Frizzled3 Shapes the Development of Retinal Rod Bipolar Cells

Ning Shen, Yibo Qu, Yankun Yu, Kwok-Fai So, Andre M. Goffinet, Noga Vardi, Ying Xu, and Libing Zhou

1Guangdong-Hongkong-Macau Institute of CNS Regeneration, Joint International Research Laboratory of CNS Regeneration, Jinan University, Guangzhou, People’s Republic of China
2Department of Anatomy and State Key Laboratory of Brain and Cognitive Sciences, The University of Hong Kong, Pokfulam, Hong Kong SAR, People’s Republic of China
3Institute of Neuroscience, University of Louvain, Brussels, Belgium
4Department of Neuroscience, University of Pennsylvania, Philadelphia, Pennsylvania, United States
5Co-innovation Center of Neuroregeneration, Nantong University, Jiangsu, People’s Republic of China

Correspondence: Libing Zhou, Guangdong-Hongkong-Macau Institute of CNS Regeneration, Jinan University, Huangpu Avenue West 601, Guangzhou 510632, P.R. China; tilbingzh@jnu.edu.cn.
Ying Xu, Guangdong-Hongkong-Macau Institute of CNS Regeneration, Jinan University, Huangpu Avenue West 601, Guangzhou 510632, P.R. China; xuying@jnu.edu.cn.
Submitted: February 3, 2016
Accepted: April 17, 2016
Citation: Shen N, Qu Y, Yu Y, et al. Frizzled3 shapes the development of retinal rod bipolar cells. Invest Ophthalmol Vis Sci. 2016;57:2788–2796. DOI:10.1167/iovs.16-19281

PURPOSE. Frizzled3 (Fzd3), a member of the core planar cell polarity (PCP) family in mammals, contributes to visual development by guiding axonal projections of some retinal ganglion cells. However, its other functions in the maturation of the visual system, especially the retina, remain elusive. The present study explores the role of Fzd3 in retinal development by focusing on rod bipolar cells (RBCs).

METHODS. Frizzled3 was conditionally removed from the retina of Isl1-Cre;Fzd3f/– mice using the Cre-loxP system. Electroretinograms (ERGs) were performed to measure the light response of retinas. Frizzled3 expression was monitored by β-galactosidase (β-gal) staining and anti-β-gal immunostaining. Immunofluorescence was used to examine cellular distribution during development, and electron microscopy was applied to visualize the dendritic invaginations of RBCs.

RESULTS. Electroretinograms showed decreased b-wave amplitudes, and lower b- to a-wave ratios in Isl1-Cre;Fzd3f/– than in control (Isl1-Cre;Fzd3f/+ ) mice. In RBCs, where Fzd3 was expressed and inactivated, the planar organization, shape, and orientation of somas were disrupted. From P10, dendrites of these RBCs displayed reduced arborization with mistargeting. Furthermore, their dendritic invaginations into rod terminals were suppressed, and the density of rod ribbons in the OPL was reduced.

CONCLUSIONS. Frizzled3 is required to shape the pattern of RBC somas and dendrites, and the structural and functional connectivity between rods and RBCs. Our results highlight novel functions for Fzd3 in regulating retinal development.

Keywords: rod bipolar cells, development, somas, dendrites, ribbons

Frizzled3 (Fzd3), a seven-transmembrane receptor with key functions in Wnt/planar cell polarity (PCP) signaling, has critical roles in neural morphogenesis, such as steering various axonal tracts by interacting with other PCP members. A recent study has shown that Fzd3 is required for central projections of retinal ganglion cells (RGCs), indicating a functional role in visual pathways. However, its role during development in other parts of the visual system and its contribution to visual maturation has not been explored in detail. In the Drosophila eye, Frizzled, the ortholog of Fzd3, regulates the relative position of photoreceptor cells R3 and R4, directing the symmetric arrangement of ommatidia relative to the equator, which suggests that Fzd3 regulates visual development not solely through axon guidance.

