and the corresponding controls were about 17 week old and the cone-less mice and corresponding controls were about 3 week old. Sections (10 μm) were cut on a cryostat and collected on glass slides. The sections were fixed for 10 min with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and washed with phosphate buffered saline (PBS). The sections were blocked for 1 h at room temperature in 0.5% normal goat serum containing 0.3% Triton X-100, incubated for 1 h at 37 °C in connexin 36 antibody (Zymed 51-6200) diluted 200 fold in PBS with 0.3% Triton X-100. This antibody has been used before for immunohistochemistry on mouse retinas and shown to be specific for connexin 36 [27]. The sections were washed with PBS, incubated for 1 h at 37 °C with goat anti-rabbit IgG conjugated with Alexa fluor 488 dye, washed with PBS, stained briefly with Hoechst dye, washed again with PBS and observed under a fluorescent microscope. Digitized pictures were obtained using a SPOT digital camera. The density of immunofluorescence was determined using the Scion Image beta v4.02 software (version 4.02 beta; Scion Corporation, Frederick, Maryland)

RESULTS

It has been reported earlier that the number of photoreceptor cells and the thickness of the outer nuclear layer are similar in the rod-less and cone-less retinas in comparison to age matched wild type retinas [24,25]. In our experiments we used retinas

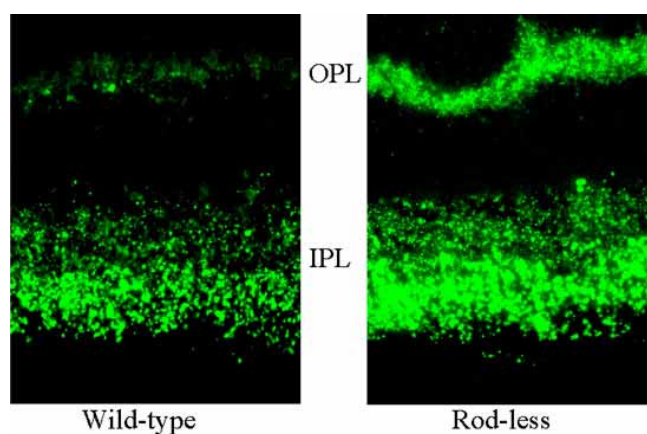


Figure 2. Marked increase of connexin 36 in the OPL of rod-less retina. Fixed sections of wild type and rod-less retinas were probed with connexin 36 antibody. Outer and inner plexiform layers are marked. Rod-less retina has a much higher connexin 36 immunofluorescence in the OPL.

from about 17 week old rod-less animals and about 21 day old cone-less animals. Figure 1 shows toluidine blue stained sections of the rod-less and cone-less retinas and their corresponding, age matched wild type retinas; in both cases, the thickness of the outer nuclear layer was comparable to that of the wild type retinas. A striking observation reported earlier [24] and seen in Figure 1 is the appearance of whorls and rosettes in the outer nuclear layer of the cone-only retina.

Figure 2 shows immunoreaction to connexin 36 antibody in sections of wild type and cone-only retinas. The antibody reacted with both the IPL and OPL in both retinas. In the wild type, most of the reaction was in the IPL; sub lamina b was more labeled than sub lamina a. The reaction in the IPL of cone-only retina was similar. The most striking difference between the wild type and cone-only retinas was seen in the OPL, where the reaction was much more prominent in the latter.

Densitometric analysis of the immunofluorescence, shown in Table 1, revealed the OPL of the cone-only retina to be 2050% of that of the wild type. The reaction in the IPL was also greater, but to a lesser degree: 127% of the wild type in sub lamina a and 144% of the wild type in sub lamina b.

Figure 3 shows immunofluorescence of cone-less and age matched wild type retinas. Here also, the fluorescence was mostly within the IPL, with sub lamina b being more reactive than sub lamina a. Densitometric analysis shown in Table 2

TABLE 1. DENSITOMETRIC ANALYSIS OF IMMUNOFLUORESCENCE IN ROD-LESS AND AGE MATCHED WILD TYPE RETINAS

	Wild type	Rod-less
OPL	8.67 ± 3.43	178.96 ± 19.92
IPL, sub lamina a	81.84 ± 6.33	104.32 ± 27.43
IPL, sub lamina b	168.58 ± 27.34	244.18 ± 8.94

Density is shown in arbitrary units. Data are mean and SD of results from six sections each of wild type and rod-less retinas.

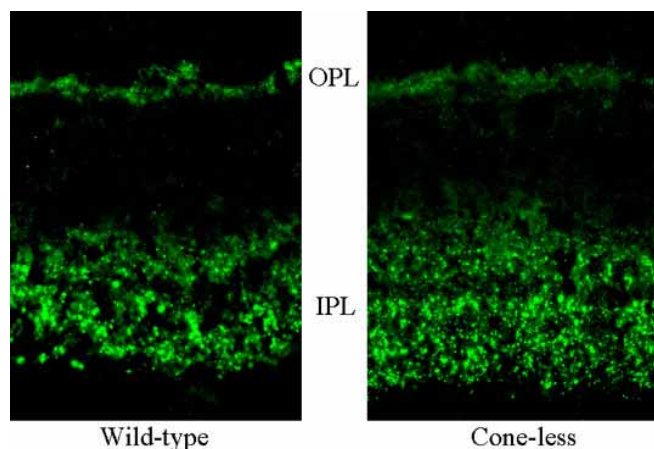


Figure 3. Reduced connexin 36 in the OPL of cone-less retina. Fixed sections of wild type and cone-less retinas were probed with connexin 36 antibody. Outer and inner plexiform layers are marked. Cone-less retina shows reduced connexin 36 immunofluorescence in OPL.

suggested that the cone-less retina has a 30% reduction in the OPL, but 40-50% increase in the IPL.

