Synaptic Plasticity in $CNGA3^{-/-}$ Mice: Cone Bipolar Cells React on the Missing Cone Input and Form Ectopic Synapses with Rods

Silke Haverkamp,¹ Stylianos Michalakis,² Ellen Claes,¹ Mathias W. Seeliger,³ Peter Humphries,⁴ Martin Biel,² and Andreas Feigenspan⁵

¹Department of Neuroanatomy, Max-Planck-Institute for Brain Research, D-60528 Frankfurt/Main, Germany, ²Department Pharmazie–Zentrum für Pharmaforschung, Ludwig Maximilians Universität, D-81377 Munich, Germany, ³Retinal Diagnostics Research Group, Department of Ophthalmology II, Eberhard-Karls University, D-72076 Tuebingen, Germany, ⁴Department of Genetics, Trinity College, Dublin 2, United Kingdom, and ⁵Department of Neurobiology, University of Oldenburg, D-26111 Oldenburg, Germany

In the mammalian retina, rods and cones connect to distinct sets of bipolar cells. Rods are presynaptic to a single type of rod bipolar cell, whereas cones connect to different types of cone bipolar cells. Synaptic rewiring between cone photoreceptor terminals and rod bipolar cell dendrites has been described as a general result of photoreceptor degeneration. To investigate whether cone bipolar cells also show synaptic plasticity in the absence of cone input, we studied the connectivity of cone bipolar cell dendrites in $CNGA3^{-/-}$ mice, a model with specific loss of cone photoreceptor function. Dendritic connections of ON and OFF cone bipolar cells were visualized using specific cell markers or by intracellular injection with fluorescent dyes. The results show that cone bipolar cells in $CNGA3^{-/-}$ mice form ectopic synapses with rods. In contrast, cone bipolar cells do not form ectopic synapses with rods in $CNGA3^{-/-} Rho^{-/-}$ mice, in which both types of photoreceptors are nonfunctional. In analogy with these results, we found that input-deprived rod bipolar cells form ectopic synapses with functional cones in $Rho^{-/-}$ mice but not with inoperable cones in the $CNGA3^{-/-} Rho^{-/-}$ mouse. Our data indicate that the formation of ectopic bipolar cell synapses in the outer plexiform layer requires a functional presynaptic photoreceptor.

Key words: retina; cones; rods; bipolar cells; dendritic plasticity; ectopic synaptogenesis

Introduction

The mammalian retina contains rod and cone photoreceptors, precisely tuned to respond to low (scotopic) and high (photopic) light levels. Rod and cone pathways are separated at their first synapse in the outer retina (Wässle, 2004). Rods connect to a single type of rod bipolar cell, whereas cones are presynaptic to at least nine different types of cone bipolar cells (Ghosh et al., 2004). OFF cone bipolar cells make flat contacts with cone pedicles and express a conventional sign conserving glutamate receptor of the AMPA or kainate subtype (DeVries, 2000). ON cone and rod bipolar cells make invaginating contacts close to the synaptic ribbons and express the sign-inverting glutamate receptor, metabotropic glutamate receptor type 6 (mGluR6) (Nakajima et al., 1993; Nomura et al., 1994; Vardi et al., 2000).

In the mouse retina, one type of OFF cone bipolar cell makes direct synaptic contacts with rods [B2 (Tsukamoto et al., 2001); this cell type is comparable with type 3 in Ghosh et al. (2004) and CB2 in Pignatelli and Strettoi (2004)]. Direct rod to OFF cone

bipolar cell connections have also been described in rat, cat, and rabbit retina (Hack et al., 1999; Fyk-Kolodziej et al., 2003; Li et al., 2004; Protti et al., 2005).

Connections between cones and rod bipolar cells have only exceptionally been reported in a normal retina (Dacheux and Raviola, 1986). However, cones make ectopic synapses with rod bipolar cells in diverse photoreceptor degeneration models [P347L pigs, *retinal degeneration* mice, Royal College of Surgeons (RCS) rats] (Peng et al., 2000, 2003) as well as in neural retina leucine zipper (Nrl) knock-out mice in which rods fail to form and only cones exist (Strettoi et al., 2004; Daniele et al., 2005).

Photoreceptors respond to light by the closure of a cyclic nucleotide-gated (CNG) cation channel, causing hyperpolarization and decrease of the synaptic glutamate release. To investigate whether cone bipolar cells form ectopic synapses in the absence of cone input, we studied the connectivity of cone bipolar cell dendrites in mice lacking $CNGA3$ (Biel et al., 1999), the A subunit of the cone CNG channel. It was demonstrated previously that these mice lack any cone-mediated photoresponses but have a normal rod photosystem (Biel et al., 1999).

Rhodopsin knock-out ($Rho^{-/-}$) mice, on the other hand, appear to have normal cone but no rod function, at least until postnatal week 6 (pw6) when cone degeneration is not yet substantial. These animals fail to form rod outer segments, leading to a sequence of primary rod and secondary cone photoreceptor loss

Received Oct. 19, 2005; revised March 31, 2006; accepted April 1, 2006.

This work was supported by Deutsche Forschungsgemeinschaft Grants SFB269/B4, Se837/4-1, and Se837/5-1. We thank B. Marshallay and G.-S. Nam for excellent technical assistance.

Correspondence should be addressed to Silke Haverkamp, Max-Planck-Institute for Brain Research, Deutschordenstrasse 46, D-60528 Frankfurt/Main, Germany. E-mail: Haverkamp@mpi-hfrankfurt.mpg.de.

DOI:10.1523/JNEUROSCI.4483-05.2006

Copyright © 2006 Society for Neuroscience 0270-6474/06/265248-08\$15.00/0

that is complete at ~3 months postnatally (Humphries et al., 1997; Jaissle et al., 2001)

We used these mice, as well as mice deficient for both CNGA3 and Rhodopsin ($CNGA3^{-/-}Rho^{-/-}$) (Claes et al., 2004), to test our hypothesis that the formation of ectopic bipolar cell synapses in the outer plexiform layer (OPL) requires a functional presynaptic photoreceptor.