We investigated the role of Fzd3 in visual system development by focusing on the retina. In mammals, the retina is composed mainly of photoreceptors, horizontal cells, bipolar cells, amacrine cells, and RGCs that are organized into the outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). Neurites of these retinal cells reach specific locations in the outer (OPL) and inner (IPL) plexiform layers where they synapse with appropriate partners to form local circuits in parallel pathways. In addition, in each tangential plane, somas of the same cell type form regular patterns and their dendritic fields cover mosaic territories of the retina. Given its highly ordered structure, the retina offers an ideal model to study the cellular and molecular mechanisms involved in assembling neural circuits and cytoarchitectural organization. So far, apart from a study demonstrating that neurites from multiple kinds of amacrine cells stratify normally in the IPL upon Fzd3 knock-out, the role of Fzd3 in shaping retinal laminar structure, planar organization, synaptic connectivity, and visual function has not been examined in detail.

As Fzd3–/– animals die at birth, we studied the retina of mice in which Fzd3 was conditionally mutated in Isl1-positive cells. In mouse retina, Isl1 is expressed in multiple types of neurons, including all ON-bipolar cells, certain amacrine cells, and RGCs, so we used Isl1 promoter to drive Cre expression and, thus, knock out Fzd3 in Isl1-Cre;Fzd3f/– mice. Using electroretinogram (ERG) recording, we first found that light
responses were impaired in Fzd3 inactivated retina. Then, after confirming that Fzd3 is expressed and inactivated in rod bipolar cells (RBCs), we studied the morphologic phenotypes of these cells in Fzd3 mutant retina, explored the developmental roles of Fzd3 in sculpting these phenotypes, and correlated these phenotypes with functional alterations.

MATERIALS AND METHODS

Animals

Mice were housed with a 12-hour light/dark cycle with access to water and food ad libitum. Isl1-Cre; Fzd3flo/flo males were crossed with Fzd3flo/flo females to obtain Isl1-Cre; Fzd3flo/flo mutant mice in which Fzd3 was conditionally inactivated in Isl1-positive cells; Isl1-Cre; Fzd3+/+/ littermates were used as controls. To monitor Fzd3 expression by β-gal staining, we used the “KO first” Fzd3 allele (Fzd3b/b). As Isl1-Cre; Fzd3flo/+ mice do not survive later than P19, P18 animals of either sex were used except when specifically indicated. All animal procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by competent ethics committees at Jinan University.

Electroretinograms

The ERG set-up and methods have been described previously.19,20 In short, mice were dark-adapted overnight, anesthetized under dim red light with tribromoethanol (25 μg/g bodyweight intraperitoneally [IP]) and placed on a platform maintained at 37°C. Pupils were dilated with a 1% tropicamide solution (Mydriacyl; Alconox, New York, NY, USA). Electroretinograms were recorded with gold-plated loop electrodes contacting the corneal surface as the active electrode. Stainless steel needle electrodes were inserted in the skin near the eye and in the tail as reference and ground leads, respectively. Control and Isl1-Cre; Fzd3flo/+ mice were recorded on the same day under the same settings and conditions, and ERG data were collected using the RETI-scan system (RETI-scan; Roland Consult, Brandenburg a.d. Havel, Germany) at a sampling rate of 2 kHz. Under dark adaptation, animals were exposed to full-field green flashes of 1.3 ms duration presented by a Ganzfeld (Roland Consult) with intensities ranging from 0.03 to 10 cd/s/°m². Electroretinographic signals were analyzed with RETIport (Roland Consult) after 50 Hz low-pass filtering. A-wave amplitude was measured from baseline to the first negative peak, and b-wave amplitude from the negative peak of the a-wave to the next positive peak. For each animal, values recorded for both eyes were averaged and treated as a single data point. We studied eight animals in each group. Data are presented as mean ± SEM and compared using independent samples t-test.

Retinal Cryosections

Mice were anesthetized with tribromoethanol (25 μg/g bodyweight IP). Eyes were removed and incised below the lens, and animals then were euthanized with an anesthetic overdose. Eyeballs were fixed in 4% paraformaldehyde for 30 minutes, rinsed in PBS, cryoprotected overnight at 4°C in 0.1 M PBS containing 30% sucrose, and embedded in optimal cutting temperature compound (OCT; Tissue Tek; Sakura Finetek USA, Inc., Torrance, CA, USA). Sections were prepared with a cryostat (Leica Biosystems, Buffalo Grove, IL, USA) at −20°C and collected on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA, USA). Approximately 15 radial cryosections (30 μm thick) were collected from the dorsal quarter of the retinas, extending from the optic disk to the periphery, and the central five were selected for analysis.