DISCUSSION

We took advantage of the availability of transgenic cone-only and rod-only mice to address the question of whether connexin 36 is expressed by rods or cones or both. The cone-only and rod-only retinas used in these experiments were reported to contain the same number of photoreceptor cells as in the wild type except that rods are absent in cone-only retina with S-cones replacing them, and all M-cones and 95% of the S-cones are missing in the rod-only retinas [24,25]. As seen in Figure 1, the outer nuclear layer in the transgenic cone-only and rod-only retinas is approximately of the same thickness as in the wild type retinas, though its appearance is distinct in the cone-only retina, displaying whorls and rosettes as reported earlier [24].

The working hypothesis behind the current experiments is that if only rods express connexin 36, there should be no reaction to connexin 36 antibody in the outer plexiform layer of the rod-less (cone-only) retina. Gap junction plaques between cone bipolar, horizontal or Müller cells could influence or interfere with our analysis, but there appears to be a consensus that horizontal and Müller cells do not express connexin 36 [14,19,28] limiting the inference to that by bipolar cells. We assumed that since rods constitute 97% of the photoreceptor cells in the mouse retina, their loss or replacement with cones should result in a substantial, discernable decrease in connexin 36 plaques if rods alone, and not cones, express the protein. Our observation that the immunofluorescence not only survived the loss of rods but was actually 20 fold higher than in the wild type was unexpected. The simplest explanation for this observation appears to be that cones express connexin 36 and that when their numbers increase, so does the number of connexin 36 plaques. In the wild type mouse retina cones constitute about 3% of the photoreceptors [17,18], but they are the only type of photoreceptors in the cone-only retina. This approximate 33 fold increase in their population resulted in a 20 fold increase in immunofluorescence, a strong indication, though not direct evidence, that cones express connexin 36. In the absence of rods, this increase in fluorescence in cone-only retina suggests that cone-cone gap junctions are assembled from connexin 36.

Conversely, would the loss of cones result in a discernable difference in the number of connexin 36 puncta in the outer plexiform layer? Here again, if rods and cones express similar

TABLE 2. DENSITOMETRIC ANALYSIS OF IMMUNOFLUORESCENCE IN CONE-LESS AND AGE MATCHED WILD TYPE RETINAS

	Wild type	Cone-less
OPL	81.68 ± 48.85	59.49 ± 48.67
IPL, sub lamina a	82.57 ± 37.12	127.50 ± 49.57
IPL, sub lamina b	118.87 ± 39.99	166.87 ± 54.08

Density is shown in arbitrary units. Data are mean and SD of results from 12 sections of wild type and 16 sections of cone-less retinas.

amounts of connexin 36, loss of cones, which make up only about 3% of the photoreceptor cells, would not be expected to yield a measurable change. However, as shown in Figure 3, the cone-less retina shows about a 30% reduction in immunolabelling within the outer plexiform layer relative to the wild type, suggesting that cones express connexin 36 more than rods. Our data do not resolve whether the residual plaques, seen in cone-less retina, about 70% of wild type, are due to rods or due to gap junctions between bipolar cells or both.

At the outset we assumed that an increase or decrease in the cone or rod population would only affect the gap junction proteins expressed by those cells, and therefore, did not anticipate changes in the connexin 36 expression in the inner plexiform layer. However, along with a 20 fold increase in the OPL, the cone-only retina also showed a smaller increase in IPL. A similar increase was noted in cone-less retina also. While these data do not reveal the cell type(s) contributing to these changes in IPL, changes in the photoreceptor population clearly influence gap junctional expression in the IPL. This was an unexpected observation which requires further investigation.

In summary, a major shift in the cone population, from 3% of photoreceptor cells to 100%, resulted in a large increase in connexin 36 immunofluorescence in the OPL suggesting strongly that cones in mouse retina express connexin 36. The loss of cones in cone-less retina resulted in a reduction in connexin 36 immunofluorescence, again consistent with the expression of connexin 36 by cones. Our results are in agreement with those of Feigenspan et al [19] who also found that cones express connexin 36. We are, however, unable to conclude from these results whether rods express connexin 36. Further investigations are required to address this question before we can fully understand how cone-rod coupling is regulated.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health (EY07158, EY11115, EY014803, EY11087), The Foundation Fighting Blindness, and Research to Prevent Blindness. We thank Dr. Janet C. Blanks for helpful suggestions on immunofluorescence and Mei Cheng for participation in preliminary investigations.

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