Materials and Methods

Animals. The generation of $CNGA3^{-/-}$ mice and $Rho^{-/-}$ mice was described previously (Humphries et al., 1997; Biel et al., 1999). Double mutants were generated by cross-breeding $CNGA3^{-/-}$ with $Rho^{-/-}$ mice (Claes et al., 2004). The α -gustducin–green fluorescent protein (GUS–GFP) transgenic mouse line was kindly provided by R. Margolske (Mount Sinai Medical Center, New York, NY) (Huang et al., 2003).

The electron microscopic study included eight $CNGA3^{-/-}$ mice of different ages (postnatal week 3.2, 4.0, 5.0, 6.1, 6.6, and 7.6 and postnatal month 4.09 and 8.29). They were raised on a 129sv background and were compared with age-matched 129sv and C57BL/6 wild-type mice. Furthermore, we investigated the retinas of three $Rho^{-/-}$ mice (postnatal week 5.1, 6.2, and 7.2) and four $CNGA3^{-/-}Rho^{-/-}$ mice (postnatal week 3.3, 4.0, 5.0, and 6.0) by preembedding immunoelectron microscopy.

Tissue preparation. Mice were anesthetized deeply with halothane and decapitated. The procedures were approved by the local animal care committees and were in accordance with the law of animal experimentation issued by the German Government (Tierschutzgesetz). The eyes were enucleated, the anterior segments removed, and the posterior eyecups immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 30 min (LM) or 60 min (EM). After fixation, the retina was dissected from the eyecup and embedded in 2–4% agar. The agar block was mounted on a vibratome, and vertical sections of 60 μ m thickness were cut. For electron microscopy, the retina was cryoprotected in graded sucrose solutions (10, 20, and 30%) and frozen and thawed before vibratome sectioning. For retinal GUS–GFP whole mounts, the tissue was cryoprotected and frozen and thawed several times before applying the antibodies. Vibratome sections and whole mounts were processed free-floating.

Light microscopic immunocytochemistry. OFF cone bipolar cells were labeled with antibodies against the neurokinin-3 receptor (rabbit anti-NK3R; 1:500; kindly provided by R. Shigemoto, National Institute, Okazaki, Japan) and rod bipolar cells with antibodies against protein kinase C α (rabbit anti-PKC α ; 1:10,000; Sigma, St. Louis, MO). Synaptic ribbons were visualized with an antibody against the cytomatrix protein bassoon (mouse anti-bassoon; 1:5000; Stressgen Biotechnologies, Victoria, British Columbia, Canada) or with antibodies against the C-terminal binding protein 2 (mouse anti-CtBP2; 1:10,000; BD Transduction, Heidelberg, Germany). The GluR5 antibody (goat anti-GluR5N; 1:100; Santa Cruz Biotechnology, Heidelberg, Germany) has been used to label cone pedicles and ectopic synapses at rod spherules. The GFP fluorescence signal has been increased with rabbit anti-GFP antibodies (1:2000; Invitrogen, San Diego, CA).

Antibodies were diluted in PBS, pH 7.4, containing 5% Chemiblocker (Chemicon, Temecula, CA), 0.5% Triton X-100, and 0.05% sodium azide. Immunocytochemical labeling was performed using the indirect fluorescence method. Vibratome sections were incubated overnight in a mixture of primary antibodies, followed by incubation (1 h) in a mixture of the secondary antibodies, which were conjugated to either Alexa TM 488 (green fluorescence; Invitrogen) or to indocarbocyanin 3 (Cy3; red fluorescence; Dianova, Hamburg, Germany). Whole mounts were incubated for 2 d in the primary and for 2 h in the secondary antibody solution.

Confocal micrographs were taken using a Zeiss (Oberkochen, Germany) LSM5 Pascal confocal microscope equipped with an argon and a helium–neon laser. High-resolution scanning was performed with a 63 $\times$ /1.4 Plan-Apochromat objective (z-axis step size, 0.8 μ m). Brightness and contrast of the final images were adjusted using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

Preembedding immunoelectron microscopy. Vibratome sections were incubated for 4 d at 4°C in a primary antibody solution (anti-NK3R, 1:1000; anti-PKC α , 1:20,000) containing 3% normal goat serum (NGS), 1% bovine serum albumin, and 0.05% sodium azide in PBS. Thereafter, the sections were rinsed in PBS and immunolabeling was detected with a biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories, Burlingame, CA) and a peroxidase-based enzymatic detection system (Vectastain Elite ABC kit; Vector Laboratories). After rinses in PBS and in 0.05 M Tris–HCl, pH 7.6, the sections were reacted in 3,3'-diaminobenzidine (DAB; 0.05% in Tris–HCl) with 0.01% H₂O₂ for 5–10 min. Subsequently, the sections were rinsed in Tris–HCl and then in 0.1 M cacodylate buffer, pH 7.4, postfixed in 2.5% glutaraldehyde in cacodylate buffer for 2 h at 4°C, and washed in cacodylate buffer overnight at 4°C. After several washes in distilled water, the DAB reaction product was silver-intensified by incubating the sections in a solution containing 2.6% hexamethylene tetramine, 0.2% silver nitrate, and 0.2% disodium tetraborate for 10 min at 60°C. The sections were then rinsed in distilled water and treated for 2 min with gold chloride (0.05% in distilled water). Finally, the sections were rinsed in distilled water and incubated for 2 min in sodium thiosulfate (2.5% in distilled water). The sections were then postfixed with 0.5% OsO₄ in cacodylate buffer for 30 min, dehydrated in a graded series of ethanol (30–100%) followed by propylene oxide, and flat-embedded in Epon 812 (Serva, Heidelberg, Germany). Serial ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM10 electron microscope.