Immunofluorescence

Sections were permeabilized and blocked with 10% normal goat serum, 3% BSA, and 0.3% Triton X-100 in 0.1 M PBS for 1 hour at room temperature (RT). After incubating overnight with primary antibodies at 4°C, sections were incubated with secondary fluorescent antibodies. For whole mount staining, eyecups were permeabilized and blocked with 5% donkey serum, 3% Triton X-100 for 1 hour, and incubated with primary antibodies for 48 hours. Retinas were washed with PBS+0.3% Triton X-100 6 times for 1 hour at RT, and incubated with secondary fluorescent antibodies at RT for 3 hours. The following primary antibodies were used: mouse anti-Isl1 (1:500; MA1502; Abnova, Taipei City, Taiwan), rabbit anti-Protein kinase Cz (PKCz; 1:20000; P4334; Sigma-Aldrich Corp., St. Louis, MO, USA), mouse anti-PKCθ (1:3000; 05-154; Merck Millipore, Billerica, MA, USA), chicken anti-β-gal (1:500; ab9361; Abcam, Cambridge, UK), goat anti-Bni3a (1:100; sc31984; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), goat anti-choline acetyltransferase (ChAT, 1:500; AB144P; Merck Millipore), rabbit anti-tyrosine hydroxylase (TH, 1:500; AB152; Merck Millipore), rabbit anti-calbindin (1:1000; ab11426; Abcam), rabbit anti-CtBP2 (1:10000; 193003; Synaptic Systems GmbH, Göttingen, Germany), and mouse anti-Cacna1s (1:500; MAB427; Merck Millipore). For secondary antibody, Alexa Fluor 405, 488 or 647 conjugated goat/donkey anti-rabbit/anti-mouse IgG (1:1000; Invitrogen, Carlsbad, CA, USA) were used.

Cell Counts and Estimation of Rod Ribbon Density

For cell counting, one field (160 × 160 μm) of each cryosection was acquired using a confocal microscope (LSM-700; Carl Zeiss Meditec, Jena, Germany) with a ×40 objective. Numbers of Isl1-positive, and Isl1-, PKCz-double positive bipolar cells were estimated. To compare the densities of ribbons in rod spherules (rod ribbons), ×40 confocal images of selected control and mutant sections were examined and the density of rod ribbons in each section was calculated as number of rod ribbons/length of the OPL in that section. Values of five sections from the same animal were averaged, and data from three pairs of control and mutant animals are presented as mean ± SEM and are compared using independent samples t-test.

Density Recovery Profile (DRP) and Nearest Neighbor (NN) Analysis

Confocal images of whole mount retinas were captured using a ×40 objective. Fields from dorsal, ventral, nasal, and temporal quarters were selected for analysis. Density recovery profile analysis was used to measure cell density and clustering by calculating packing factor. This factor compares a given distribution of cells to the most highly spaced distribution that is possible with that cell number, and it ranges from 0 for a random distribution to 1 for a packed hexagonal array (i.e., maximal packing of nonoverlapping disks).21 Nearest neighbor analysis was used to estimate the regularity of the distribution of a given cell type. A lower nearest neighbor regularity index (NNRI), calculated based on the distance between each cell and its nearest neighbor, indicates an irregular cell distribution. The parameters were estimated using the Win DPR 1.6.4 software with functions “Win DRP/Autocorrelation” for DRP analysis, and “Win DRP/Nearest Neighbor” for NN analysis. Retinas from three animals of each genotype were analyzed for packing factor and NNRI, and the independent

Downloaded from tvst.arvojournals.org on 03/28/2019
samples t-test was used to compare control and mutant samples. Data are presented as mean ± SEM.

Comparison of Shape and Orientation of RBC Somas

To analyze cell shape, we took the longest diameter of cell bodies as the height and the longest diameter perpendicular to the height as the width, and calculated the height/width ratio as the “aspect ratio.” Soma orientation was estimated by measuring the angle (<90˚) between the plane of the OPL and a line joining the initiation of the axon and that of the primary dendrites. Differences were compared with independent samples t-test, and data are presented as mean ± SEM.

