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Rod Bipolar Cells and Horizontal Cells Form Displaced Synaptic Contacts With Rods in the Outer Nuclear Layer of the *nob2* Retina

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Abstract

The *nob2* mouse carries a null mutation in the *Cacna1f* gene, which encodes the pore-forming subunit of the L-type calcium channel, $Ca_v1.4$. The loss of the electroretinogram b-wave in these mice suggests a severe reduction in transmission between photoreceptors and second-order neurons in the retina and supports a central role for the $Ca_v1.4$ calcium channel at photoreceptor ribbon synapses, to which it has been localized. Here we show that the loss of $Ca_v1.4$ leads to the aberrant outgrowth of rod bipolar cell dendrites and horizontal cell processes into the outer nuclear layer (ONL) of the *nob2* retina and to the formation of ectopic synaptic contacts with rod photoreceptors in the ONL. Ectopic contacts are predominantly between rods and rod bipolar cells, with horizontal cell processes also present at some sites. Ectopic contacts contain apposed pre- and postsynaptic specializations, albeit with malformed synaptic ribbons. Cone photoreceptor terminals do not participate in ectopic contacts in the ONL. During retinal development, ectopic contacts appear in the days after eye opening, appearing progressively farther into the ONL at later postnatal stages. Ectopic contacts develop at the tips of rod bipolar cell dendrites and are less frequently associated with the tips of horizontal cell processes, consistent with the adult phenotype. The relative occurrence of pre- and postsynaptic markers in the ONL during development suggests a mechanism for the formation of ectopic synaptic contacts that is driven by the retraction of rod photoreceptor terminals and neurite outgrowth by rod bipolar cell dendrites.

Indexing terms

bipolar cell; horizontal cell; rod retraction; second-order neuron outgrowth

L-type voltage-dependent calcium channels, including $Ca_v1.4$ and the closely related $Ca_v1.3$, regulate calcium entry into the synaptic terminals of retinal photoreceptors (Yagi and Macleish, 1994; Schmitz and Witkovsky, 1997; Taylor and Morgans, 1998; Cia et al., 2005). These calcium channels are distinct from the N- and P/Q-type calcium channels that support fast neurotransmitter release in spiking neurons. Instead, at the photoreceptor dark resting potential of -40 mV (Schneeweis and Schnapf, 1999), the L-type calcium channels

are tonically activated, supporting continuous glutamate release from photoreceptors. On exposure to light, photoreceptors progressively hyperpolarize, closing calcium channels and reducing glutamate release. The properties of the photoreceptor L-type channels—low-voltage activation, with little voltage dependent inactivation and rapid activation and deactivation kinetics (Barnes and Hille, 1989; Taylor and Morgans, 1998; Koschak et al., 2003; McRory et al., 2004)—allow continuous modulation of the rate of glutamate release to track changing light conditions. Furthermore, the high voltage sensitivity of the channels means that even the absorption of a single photon by a rod photoreceptor, which hyperpolarizes the membrane by ~1 mV, causes a signal that is reliably transmitted to the postsynaptic rod bipolar cell (Field and Rieke, 2002; Berntson et al., 2004).

Immunolabeling has localized $\text{Ca}_v1.4$ to the synaptic layers of the retina—the outer and inner plexiform layers (OPL and IPL)—and more specifically to the ribbon synapses of rod and cone photoreceptors and bipolar cells (Morgans, 2001; Morgans et al., 2001, 2005; Berntson et al., 2003). Immunolabeling for $\text{Ca}_v1.4$, with either pan- α_1 - or α_{1F} -specific antibodies, colocalizes with the synaptic ribbon proteins Bassoon and RIBEYE (Brandstätter et al., 1999; Morgans et al., 2005; tom Dieck et al., 2005) and is nested within the crescent-shape of the ribbon, suggesting a localization of $\text{Ca}_v1.4$ to the plasma membrane at the ribbon's base. $\text{Ca}_v1.4$ forms part of the presynaptic cytomatrix at the photoreceptor active zone, where it colocalizes with the proteins RIM2, Munc13-1, and CAST1 (tom Dieck et al., 2005). $\text{Ca}_v1.4$ in the photoreceptor synapse is therefore in a position, both structurally and functionally, to coordinate presynaptic calcium entry with synaptic vesicle fusion.