Quantitative analysis. To quantify the amount of flat contacts of NK3R-labeled OFF bipolar cell dendrites at the rod spherules, single ultrathin sections of three $CNGA3^{-/-}$ and three wild-type retinas were analyzed. Photoreceptor somas and synaptic terminals stack in multiple tiers in the mouse retina (Tsukamoto et al., 2001) (see Fig. 5). The densely packed somas form ~10 tiers; the underlying synaptic terminals form three to four tiers. The NK3R-labeled dendrites reach the innermost tier of synaptic terminals, where cone pedicles and rod spherules intermingle (see Fig. 2B,C). Because the dendrites mostly stop there and barely penetrate the remaining tiers of rod spherules, we counted only the rod spherules in this innermost tier.

We did not count the number of PKC α -labeled rod bipolar cell dendrites at cone pedicles in the $Rho^{-/-}$ mouse, because the penetration of the PKC α -antibody was not optimal and we only found labeling at the surface, not enough to find a sufficient number of examples for quantification.

Intracellular injection of ON cone bipolar cells. For the preparation of vertical sections, the retinas of C57BL/6 wild-type and $CNGA3^{-/-}$ mice were removed from the eyecup and embedded in 2% agarose in Ames medium (Sigma, Deisenhofen, Germany). Agar blocks were mounted on a vibratome (Leica, Nussloch, Germany) and cut into slices of 200 μ m thickness. After cutting, the slices were fixed in 4% paraformaldehyde for 10 min, washed in PB, and subsequently transferred to the stage of an upright microscope (Leica) for intracellular injections.

Intracellular injections were performed with sharp borosilicate microelectrodes (Hilgenberg, Malsfeld, Germany) filled with 10 mM Alexa Fluor 488 hydrazide (Invitrogen). Alexa 488 was injected iontophoretically (2–3 min, –1 nA) into the somata of morphologically identified bipolar cells using the current-clamp circuit of the EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). After injection, slices were postfixed in 4% paraformaldehyde for 20 min, washed in PB, and processed for immunocytochemistry as described below.

To block unspecific labeling, vertical sections were incubated in a solution containing 5% NGS and 0.3% Triton X-100 in PB for 1 h. Dendritic tips of ON cone bipolar cells were labeled with polyclonal antibodies against mGluR6 (rabbit anti-mGluR6; 1:500; Neuromics, Minneapolis, MN), and presynaptic ribbons were labeled with monoclonal antibodies against kinesin (mouse anti-kinesin II; 1:50; Babco, Richmond, CA).

Primary antibodies were diluted in 3% NGS and 0.3% Triton X-100 in PB and applied overnight at 4°C. After several washes in PB, secondary antibodies, dissolved in 1% NGS and 0.3% Triton X-100 in PB, were applied for 2 h at room temperature. Secondary antibodies were conjugated to Alexa Fluor 568 (Invitrogen) and Cy5 (Dianova). In all experi-

ments, sections were incubated in a mixture of primary antibodies, followed by a mixture of secondary antibodies.

Confocal micrographs of fluorescent specimens were taken with a Leica TCS SL confocal microscope equipped with an argon and a helium–neon laser. Scanning was performed with a 40×/1.25 Plan-Apochromat and a 63×/1.32 Plan-Apochromat objective at a resolution of 1024 × 1024 pixels (z-axis step size, 0.5 μm). Different wavelength scans were performed sequentially to rule out cross-talk between red, green, and blue channels.

Results

The retina of *CNGA3*^{−/−} mice displays a normal general morphology and lamination (Biel et al., 1999). Only a degeneration of cones is evident over a time course of several months (Michalakakis et al., 2005). To investigate the connectivity of OFF cone bipolar cells in the *CNGA3*^{−/−} retina, we used a specific antibody directed against the neurokinin-3 receptor (Ding et al., 1996). NK3R labels type 1 and type 2 bipolar cells in the mouse retina but not the rod contacting type 3 cell, which can be labeled by antibodies against the calcium-binding protein 5 (CaB5) (Haverkamp et al., 2003; Ghosh et al., 2004).

NK3R immunoreactivity was present in bipolar cells with axons terminating in the outermost part of the inner plexiform layer (IPL) (Fig. 1A). The processes in the inner IPL in Figure 1A belong to NK3R-immunoreactive amacrine cells (Haverkamp et al., 2003). There was no difference between wild-type and *CNGA3*^{−/−} retinas except for small NK3R-labeled dendrites extending into the outer part of the outer plexiform layer (Fig. 1A, arrows). To determine potential contact sites of these dendrites, we double labeled vibratome sections with antibodies against NK3R and the cytomatrix protein bassoon. Bassoon labels the photoreceptor ribbons in both cone pedicles and rod spherules (Brandstätter et al., 1999). They show a horseshoe-shaped structure in rod spherules (Fig. 1B–F), whereas they are clustered in a row in cone pedicles (Fig. 1B, frame). We found in all *CNGA3*^{−/−} mice tested (10 mice between pw3.2 and postnatal month 12) clear examples of dendrites extending into the outer OPL (Fig. 1C–F). In contrast, this was almost never the case in age-matched wild-type mice (Fig. 1B). Figure 1C–F shows examples in which the tips of outgrowing bipolar cell dendrites are in close relationship to bassoon-labeled ribbons, indicating that they are in contact with rod spherules.

OFF cone bipolar cells in the *CNGA3*^{−/−} mouse contact rod spherules

At the ultrastructural level, ON and OFF bipolar cells can be clearly distinguished with respect to their contacts with cone pedicles. OFF cone bipolar cells make flat contacts at the cone pedicle base, whereas ON cone bipolar cells make invaginating contacts at the ribbons (Kolb and Nelson, 1995). In the case of the NK3R-immunolabeled OFF cone bipolar cells, we found many examples of flat contacts at the cone pedicle base of *CNGA3*^{−/−} mice (Fig. 2A). In addition, labeled dendrites contacting rod spherules were seen frequently (Fig. 2B,C). To demonstrate that