Electron Microscopy

Retinas were isolated from eye cups and fixed in 0.1 M PBS containing 2% paraformaldehyde and 2.5% glutaraldehyde for 6
to 12 hours. Tissue was postfixed in 2% osmium tetroxide, dehydrated in ethanol and acetone, and embedded in Embed 812. (Electron Microscopy Sciences, Hatfield, PA, USA). Sections 90 nm thick were prepared with an ultramicrotome (Reichert & Co., Vienna, Austria) and stained with uranyl acetate and lead citrate. Images were taken with a transmission electron microscope (Philips CM 10, Eindhoven, The Netherlands).

**RESULTS**

**Lack of Fzd3 in the Retina Impairs Light Response**

First, we tested if inactivation of Fzd3 impacts light responses in RBCs by performing ERG recordings. In ERG recordings, the a-wave reflects the response of photoreceptors, and the b-wave mainly reflects the activity of ON-bipolar cells. Under dark adaptation, at almost all flash intensities, Isl1-Cre;Fzd3f/- mice displayed significantly smaller amplitudes of b-waves than their control littermates (Figs. 1A–C), suggesting that inactivation of Fzd3 impairs dramatically the light response of ON-bipolar cells. Even though the amplitudes of a-waves also were reduced in these mutant mice, the ratio of b-to a-wave was significantly decreased at high flash intensities (Fig. 1D). This result demonstrates that the b-wave suffers a greater amplitude reduction than the a-wave, thus indicating impaired transmission from photoreceptors to ON-bipolar cells in Fzd3-inactivated mice.

**Fzd3 is Highly Expressed in the Retina During Development**

To understand why inactivation of Fzd3 decreases the ERG response, we used immunohistochemistry (IHC) to identify retinal cells and structures that are affected by Fzd3 inactivation. We first looked at Fzd3 expression. As there is no antibody against Fzd3 that works well in IHC, we took advantage of the LacZ reporter inserted in the KO-first Fzd3+/- allele. At E13.5, β-gal histochemistry was widely distributed in retinal cells, particularly RGCs and their axons (Fig. 2A). At P0, β-gal IHC showed strong expression in the GCL and some amacrine cells in the neuroblast layer (NBL; Fig. 2B). At P7, in addition to ganglion cells, β-gal was detected in the INL (Fig. 2C), consistent with a previous study, suggesting that Fzd3 is expressed in multiple retinal interneurons. At P14 and P18, this staining pattern was defined more clearly (Figs. 2D, 2E) and no β-gal immunoreactivity was detected in the photoreceptor layer (Fig. 2F). To further identify the cell types that express Fzd3, we double-labeled P18 sections with antibodies to β-gal and specific cell markers. All PKCa-positive RBCs (Fig. 3A), all calbindin-positive horizontal cells (Fig. 2G), and some Brn3a-positive RGCs (Fig. 2H) expressed β-gal. Among amacrine cells, ChAT-positive ON-starburst amacrine cells were β-gal positive (Fig. 2I, arrow), whereas ChAT-positive OFF-starburst amacrine cells and TH-positive dopaminergic amacrine cells did not coexpress β-gal (Figs. 2I, 2J). Triple IHC staining for β-gal, Isl1 and PKCa showed that all RBCs expressed Fzd3 (β-gal and Isl1 (Fig. 3), suggesting that expression of Fzd3 in RBCs should be abrogated in Isl1-Cre;Fzd3f/- mutant retina.

**Inactivation of Fzd3 Compromises Soma Patterning of RBCs**

Given that ERGs test the responses of photoreceptors and ON-bipolar cells, and that Fzd3 is not expressed in photoreceptors, we investigated how deletion of Fzd3 may change the phenotype of RBCs. Double IHC labeling for PKCa and Isl1 showed that the numbers of RBCs (PKCa- and Isl1-positive, arrow in Fig. 4A) and ON-cone bipolar cells (Isl1-positive, arrow in Fig. 4B) were significantly decreased at high flash intensities (Fig. 1D). This result demonstrates that the b-wave suffers a greater amplitude reduction than the a-wave, thus indicating impaired transmission from photoreceptors to ON-bipolar cells in Fzd3-inactivated mice.