Mutations in the *CACNA1F* gene, which encodes the α_{1F} pore-forming subunit of $\text{Ca}_v1.4$, cause the X-linked heritable disease incomplete congenital stationary night blindness (CSNB2) in humans (Bech-Hansen et al., 1998; Boycott et al., 2001). The same gene in mouse, *Cacna1f*, is mutated in the *nob2* (no b-wave 2) strain, which is a model for CSNB2. In the *nob2* mouse, the *Cacna1f* gene is disrupted by a naturally occurring transposable element insertion in exon 2 (Chang et al., 2006), resulting in a nonfunctional, truncated protein product. The *nob2* mouse is therefore null for *Cacna1f* (Chang et al., 2006).

A characteristic of CSNB2 is a normal a-wave but a severely reduced b-wave in the dark-adapted electroretinogram (ERG; Miyake et al., 1986, 1987; Nakamura et al., 2001). The normal a-wave indicates intact phototransduction in the photoreceptor outer segments of CSNB2 patients, but the reduced b-wave indicates disrupted synaptic transmission between photoreceptors and ON-bipolar cells (Miyake et al., 1986; Tremblay et al., 1995). Similarly, the *nob2* mouse has a no-b-wave ERG phenotype much like that of CSNB2, displaying a normal a-wave but severely reduced b-wave in both the dark-adapted and the light-adapted ERG (Chang et al., 2006). Effects on both the dark- and the light-adapted ERG suggest that neurotransmission from both rod and cone photoreceptors is affected. This is supported by data from a $\text{Ca}_v1.4$ knockout mouse, which shows a dramatic decrease in depolarization-induced calcium influx into photoreceptor terminals (Mansergh et al., 2005). Preliminary characterization of the *nob2* mouse also indicates that the loss of $\text{Ca}_v1.4$ results in altered retinal ganglion cell ON-responses and also in abnormal bipolar and horizontal cell morphology (Chang et al., 2006).

The elimination of both electrophysiological and structural functions of Ca_v1.4 in CSNB2 patients and in the *nob2* mice is likely to have developmental consequences, particularly for ribbon synapse formation. The formation of the invaginating photoreceptor ribbon synapse in mouse begins early in postnatal development, at about postnatal day 3 or 4 (P3/P4; Blanks et al., 1974; Rich et al., 1997), when cone photoreceptor terminals first contact horizontal cell processes in the future OPL. Contact of the cone by a second horizontal cell process coincides with invagination of the postsynaptic processes into the photo-receptor terminal, and the recruitment of the formed synaptic ribbon to the future active zone at about P6. Finally, at P7–P12 for cones, ON-bipolar cell dendrites invade the invagination to assume their position under the presynaptic ribbon (Blanks et al., 1974). Rods follow a very similar maturational sequence but are delayed with respect to cones by about 3–4 days, forming mature synapses only by P10–P14 (Rich et al., 1997).

It is presently unknown whether normal, light-regulated glutamate release is necessary for the development of the photoreceptor-bipolar cell or photoreceptor-horizontal cell synapse. Synapse formation in the inner retina requires neuronal activity and neurotransmitter release (for review see Mumm et al., 2005), but little is known about this process in the outer retina. Study of the *nob2* mouse will help to elucidate the role of the Ca_v1.4 calcium channel in the formation of OPL ribbon synapses.

Here we examine in detail the reported aberrant outgrowth of bipolar and horizontal cells in the *nob2* retina (Chang et al., 2006), focusing on the constituents of the ectopic contacts that form between rods and rod bipolar cells at the tips of aberrant bipolar cell dendrites. Furthermore, we characterize the developmental time course of the outgrowth of rod bipolar and horizontal cell processes in the *nob2* retina and present data suggesting that it is rod bipolar cells, rather than horizontal cells, that are involved in the formation of ectopic synaptic contacts with rod photoreceptors in the *nob2* retina in the days following eye opening.