Figure 1. Confocal images of NK3R-immunoreactive bipolar cells from vertical sections of *CNGA3*^{−/−} and wild-type retinas. **A**, *CNGA3*^{−/−} mouse retina at pw8. The retinal layers are indicated. INL, Inner nuclear layer; GCL, ganglion cell layer; pm, postnatal month. NK3R immunoreactivity is visible in bipolar cells with axons terminating in the outer IPL. The arrows indicate terminal dendrites, which extend into the outer part of the OPL. **B**, High-power image of NK3R (green) and bassoon (red) immunoreactivity in the OPL of a wild-type mouse. Bassoon labels the photoreceptor ribbons in rod spherules, where they show a horseshoe-shaped structure, and in cone pedicles, where they are clustered in a row (frame). **C–F**, NK3R (green) and bassoon (red) immunoreactivity in the OPL of *CNGA3*^{−/−} mice at the ages indicated. In all cases, NK3R-labeled bipolar cell dendrites extending into the outer part of the OPL can be found. The arrows in **C**, **E**, and **F** mark examples in which the tips of single outgrowing bipolar cell dendrites are in close relationship to bassoon-labeled ribbons, indicating that they are in contact with a rod spherule. The circle in **D** encloses an example in which three outgrowing dendrites apparently make contact with three rod spherules. Scale bar: (in **B**) **A**, 20 μm; **B–F**, 10 μm.

these contacts were flat, noninvaginating synaptic contacts, we performed serial sectioning and followed the labeled dendrites in 8–10 sections (Fig. 2C,D). Altogether, eight *CNGA3*^{−/−} mice of different age have been investigated, and in all cases, numerous ectopic synapses at rod spherules have been found. In contrast, almost no ectopic contacts were found in wild-type mouse retinas. For quantification, we counted the number of flat contacts at rod spherules from single sections of three *CNGA3*^{−/−} and three wild-type animals (see Materials and Methods). In the case of the *CNGA3*^{−/−} tissue, 207 of 1282 rod spherules made ectopic synapses with NK3R-labeled dendrites (16%); in the case of the wild-type tissue, only 6 of 867 rod spherules were possibly contacted by NK3R-labeled dendrites (0.67%).

We now asked whether the presence of these ectopic synapses depends on the activity of rod photoreceptors. To this end, we looked for ectopic synapses in retinas of *CNGA3*^{−/−} *Rho*^{−/−} mice, in which both cones and rods are nonfunctional. In all double-mutant mice, we investigated (four mice between postnatal week 3.3 and 6.6), the NK3R-labeled OFF cone bipolar cells did not contact rod spherules. Apparently, cone bipolar cells form ectopic synapses with functional rods but not with rods sending no light-driven output onto second-order neurons, suggesting that the formation of ectopic synapses in the OPL requires a functional presynaptic photoreceptor.

GluR5 expression at ectopic synapses in the *CNGA3*^{−/−} mouse

Connections between rods and OFF cone bipolar cells have been described in rodent and rabbit retina, and the synaptic nature of these contacts was confirmed by the presence of the GluR1 and GluR2 receptors (Hack et al., 1999; Li et al., 2004). To proof the functional relevance of the ectopic bipolar cell synapses in the

Figure 2. Electron micrographs of a 7-week-old *CNGA3*^{−/−} mouse retina immunostained with anti-NK3R antibody. **A**, Cone pedicle (cp) containing one presynaptic ribbon (arrowhead) and two lateral postsynaptic horizontal cell elements (h). Two NK3R-immunoreactive dendrites make flat contacts at the cone pedicle base (arrows). **B**, Labeling is seen in a process making a flat contact at the cone pedicle base (cp; arrow) and another one onto a rod spherule (rs; arrow). **C**, Row of rod spherules with potential contacts to NK3R-immunoreactive dendrites (arrows). **D**, Higher magnification of the second rod spherule (RS) shown in **C**, but two sections further. Now the flat contact is clearly visible (arrow). Scale bar: (in **A**) **A**, **B**, **D**, 1 μ m; **C**, 1.43 μ m.

Figure 3. Confocal images of vertical sections through the outer plexiform layer of wild-type and *CNGA3*^{−/−} retina double labeled for GluR5 (red) and bassoon (green). **A**, Wild-type retina with strong punctate immunofluorescence of GluR5 at three cone pedicles. **B**, **C**, GluR5 immunoreactivity in the OPL of *CNGA3*^{−/−} mice. Four examples of red GluR5 puncta (arrows in **B**, circle in **C**) at the locations where OFF bipolar cell dendrites contact rod spherules (compare with Fig. 1C–F). Scale bar: (in **C**) 5 μ m.

CNGA3^{−/−} retina, we studied the localization of the glutamate receptor subunit GluR5. GluR5 has been shown to be expressed by OFF cone bipolar cell dendrites at their contacts with cone pedicles (Haverkamp et al., 2001); however, it was not expressed at the dendritic tips of NK3R-immunoreactive bipolar cells (Haverkamp et al., 2003). We double labeled sections for GluR5 and bassoon and found a clear difference between wild-type and

CNGA3^{−/−} retina (Fig. 3). In the wild-type retina, GluR5 was exclusively aggregated in postsynaptic clusters at the cone pedicle base (Fig. 3A) [Haverkamp et al. (2003), their Fig. 8B–D]. In contrast, in the *CNGA3*^{−/−} retina, clear examples of GluR5 puncta were found at the positions where OFF bipolar cell dendrites contact rod spherules (compare Figs. 3B, C, 1C–F). The localization of GluR5 puncta at ectopic contact sides strongly indicates that the rod–cone bipolar cell synapses are functional. In addition, it shows that OFF cone bipolar cells other than NK3R-positive cells are engaged in ectopic synapses with rod spherules.

Plasticity of ON cone bipolar cells in the *CNGA3*^{−/−} mouse

Direct synaptic contacts of ON cone bipolar cells with rods have not been described so far. This could be because of a technical problem, because immunocytochemical markers for specific ON cone bipolar cell types are not available, and chances of analyzing all ON types in a sufficient number by intracellular dye injection are low. Therefore, we concentrated on a transgenic mouse line with strong GFP expression in a single type of ON cone bipolar cell. GFP expression was also present in rod bipolar cells but was significantly weaker than in ON cone bipolar cells (GUS–GFP mouse) (Huang et al., 2003). The labeled ON cone bipolar cells resemble those termed type 7 by Ghosh et al. (2004). Figure 4 shows the dendritic trees of two type 7 cells double labeled with antibodies against GluR5 and GFP. It can be seen that all the bipolar dendrites terminate at clusters of GluR5 puncta, which represent individual cone pedicles; none of the dendrites terminates at a rod spherule (Fig. 4B, D). This was the case for all type 7 cells we examined ($n = 36$). Their dendrites contacted all the cone pedicles (between 6 and 10 per cell) within their dendritic field. None of the cells made a potential contact with a rod spherule.