**Figure 3.** Frizzled3 and Isl1 are expressed in RBCs. Triple IHC staining for β-gal (Fzd3, A), Isl1 (B), and PKCa, a marker of RBCs (C) in Fzd3f/- retinal sections shows colocalization of all three markers (D) in all PKCa-positive RBCs, suggesting Fzd3 and Isl1 are coexpressed in these cells. Inset (D’) shows enlargement of box outlined in (D). Scale bars: 20 μm (A–D) and 2.5 μm (D’).

**Figure 4.** Rod bipolar and ON-cone bipolar cells are reduced and abnormally distributed in Isl1-Cre;Fzd3f/- retina. (A, B) Double IHC labeling for PKCa (green) and Isl1 (red) shows the distribution of RBCs (positive for Isl1 and PKCa, arrow) and ON-cone bipolar cells (CBCs, Isl1-positive and PKCa-negative, arrowhead) in both genotypes. Abnormal soma clumps are observed frequently in Isl1-Cre;Fzd3f/- retinal sections ([B], *) but seldom in controls (A). DAPI (blue) is used as general nuclear stain. Scale bar: 20 μm (A–D) and 2.5 μm (D’).
positive and PKCα-negative, arrowhead in Fig. 4A) were modestly reduced in mutant than in control retinal sections (Figs. 4C, 4D). Moreover, the somas of mutant RBCs were clustered in some areas (asterisk in Fig. 4B), a pattern seldom observed in control retina. To further define this irregular distribution, we imaged RBC somas in the INL of control and mutant whole mount preparations (Figs. 5A, 5B), then performed density recovery profile and nearest neighbor analyses. Even though Isl1-Cre;Fzd3f/- C0 retinas showed a decreased density of RBCs (Fig. 5C), their packing factor (Fig. 5D) and NNRI (Fig. 5E) were drastically decreased, showing that abrogation of Fzd3 promotes RBC aggregation (decreased packing factor) and reduces the regularity of their distribution (decreased NNRI). On the cellular level, mutant somas appeared more rounded, with lower aspect ratios than control somas (Figs. 6A, 6B), and they did not orientate as perpendicularly to the OPL as did the control somas (Figs. 6C, 6D). These results suggest that Fzd3 is required for planar distribution, shape, and radial orientation of RBC somas.

**Inactivation of Fzd3 Disturbs Maturation of RBC Dendrites**

We next explored if Fzd3 is necessary for maturation of RBC neurites during development. As rodent RBCs achieve differentiation at approximately P7,24,25 we first examined laminar targeting of RBC dendrites at P7, then P10, P14, and P18. At P7, the apical processes of control (Fig. 7A) and mutant (Fig. 7E) retinas overshot into the ONL frequently, but not clear enough to count and calculate the dendritic extension percentage (cells with overshooting dendrites into the ONL/total cells counted). The observable difference in dendritic overextension arose at P10 (Figs. 7B, 7F) when rod bipolar dendrites were prevalently undergoing vertical retraction. At this age, most RBC dendrites in control retina retracted to the OPL, although some still protruded into the ONL (Fig. 7B), while dendrites in the OPL of mutant retina were more diffused vertically. The percentage of RBCs with overshooting dendrites in mutant retina (Figs. 7F, 7I; 17.18 ± 1.95%) was significantly higher than that in control samples (Figs. 7B, 7I; 7.72 ± 0.83%). By P14, the dendrites of RBCs in control retina
continued retracting and became restricted to the OPL (Fig. 7C) with the percentage of overshooting RBCs decreasing to 5.16 ± 0.91% (Figs. 7C, 7I), similar to the percentage at P18 (Figs. 7D, 7I; 5.83 ± 0.79%). In contrast, this percentage in mutant retina remained high at P14 (Figs. 7G, 7I; 14.09 ± 2.32%) and P18 (Figs. 7H, 7I; 13.15 ± 1.83%). These findings indicated that the retraction of RBC dendrites is suppressed in the absence of Fzd3, and the resulting defects are observable as early as P10 and last up to P18. Intriguingly, even though Fzd3 is a critical axon guidance cue, the axons of RBCs terminated in the sublamina (S)5 of the IPL for control (Fig. 7J) and mutant (Fig. 7K) samples, suggesting that Fzd3 does not have an instructive role in the stratification of RBC axons.