MATERIALS AND METHODS

All procedures were carried out according to protocols approved by IACUC at OHSU and in accordance with National Institutes of Health guidelines. AXB6/PgnJ (*nob2*) mice were obtained from Dr B. Chang at The Jackson Laboratory (Bar Harbor, ME). This recombinant inbred line resulted from a cross between A/J and C57BL/6 lines and contains a naturally occurring ETn transposon insertion in the second exon of the *Cacna1f* gene (Chang et al., 2006). The location and size of the transposon results in a null allele of *Cacna1f* (Chang et al., 2006). All experiments were performed with either age-matched AXB6/PgnJ mutant animals and C57BL/6J controls or with siblings from crosses between AXB6/PgnJ and C57BL/6J lines. In the latter case, animals were PCR genotyped with the primers 1F-6 and 1F-2 for the wild-type (WT) allele of *Cacna1f* and 1F-5 and 1F-2 for the mutant allele. Primer sequences were as follows: 1F-6 (fwd): 5'-AGTTGAAATG-CACAGCATGG; 1F-5 (fwd): 5'-CACGTGCACCTTTC-TACTGG; 1F-2 (rev): 5'-GCTGGTATCAGTCCCCACAG. Results were consistent irrespective of strain background, and all adult animals used were between 3 and 7 months of age.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Berntson et al., 2003). Briefly, eyes were removed following death by CO₂ inhalation. Eyecups were prepared by cutting behind the ora serrata and removing the cornea and lens and were fixed in freshly made ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), for 5–30 minutes. Fixed eyecups were rinsed in PB and cryoprotected through a series of 10%, 20%, and 30% sucrose solutions in phosphate-buffered saline (PBS), before being frozen in OCT (Sakura Finetek, Torrance, CA) in a dry-ice-cooled isopentane bath. Eighteen-micrometer retinal sections were cut on a cryostat, collected on Super-Frost glass slides, and stored at –20°C until use.

For immunohistochemistry, sections were blocked for 30 minutes in antibody incubation solution (AIS; 3% horse serum, 0.5% Triton X-100, 0.025% NaN₃ in PBS), before being incubated in primary antibody diluted in AIS for 3 hours at room temperature. The primary antibodies used are reported in detail in Table 1.

Sections were rinsed three times in PBS and incubated in secondary antibodies diluted in PBS: anti-mouse Cy3, 1:500 (Jackson ImmunoResearch, West Grove, PA); anti-rabbit Alexa Fluor 488, 1:1,000 (Molecular Probes, Eugene, OR); anti-sheep Alexa Fluor 594, 1:1,000 (Molecular Probes). Sections were rinsed three times in PBS and coverslipped with GelMount (Biomedex, Foster City, CA).

Samples were surveyed on a Nikon E800 epifluorescence microscope, and then imaged on a Zeiss LSM510 confocal microscope. Optical slices of 1 µm thickness were acquired with a ×63/1.8 NA objective, and maximal intensity projections of stacks of 8–10 µm were used for figures. Minimal image processing for brightness and contrast was carried out in Adobe Photoshop.

For quantification of immunohistochemistry, aberrant processes of RBCs or HCs were defined as those that extended into the outer nuclear layer, as assessed by brightfield imaging. Puncta of immunostaining were de-fined as being at the tips or along the shafts of aberrant processes if they lay within a 0.5 µm radius of the tip or within 0.5 µm of the shaft, respectively. The same distance criterion was applied for quantifying the apposition of mGluR6 and RIBEYE puncta (see Fig. 2). Statistical analysis was carried out with a Student's two-tailed *t*-test on three samples of each condition, with significance set at the *P* 0.05 level.

Transmission electron microscopy

Eyecups were prepared as described above and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 hours. Samples were rinsed; osmicated in 2% OsO₄ in 0.1 M phosphate buffer, pH 7.4, for 1 hour; and dehydrated through an ethanol series and propylene oxide. Samples were incubated overnight in 1:1 propylene oxide:Embed 812 (Electron Microscopy Sciences, Hatfield, PA) and then embedded in Embed 812 and cured in lozenge-shaped molds (Ted Pella, Redding, CA). Blocks were cut at 75 nm on a Leica ultramicrotome, and sections loaded onto 400 thin-bar copper grids. Sections were contrasted with uranyl acetate and lead citrate before being viewed on a FEI Tecnai 12

microscope. Images were captured digitally by using a Hammamatsu camera and were minimally processed in Adobe Photoshop.

RESULTS

In the WT mouse retina, second-order neurons—bipolar cells and horizontal cells—form synapses with photoreceptor terminals in the outer plexiform layer (OPL). In the adult *nob2* mouse, both rod bipolar cells (RBCs) and horizontal cells (HCs) extend aberrant processes into the outer nuclear layer (ONL) of the retina (Chang et al., 2006; Fig. 1B,D), a layer normally occupied by photoreceptor cell bodies and devoid of synapses. RBCs, labeled by an antibody to PKC α , form synapses in the OPL of the WT retina (Fig. 1A) but in the *nob2* retina extend thick, branching processes up to ~30 μ m into the ONL (Fig. 1B). HCs, labeled by an antibody to calbindin D28K, also normally form synapses in the OPL (Fig. 1C) but in the *nob2* retina extend processes that are even more extensive than those from RBCs (Fig. 1D). HC processes frequently reach the outer limiting membrane of the retina before returning in a vitreal direction and vary in thickness, tracing convoluted paths with extensive branching and braiding. Labeling both RBC and HC populations indicates that aberrant *nob2* bipolar cell and horizontal cell processes frequently exit the OPL at similar points and in some instances appear to cofasciculate in the ONL (Fig. 1E).