Because ON cone bipolar cells resembling type 7 in the GUS–GFP mouse are exclusively connected to cones, we injected this bipolar cell type in the *CNGA3*^{−/−} mouse retina. In addition, we also studied the presynaptic contacts of type 5 ON cone bipolar cells. Figure 5A shows a projection of a type 5 ON cone bipolar cell with its axon terminating in stratum 3 of the IPL. When double-labeled with antibodies against kinesin and mGluR6, the presynaptic and postsynaptic components of the photoreceptor to bipolar cell synapse can be visualized. In single optical sections, cone pedicles can be easily identified according to the row-like clustering of the synaptic ribbon marker kinesin. As expected, all type 5 cells examined ($n = 6$) contacted cone pedicles (Fig. 5B). However, this cell type also extended dendrites into close proximity of rod spherules, suggesting potential synaptic contact sites (Fig. 5C). We observed putative ectopic synapses between type 5 ON cone bipolar cells and rod spherules in all cells injected ($n = 6$).

Injection and double-labeling of type 7 ON cone bipolar cells in the *CNGA3*^{−/−} mouse retina (Fig. 5D) also provided evidence for ectopic contacts with rod spherules. Again, the clustering of

Figure 4. ON cone bipolar cells (type 7) in the GUS–GFP mouse retina. **A, C**, Horizontal view of two GFP-labeled bipolar cells in a whole-mounted retina (confocal stack from the inner nuclear layer to the OPL; dendritic trees were constructed by collapsing the stacks into a single plane). **B, D**, Same cells as above double labeled for GFP and for the glutamate receptor subunit GluR5. The clusters of GluR5 puncta represent individual cone pedicles (circles). The dendrites of the type 7 bipolar cell in **B** contact eight cone pedicles; the cell in **D** contacts nine cone pedicles. Scale bar: (in **B**) 10 μ m.

kinesin and mGluR6 immunoreactivity is indicative for cone pedicles, and dendrites of type 7 ON cone bipolar cells clearly make synapses with cones (Fig. 5E). However, individual contact sites (in contrast to the cone pedicle clusters) suggest also putative synapses with rod spherules (Fig. 5F). Dendrites extending from type 7 ON cone bipolar cells to rod spherules were observed in six of seven injected cells (86%).

Cone contacts of rod bipolar cells in the *Rho*^{−/−} mouse

We have shown so far that cone bipolar cells react to the loss of light-driven output by establishing synaptic contacts to rod spherules. We now asked whether rod bipolar cells do also show a corresponding behavior when the rod photoreceptor output is missing. It has been shown that rod bipolar cells form ectopic synapses with cones in diverse photoreceptor degeneration models (Peng et al., 2000, 2003), and one would expect to find the same in the *Rho*^{−/−} mouse.

To analyze their synaptic contacts at the light microscopic level, rod bipolar cells were labeled with antibodies against PKC α (Haverkamp and Wässle, 2000) and photoreceptor ribbon synapses with antibodies against CtBP2 (tom Dieck et al., 2005) (Fig. 6). We did not find any PKC α -labeled dendrites closely associated with cone pedicles in the wild-type retina (Fig. 6B), and we found hardly any examples of rod bipolar cell dendrites making potential contact with cone pedicles in the *Rho*^{−/−} retina (Fig. 6C).

Analyzing the contacts of rod bipolar cell dendrites at the ultrastructural level was much more promising. We found a number of examples of PKC α -labeled dendrites at cone pedicles in the *Rho*^{−/−} mouse (Fig. 7). In the wild-type retina, rod bipolar cells make invaginating synaptic connections with rod spherules (Fig. 7A) but not with cone pedicles (Peng et al., 2000). In the *Rho*^{−/−} mouse, most of the rod bipolar cell dendrites, which were followed through a series of sections at the cone pedicle base, made invaginating synaptic contact (Fig. 7B–E). This result is in contrast to the findings in rhodopsin transgenic pigs and RCS rats, in which nearly all of the ectopic cone–rod bipolar cell synapses had flat, noninvaginating characteristics (Peng et al., 2000, 2003).

When looking for ectopic rod bipolar cell synapses in the

CNGA3^{−/−}*Rho*^{−/−} mouse, the success rate, again, was extremely low. We found only one positive example in three animals, indicating that rod bipolar cells form ectopic synapses with functional cones but not with those lacking cone function.

Discussion

In the wild-type mouse retina, most of the cone bipolar cells do not form connections with rods. We analyzed the dendritic connections made by cone bipolar cells in the retina of *CNGA3*-deficient mice, in which the cone light input is specifically switched off by genetic deletion of the A subunit of the cone CNG channel. Interestingly, we found that most of the cone bipolar cells in these mice show synaptic plasticity and form ectopic synapses with rods.

The demonstration of ectopic synapses between rod bipolar cells and cones in the OPL of RCS rats has been interpreted as a process of synaptic rewiring during retinal degeneration (Peng et al., 2003). Our results demonstrate that also cone bipolar cell dendrites have the capability to make alternative connections when the preferred contacts are out of function. Hence, the rules that govern synaptic partnering between rods and rod bipolar cells and between cones and cone bipolar cells are not absolute.