Besides overextending into the ONL, in the OPL of whole mount preparations, the dendrites of mutant RBCs assumed a blunted shape (Fig. 8B), in contrast with the well ramified dendrites of control RBCs (arrows in Fig. 8A). To quantify this morphologic difference, we chose random RBCs that were well separated from others, traced the dendrites of each cell through a thickness of 3 μm (or perhaps slightly more due to collection from out of focus planes), and counted the number of dendritic tips within this range. For simplicity, we refer to this index as dendritic tip number per RBC. As dendritic arbors of RBCs cannot be identified clearly at P7, we studied them from P10 on. At P10, arborizations of RBC dendrites were limited with no significant difference in dendritic tip number between control and mutant retinas (Figs. 8C, 8F, 8I; 13.13 ± 0.89 per cell in control versus 11.14 ± 0.91 per cell in mutant). However, by P14, these dendrites in control retinas branched profusely (Figs. 8D, 8I; 16.86 ± 0.93 per cell), whereas mutant RBC primary dendrites adhered to each other and fasciculated with fewer arbors (Figs. 8G, 8I; 11.86 ± 0.63 per cell). At P18, control dendrites ramified further with dendritic tip number growing to 19.90 ± 0.96 per cell (Figs. 8E, 8I), whereas mutant dendrites displayed dendritic tip numbers (Figs. 8H, 8I; 12.80 ± 0.61 per cell) similar to those at P10 (Figs. 8G, 8I) and P14 (Figs. 8G, 8I). Thus, Fzd3 is indispensable for the branching of RBC dendrites.

Inactivation of Fzd3 Suppresses Connections Between Rods and RBCs

The altered dendrites of mutant RBCs prompted us to study their contacts with rods. As the synapses between bipolar cells and photoreceptors in the OPL are mostly ribbons,26 we immunostained for CtBP2 so that ribbons in rod spherules (rod ribbons) could be identified by their horse-shoe shapes (arrow...
in Fig. 9A). We found that the density of rod ribbons decreased from 338.33 ± 26.34 ribbons/mm in control to 181.67 ± 20.07 ribbons/mm in mutant retinas (Figs. 9A–C). Furthermore, double labeling with CtBP2 and Cacna1s, which labels the tips of RBC dendrites,27 showed that the apposition of these two proteins was significantly less frequent in Isl1-Cre;Fzd3f/− animals at P10 (C, F), P14 (D, G), and P18 (E, H). Control RBCs ramify their dendrites gradually from P10 to P18 (C–E), but this growth is constrained in mutant samples (F–H). At P14 and P18 (G–H), the proximal dendrites of mutant RBCs remain compact as at P10 (F), and the numbers of dendritic tips (exampled by asterisks in [C]–[H]) are significantly lower than those in control cells, as quantified in (I). Scale bar: 5 μm (A–H); 20 cells/animal, 3 animals of each genotype at each stage. **P < 0.01.

**FIGURE 8.** Dendritic branching of RBCs is impaired in Isl1-Cre;Fzd3f/− retina. (A–B) Confocal tangential images of the OPL in control (A) and Isl1-Cre;Fzd3f/− (B) retinas stained with PKCa. Mutant RBC dendrites show elimination of arbors seen in controls (arrows in [A]). (C–H) Confocal images of single RBCs in sections stained with PKCa from control (C–E) and mutant (F–H) animals at P10 (C, F), P14 (D, G), and P18 (E, H). Control RBCs ramify their dendrites gradually from P10 to P18 (C–E), but this growth is constrained in mutant samples (F–H). At P14 and P18 (G–H), the proximal dendrites of mutant RBCs remain compact as at P10 (F), and the numbers of dendritic tips (exampled by asterisks in [C]–[H]) are significantly lower than those in control cells, as quantified in (I). Scale bar: 5 μm (A–H); 20 cells/animal, 3 animals of each genotype at each stage. **P < 0.01.

**DISCUSSION**

In Wnt/PCP signaling, Wnt proteins bind to Frizzled receptors that then interact with other core PCP members and contribute to morphogenesis by reorganizing the cytoskeleton.28 Together with Fzd6, Fzd3 is a main mammalian Frizzled known to have a key role in Wnt/PCP signaling, and controls a wide variety of developmental events, such as organization of the epithelium29 and establishment of axonal projections.2,3 Even though Fzd3 guides some optical tracts, little evidence has demonstrated its specific role in regulating the morphologic development of certain retinal neurons. Here, to our knowledge we showed for the first time that targeted Fzd3 inactivation in retinal RBCs disrupts their soma patterning, perturbs their dendritic targeting and arborization, and impairs their synaptic connections with rod photoreceptors. These defects are functionally relevant as ERG recordings display a reduced light response.