Among the bipolar cell subtypes, only rod bipolar cells extend aberrant dendrites in *nob2*. Double labeling for PKC α (to label RBCs) and the G-protein subunit G $_{\text{O}\alpha}$, a marker for both rod and cone ON-bipolar cells, indicates that there are no G $_{\text{O}\alpha}$ -labeled aberrant processes that are not also labeled by PKC α (Fig. 1F); i.e., ON-cone bipolar cells, as with OFF-cone bipolar cells (Chang et al., 2006), terminate in the OPL of the *nob2* retina and do not extend aberrant dendrites into the ONL. The anatomical defects of second-order neurons in the *nob2* retina are therefore restricted to RBCs and HCs, suggesting a predominant effect on the anatomy of the rod pathway in *nob2*.

At the rod synapse, a single presynaptic ribbon docked with glutamate-filled vesicles lies juxtaposed to postsynaptic HC processes and RBC dendrites that protrude into an invagination of the photoreceptor terminal (Rao-Mirotznik et al., 1995; Migdale et al., 2003). The depolarizing response of RBCs to increases in light is mediated by the metabotropic glutamate receptor mGluR6, which is localized to the tips of RBC dendrites (Masu et al., 1995; Fig. 2A). In the *nob2* retina, aberrant RBC dendrites continue to express mGluR6 at their tips, despite their location in the ONL (Fig. 2B). Furthermore, double labeling for mGluR6 and for the presynaptic ribbon marker RIB-EYE (using the CtBP2 antibody, which additionally labels the nuclei of inner retinal neurons) indicates a close apposition of these pre- and postsynaptic specializations not only in the WT and *nob2* OPL (Fig. 2C,D) but additionally at ectopic locations in the *nob2* ONL (Fig. 2D). In the *nob2* retina, $85.43\% \pm 9.15\%$ of all ectopic mGluR6 puncta are associated with RIBEYE labeling, and $80.87\% \pm 3.98\%$ of all ectopic RIBEYE puncta are associated with mGluR6 labeling (mean $\pm$ SD; $n = 3$), indicating a high degree of apposition of these two markers in the *nob2* ONL. Double labeling for either RBCs or HCs with presynaptic ribbon markers additionally indicates that puncta of ribbon protein, either RIBEYE (Fig. 2E–H) or Bassoon (data not shown, but is as for RIBEYE), are more frequently associated with the tips of aberrant RBC

dendrites than with the tips of aberrant HC processes (compare Figs. 2F and H; summarized in Fig. 2I). In the *nob2* retina, $81.10\% \pm 9.92\%$ of aberrant RBC dendrites terminate with one or more RIBEYE puncta, whereas the same is true for only $48.65\% \pm 12.32\%$ of aberrant HC processes (mean $\pm$ SD; $n = 3$; Student's *t*-test $P < 0.05$). A significantly higher proportion of RIBEYE puncta is associated with the tips of aberrant RBC dendrites than with the tips of aberrant HC processes: $44.05\% \pm 12.17\%$ vs. $11.35\% \pm 1.37\%$ respectively (mean $\pm$ SD; $n = 3$; Student's *t*-test $P < 0.05$). The proportion of RIBEYE puncta associated with the shafts of aberrant processes, or alone (not associated with an aberrant process), is not significantly different for RBC dendrites and HC processes [shaft: $38.60\% \pm 4.64\%$ (RBC), $44.16\% \pm 15.53\%$ (HC); alone: $17.35\% \pm 11.97\%$ (RBC), $44.48\% \pm 15.86\%$ (HC)]. Aberrant RBC dendrites, therefore, are more likely than aberrant HC processes to terminate apposed to RIBEYE puncta, but both are extensively flanked by RIBEYE puncta along their shafts.