The molecular events that mediate the formation of normal rod and cone synapses during retinal development are poorly understood; however, an inherent molecular flexibility for forming synaptic connections may provide an adaptive advantage for the visual system. Molecular flexibility in forming synaptic contacts at the photoreceptor terminals have been explored in retinas in which the cones are genetically eliminated (Soucy et al., 1998) or in retinas in which the rods fail to form and all photoreceptors are cone-like (*Nrl*^{−/−} mouse) (Strettoi et al., 2004; Daniele et al., 2005). The direct connections between rods and cone bipolar cells in the “coneless” mouse can be interpreted as a reaction caused by the genetic manipulation, such that cone bipolar cells search for and make inappropriate contacts with rods in the absence of the normal synaptic target. The same holds true for the *Nrl*^{−/−} mouse, in which rod bipolar cells form synaptic connections with cone-like cells that presumably were supposed to further develop into rods.

Although a large number of cones in *CNGA3*^{−/−} mice degenerate in the first postnatal months, the ultrastructure of the remaining ones appears normal (Michalakakis et al., 2005). OFF cone bipolar cells make the usual flat contacts at the cone pedicle base (Fig. 2A,B); ON cone bipolar cells and horizontal cells make invaginating contacts at the ribbon synapses [Michalakakis et al. (2005), their Fig. 7]. In this study, we found several clear examples of OFF cone bipolar cells making flat contacts with rod spherules. ON cone bipolar cells also made putative contact with rod spherules, but EM reconstruction will be necessary to show whether these contacts are flat or invaginating ones. Most of the ectopic rod bipolar cell contacts we found in the *Rho*^{−/−} mouse were invaginating contacts, which shows that bipolar cells keep their synaptic features at ectopic synapses in the *CNGA3*^{−/−} and *Rho*^{−/−} mouse.

We have no explanation for the contrary findings of Peng and his colleagues (2000, 2003). They showed in different retinal degeneration models that most of the ectopic cone–rod bipolar cell synapses had flat, noninvaginating characteristics.

Interestingly, we found no evidence of cone bipolar cell sprouting into the ONL (NK3R immunostaining in *CNGA3*^{−/−} and *CNGA3*^{−/−}*Rho*^{−/−} mice; data not shown), whereas rod bipolar cell and horizontal cell sprouting has been demonstrated in several animal models. Horizontal and rod bipolar cell processes

Figure 5. Confocal images of type 5 and type 7 ON cone bipolar cells in the *CNGA3*^{−/−} mouse retina. **A**, Overall morphology of a type 5 ON cone bipolar cell as visualized by projecting a z-stack of optical images and superimposed onto the differential interference contrast image of the corresponding vertical section. The horizontal lines indicate strata S1 through S5 in the IPL. **B**, Double-labeling immunocytochemistry with antibodies against mGluR6 (red) and kinesin (blue) reveals a contact site with a cone pedicle (CP; circle). **C**, In a different optical section, a putative synapse with a rod spherule (RS; circle) is visible. **D**, Overall morphology of a type 7 ON cone bipolar cell. **E**, In a single optical section, a dendrite of this bipolar cell makes synaptic contacts with two cone pedicles (circles). **F**, In a different section, three contact sites with rod spherules (circles) are clearly visible. Double-labeling immunocytochemistry same as in **B** and **C**. Scale bars: (in **A**) **A**, **D**, 20 μ m; **B**, **C**, 12 μ m; **E**, **F**, 10 μ m.

Figure 6. Vertical sections of *Rho*^{−/−} and wild-type retinas double labeled for PKC α (green) and CtBP2 (red). **A**, Low-power image of a *Rho*^{−/−} mouse retina at pw5. PKC α immunoreactivity is visible in rod bipolar cells with axons terminating deep within the IPL. Antibodies against CtBP2 label all photoreceptor ribbons in the OPL and all bipolar cell ribbons in the IPL. INL, Inner nuclear layer. **B**, High-power image of PKC α and CtBP2 immunoreactivity in the OPL of a wild-type (wt) mouse. Frames indicate two cone pedicles. PKC-labeled dendrites are in close proximity to CtBP2-labeled ribbons in rod spherules (horseshoe-shaped structure) but not to the labeled ribbons of the two cone pedicles. **C**, **D**, OPL of *Rho*^{−/−} mouse retina at pw5. Two examples of PKC-labeled dendrites with potential contact to cone pedicles (arrows) are shown. Scale bar: (in **D**) **A**, 24 μ m; **B**–**D**, 10 μ m.

grow into the ONL and form ectopic synapses with photoreceptors as a result of photoreceptor degeneration (Claes et al., 2004), after retinal detachment (Lewis et al., 1998), or in mutant mice deficient for bassoon (Dick et al., 2003) or CaBP4 (Haeseleer et al., 2004), which are important proteins for ribbon synapse formation or transmitter release. The molecular mechanisms mediating neurite outgrowth and the formation of ectopic synapses in these diverse animal models are unknown.

We found ectopic bipolar synapses in *CNGA3*^{−/−} and *Rho*^{−/−} single knock-out mice but not in *CNGA3*^{−/−}*Rho*^{−/−} double knock-out mice, which clearly shows that the formation of ectopic synapses between rod and cone bipolar cells requires functional rods and between cone and rod bipolar cells requires functional cones. Obviously, heterologous gap junctions between rods and cones (Smith et al., 1986) cannot substitute for the endogenous light-driven input mediated by CNG channels or activation of rhodopsin. This is in line with the observed degeneration of rods and cones in the respective transgenic models. In addition, we found no altered expression of connexin36 (Cx36) in transgenic animals when compared with wild-type mice (data not shown). Therefore, it seems unlikely that upregulation of Cx36 would compensate for defects in the visual transduction cascade. It is possible that the lateral spread of the electrical signal from the network of functional rods into the inoperable cone system (and vice versa) is not sufficient, because the signal is too small to mimic by itself the endogenous photoreceptor response. Although it seems reasonable to assume, we currently do not know whether or not Cx36-mediated gap junctions are functional in the transgenic animals. Because gap junctional channels respond in a very sensitive manner to changes in the concentration of various intracellular metabolites, the entire loss of the signal transduction cascade could also have profound functional implications for the electrical coupling between rods and cones.