The observed anomalies of planar regularity upon Fzd3 inactivation of RBCs suggest that Fzd3 might mediate their planar organization. This mediation by Fzd3 could work similarly to that by Fzd6, which shares redundant functions with Fzd3 and regulates the planar arrangement of hair follicles and associated structures.31,32 Moreover, the disturbed orientation of RBC somas observed in Fzd3 mutant retina...
abnormal morphogenesis contribute to a decreased light transmission from rods to RBCs. One possible reason for the decrease in dendritic numbers of RBCs is the decrease in dendritic targeting, which may result from different subcellular expressions of Frizzled3. In retina, the neurite maturation of a given cell in the plexiform layers is controlled by guidance cues from diverse sources through a variety of mechanisms including ligand-receptor signaling. Frizzled3 interacts with Wnts and induces a repulsive signal that promotes dendritic withdrawing and branching of RBCs in control OPL, dendrites of RBCs are significantly reduced, proximal to the ribbons. However, in mutant retina, the dendritic invaginations of RBCs were more frequently observed in mutant than in control retina, but some ectopic rod bipolar dendrites in the ONL still invaginate rod terminals (asterisk in B). (C) Density of rod ribbons decreases significantly in Isl1-Cre; Fzd3f/− retinas. DAPI (blue) is used as general nuclear stain. (D) Percentages of apposed rod ribbons (number of rod ribbons/total number of rod ribbons ×100%) in control and mutant retinas. Three animals of each genotype. *P < 0.05, **P < 0.01. (E, F) Electron micrographs of rod terminals (RT) in control (D) and Isl1-Cre; Fzd3f/− (E) OPL. Profiles of RTs are outlined with white dotted lines, and ribbons are marked “r.” Horizontal invaginations (H) are in register with ribbons like butterfly wings, while RBC invaginations (R) are surrounded by thick membranes. Scale bars: 5 μm (A, B); 200 nm (E, F). (G, H) Percentages of RT profiles with 2, 1 or 0 rod bipolar (G) or horizontal (H) cell invaginations for both genotypes. Number of profiles analyzed: 144 from 3 control animals, and 149 from 3 mutant animals. χ2, P < 0.001 in (G) and P = 0.15 in (H).

Our results and previous studies show that neither Fzd3 nor Isl1 is expressed in photoreceptors so the light response of photoreceptors could not be directly suppressed upon Fzd3 conditional knock-out. Therefore, the observed decrease in a-wave amplitude is probably due to reduced light transmission into the retina because Fzd3 is inactivated in certain tissues in the front of the eye (data not shown). Interestingly, our results suggest that the mutation of Fzd3 in postsynaptic RBCs affects the synaptic structure of the presynaptic rods. This interplay between pre- and postsynaptic partners must be further explored.

In conclusion, we demonstrated a novel role for Fzd3 in shaping the development of retinal RBCs. Fzd3 regulates their soma patterning, refines their dendritic maturation and promotes their connections with rods, thereby contributing to the light transmission from rods to RBCs and to the light response of RBCs. In addition to the role of Fzd3 in axonal guidance, our results reveal previously unsuspected functions in the maturation of the central nervous system. It will be intriguing to further explore the role of Fzd3 in the development and function of other retinal neurons, and to elucidate the underlying molecular and cellular mechanisms.

Acknowledgments

The authors thank Sylvia Evans for Isl1-Cre mice, Xin Duan and Fadel Tissir for critical comments, Shengyan Yang for ERG instruction, Mi Huang for experimental assistance, and Rukki Mirotznik from Mirotznik Editing Services for English editing.

Supported by Key project of Natural Science Foundation of Guangdong Province (2016A030311053, LZ), National Basic Research Program of China (973 Program, 2014CB542205, LZ), National Natural Science Foundation of China (31371101, 31070955, 81470656), Project of Internation as well as Hong Kong, Macao & Taiwan Science and Technology Cooperation Innovation Platform in Universities in Guangdong Province (2013gjh20002), 111 project (B14056), Science & Technology
References