The expression of GluR5 at locations where OFF bipolar cells contact rod spherules (Fig. 3B,C) indicates that the ectopic rod–cone bipolar cell synapses are functional. If this is the case, the rods could provide input to both the rod-mediated and cone-mediated signaling pathways. A study of the functional properties of the ectopic synapses will require a detailed physiological analysis at the cellular level.

Three different pathways responsible for the transmission of rod signals have been postulated (Völgyi et al., 2004) (for review, see Wässle, 2004), and the gap junction protein Cx36 is essential for two of them (Deans et al., 2002). In *Cx36*^{−/−}

retinas, the coupling between AII amacrine and ON cone bipolar cells (primary rod pathway with the highest sensitivity) and between rods and cones (secondary rod pathway with intermediate sensitivity) is lost (Güldenagel et al., 2001). Responses of low-intermediate sensitivity OFF ganglion cells survive in the *Cx36*^{−/−} mouse retina (Völgyi et al., 2004), and they are carried by the tertiary pathway (direct contact of OFF bipolar cells with rods). Although the rod-mediated electroretinogram of

CNGA3^{-/-} mice shows no anomalous features (Biel et al., 1999), the generation of *CNGA3*^{-/-}*Cx36*^{-/-} double knock-out mice combined with single unit (Deans et al., 2002) or multineuron (Meister et al., 1994) recordings might be a way to study the functional properties of the ectopic rod–cone bipolar cell synapses in more detail. Given that functional ectopic synapses between ON cone bipolar cells and rods do form in *CNGA3*^{-/-}*Cx36*^{-/-} mice, we would expect to find low-intermediate sensitivity ON ganglion cells, which do not exist in the normal ON system (Völgyi et al., 2004). Furthermore, the data of multielectrode recordings from coneless mice strongly suggest the existence of functional rod–cone bipolar cell contacts (Soucy et al., 1998). In wild-type retina, the APB-resistant OFF pathway relies mainly on electrical coupling between rods and cones, whereas in the coneless mouse retina, the APB-resistant responses can be explained if rods connect directly to OFF bipolar cells. The fact that all ganglion cells with OFF responses were APB resistant indicates that not only the type 3 OFF bipolar cell makes functional synapses with rods (Tsukamoto et al., 2001) in the coneless mouse, but also the other OFF bipolar cell types. This would be comparable with our model in which the cone bipolar cells react after the missing cone input and form ectopic and most likely functional synapses with rods.

References

- Biel M, Seeliger M, Pfeifer A, Kohler K, Gerstner A, Ludwig A, Jaissle G, Fauser S, Zrenner E, Hofmann F (1999) Selective loss of cone function in mice lacking the cyclic nucleotide-gated channel CNG3. *Proc Natl Acad Sci USA* 96:7553–7557.
- Brandstätter JH, Fletcher EL, Garner CC, Gundelfinger ED, Wässle H (1999) Differential expression of the presynaptic cytomatrix protein bassoon among ribbon synapses in the mammalian retina. *Eur J Neurosci* 11:3683–3693.
- Claes E, Seeliger M, Michalakakis S, Biel M, Humphries P, Haverkamp S (2004) Morphological characterization of the retina of the *CNGA3*^{-/-}*Rho*^{-/-} mutant mouse lacking functional cones and rods. *Invest Ophthalmol Vis Sci* 45:2039–2048.
- Dacheux RF, Raviola E (1986) The rod pathway in the rabbit retina: a depolarizing bipolar and amacrine cell. *J Neurosci* 6:331–345.
- Daniele LL, Lillo C, Lyubarsky AL, Nikonov SS, Philp N, Mears AJ, Swaroop A, Williams DS, Pugh Jr EN (2005) Cone-like morphological, molecular, and electrophysiological features of the photoreceptors of the *Nrl* knockout mouse. *Invest Ophthalmol Vis Sci* 46:2156–2167.
- Deans MR, Völgyi B, Goodenough DA, Bloomfield SA, Paul DL (2002) Connexin36 is essential for transmission of rod-mediated visual signals in the mammalian retina. *Neuron* 36:703–712.
- DeVries SH (2000) Bipolar cells use kainate and AMPA receptors to filter visual information into separate channels. *Neuron* 28:847–856.
- Dick O, tom Dieck S, Altmann WD, Ammermüller J, Weiler R, Garner CC, Gundelfinger ED, Brandstätter JH (2003) The presynaptic active zone protein bassoon is essential for photoreceptor ribbon synapse formation in the retina. *Neuron* 37:775–786.
- Ding YQ, Shigemoto R, Takada M, Ohishi H, Nakanishi S, Mizuno N (1996) Localization of the neuromedin K receptor (NK3) in the central nervous system of the rat. *J Comp Neurol* 364:290–310.
- Fyk-Kolodziej B, Qin P, Pourcho RG (2003) Identification of a cone bipolar cell in cat retina which has input from both rod and cone photoreceptors. *J Comp Neurol* 464:104–113.
- Ghosh KK, Bujan S, Haverkamp S, Feigenspan A, Wässle H (2004) Types of bipolar cells in the mouse retina. *J Comp Neurol* 469:70–82.
- Güldenagel M, Ammermüller J, Feigenspan A, Teubner B, Degen J, Sohl G, Willecke K, Weiler R (2001) Visual transmission deficits in mice with targeted disruption of the gap junction gene connexin36. *J Neurosci* 21:6036–6044.
- Hack I, Peichl L, Brandstätter JH (1999) An alternative pathway for rod signals in the rodent retina: rod photoreceptors, cone bipolar cells, and the localization of glutamate receptors. *Proc Natl Acad Sci USA* 96:14130–14135.
- Haeseleer F, Imanishi Y, Maeda T, Possin DE, Maeda A, Lee A, Rieke F, Palczewski K (2004) Essential role of Ca²⁺-binding protein 4, a Cav1.4 channel regulator, in photoreceptor synaptic function. *Nat Neurosci* 7:1079–1087.
- Haverkamp S, Wässle H (2000) Immunocytochemical analysis of the mouse retina. *J Comp Neurol* 424:1–23.
- Haverkamp S, Grünert U, Wässle H (2001) Localization of kainate receptors at the cone pedicles of the primate retina. *J Comp Neurol* 436:471–486.
- Haverkamp S, Ghosh KK, Hirano AA, Wässle H (2003) Immunocytochemical description of five bipolar cell types of the mouse retina. *J Comp Neurol* 455:463–476.
- Huang L, Max M, Margolske RF, Su H, Masland RH, Euler T (2003) G protein subunit G gamma 13 is coexpressed with G alpha o, G beta 3, and G beta 4 in retinal ON bipolar cells. *J Comp Neurol* 455:1–10.
- Humphries MM, Rancourt D, Farrar GJ, Kenna P, Hazel M, Bush RA, Sieving PA, Sheils DM, McNally N, Creighton P, Erven A, Boros A, Gulya K, Capocchi MR, Humphries P (1997) Retinopathy induced in mice by targeted disruption of the rhodopsin gene. *Nat Genet* 15:216–219.
- Jaissle GB, May CA, Reinhard J, Kohler K, Fauser S, Lutjen-Drecoll E, Zrenner E, Seeliger MW (2001) Evaluation of the rhodopsin knockout mouse as a model of pure cone function. *Invest Ophthalmol Vis Sci* 42:506–513.
- Kolb H, Nelson R (1995) The organization of photoreceptor to bipolar cell synapses in the outer plexiform layer. In: *Neurobiology and clinical aspects of the outer retina* (Djamgoz MBA, Archer SN, Vallerger S, eds), pp 273–296. London: Chapman and Hall.
- Lewis GP, Linberg KA, Fisher SK (1998) Neurite outgrowth from bipolar

Figure 7. Electron micrographs of PKC α immunoreactivity in the outer plexiform layer of a 5-week-old *Rho*^{-/-} mouse retina. Synaptic ribbons are marked by arrowheads and horizontal cell processes by “h.” **A**, Rod spherule (rs) with a presynaptic ribbon, two lateral horizontal cell processes, and a PKC α -labeled invaginating rod bipolar cell dendrite. **B**, **C**, Section numbers 1 and 6 of a series of ultrathin sections showing a cone pedicle (cp) with a heavily stained dendrite making flat contact in **B** (arrow) and invaginating contact in **C** (asterisk). **D**, **E**, Two additional examples of PKC α -immunoreactive dendritic tips making invaginating synaptic contact with the cone pedicle. Scale bar: (in **D**) 1 μ m.

- and horizontal cells after experimental retinal detachment. *Invest Ophthalmol Vis Sci* 39:424–434.
- Li W, Keung JW, Massey SC (2004) Direct synaptic connections between rods and OFF cone bipolar cells in the rabbit retina. *J Comp Neurol* 474:1–12.
- Meister M, Pine J, Baylor DA (1994) Multi-neuronal signals from the retina: acquisition and analysis. *J Neurosci Methods* 51:95–106.
- Michalakis S, Geiger H, Haverkamp S, Hofmann F, Gerstner A, Biel M (2005) Impaired opsin targeting and cone photoreceptor migration in the retina of mice lacking the cyclic nucleotide-gated channel CNGA3. *Invest Ophthalmol Vis Sci* 46:1516–1524.
- Nakajima Y, Iwakabe H, Akazawa C, Nawa H, Shigemoto R, Mizuno N, Nakanishi S (1993) Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphonobutyrate. *J Biol Chem* 268:11868–11873.
- Nomura A, Shigemoto R, Nakamura Y, Okamoto N, Mizuno N, Nakanishi S (1994) Developmentally regulated postsynaptic localization of a metabotropic glutamate receptor in rat rod bipolar cells. *Cell* 77:361–369.
- Peng YW, Hao Y, Petters RM, Wong F (2000) Ectopic synaptogenesis in the mammalian retina caused by rod photoreceptor-specific mutations. *Nat Neurosci* 3:1121–1127.
- Peng YW, Senda T, Hao Y, Matsuno K, Wong F (2003) Ectopic synaptogenesis during retinal degeneration in the royal college of surgeons rat. *Neuroscience* 119:813–820.
- Pignatelli V, Strettoi E (2004) Bipolar cells of the mouse retina: a gene gun, morphological study. *J Comp Neurol* 476:254–266.
- Protti DA, Flores-Herr N, Li W, Massey SC, Wässle H (2005) Light signaling in scotopic conditions in the rabbit, mouse and rat retina: a physiological and anatomical study. *J Neurophysiol* 93:3479–3488.
- Smith RG, Freed MA, Sterling P (1986) Microcircuitry of the dark-adapted cat retina: functional architecture of the rod–cone network. *J Neurosci* 6:3505–3517.
- Soucy E, Wang Y, Nirenberg S, Nathans J, Meister M (1998) A novel signaling pathway from rod photoreceptors to ganglion cells in mammalian retina. *Neuron* 21:481–493.
- Strettoi E, Mears AJ, Swaroop A (2004) Recruitment of the rod pathway by cones in the absence of rods. *J Neurosci* 24:7576–7582.
- tom Dieck S, Altrock WD, Kessels MM, Qualmann B, Regus H, Brauner D, Fejtova A, Bracko O, Gundelfinger ED, Brandstätter JH (2005) Molecular dissection of the photoreceptor ribbon synapse: physical interaction of Bassoon and RIBEYE is essential for the assembly of the ribbon complex. *J Cell Biol* 168:825–836.
- Tsukamoto Y, Morigiwa K, Ueda M, Sterling P (2001) Microcircuits for night vision in mouse retina. *J Neurosci* 21:8616–8623.
- Vardi N, Duvoisin R, Wu G, Sterling P (2000) Localization of mGluR6 to dendrites of ON bipolar cells in primate retina. *J Comp Neurol* 423:402–412.
- Völgyi B, Deans MR, Paul DL, Bloomfield SA (2004) Convergence and segregation of the multiple rod pathways in mammalian retina. *J Neurosci* 24:11182–11192.
- Wässle H (2004) Parallel processing in the mammalian retina. *Nat Rev Neurosci* 5:747–757.