The appearance of the presynaptic marker RIBEYE at the tips and along the shafts of aberrant RBC and HC processes is suggestive of retracted photoreceptor terminals in the *nob2* ONL. To see whether ectopic puncta of synaptic ribbon protein colocalized with synaptic vesicles, as would be expected if the ribbons were located in retracted synaptic terminals, we labeled retinal sections with an antibody to the vesicular glutamate transporter VGLUT1 (Fig. 2J,K). VGLUT1 is responsible for loading glutamate into synaptic vesicles at retinal ribbon synapses (Johnson et al., 2003). Whereas, in the WT retina, VGLUT1 and RIBEYE labeling were confined to the OPL (Fig. 2J), in the *nob2* retina, puncta of VGLUT1 were additionally evident in the ONL, where they colocalized with puncta of RIBEYE (Fig. 2K). The presence of synaptic vesicles and ribbon protein confirms that photoreceptor presynaptic terminals are mislocalized in the ONL of the *nob2* retina.

The localization of presynaptic (ribbon proteins) and postsynaptic (mGluR6) markers to ectopic locations is suggestive of the presence of ectopic synaptic contacts in the ONL. In the absence of evidence relating to transmission at these potential synapses, we use the term *contacts* to designate a structure with apposed pre- and postsynaptic specializations. To examine the ultrastructure of these ectopic contacts, we performed transmission electron microscopy of *nob2* and WT retinæ. In WT tissue, rod photoreceptor terminals are found exclusively in the OPL (as in Fig. 3A). Areas of the ONL between photoreceptor nuclei are filled with the profiles of photoreceptor axons, Müller cell processes, and extracellular matrix but show no evidence of synaptic terminals (not shown). However, in the *nob2* ONL, membrane-enclosed structures resembling synaptic terminals are evident in the regions between photoreceptor nuclei (Figs. 3B,C). In Figure 3B', the magnification of a region of the ONL shown in Figure 3B reveals an ectopic presynaptic terminal containing presumed synaptic vesicles and an aggregate of electron-dense material that may comprise synaptic ribbon protein (Fig. 3B'). A second ectopic presynaptic terminal (Fig. 3C,C') has a crescent morphology, which we frequently observed, and is apposed to a photoreceptor nucleus. This terminal is also full of synaptic vesicles and contains the profiles of potential invaginating postsynaptic processes (normal invaginations were rarely observed). The general morphology and size of the ectopic terminals in the *nob2* suggest that these are displaced rod

terminals. In contrast, cone terminals remained in the *nob2* OPL, the position of cone terminals in WT retina.

To confirm that cone terminals are not displaced to the ONL in the *nob2* retina, we labeled cone photoreceptors with a fluorescent-conjugated peanut agglutinin (PNA). As well as labeling cone outer segments, PNA labels cone terminals (pedicles) in the OPL of both the WT and the *nob2* retina (Fig. 4). No PNA labeling is observed in the *nob2* ONL, confirming that all identifiable cones remain in their normal position in the OPL in the *nob2* retina. This evidence further reinforces the idea that the displaced photoreceptor terminals in the ONL are those of rods.

The localization of presynaptic (synaptic ribbon proteins; synaptic vesicle proteins) and postsynaptic (mGluR6) specializations at the ectopic *nob2* contacts raises the question of which proteins may coordinate the apposition of these specializations. Members of the dystrophin/dystroglycan complex maintain synaptic integrity at peripheral and central synapses and have been localized to the photoreceptor synapse (Blank et al., 1999; Jastrow et al., 2006). Although the function of the dystrophin/dystroglycan complex at the photoreceptor synapse is presently unknown, mouse knockouts affecting this complex show a reduction in the ERG b-wave (for review see Pillers, 1999). Dystrophin itself is intracellular, but it binds to dystroglycan (a component of which is transmembrane), which in turn binds to extracellular laminins. We localized the dystrophin protein to the OPL of the WT retina, juxtaposed to the postsynaptic ON-bipolar cell marker mGluR6 (Fig. 5A–C). Dystrophin is localized to cone synapses in the *nob2* OPL and additionally to ectopic contacts in the ONL, where it localizes with ectopic puncta of mGluR6 (Fig. 5D–F). Thus a dystrophin-dystroglycan complex may be involved in maintaining the pre- and postsynaptic components in register at ectopic locations.

Having characterized *nob2* ectopic synaptic contacts in the adult retina, we wanted to establish how and when these contacts develop. We studied animals from postnatal day 7 (P7) to P42 by immunolabeling with markers of rod bipolar cells (PKC α) and horizontal cells (calbindin) as well as for synaptic ribbons and for mGluR6. From P7 to the time of eye opening, at about P13, staining with PKC α for the RBC population (Fig. 6A–F,M,N) did not reveal the presence of aberrant dendrites in the ONL (data not shown, and Fig. 6A,M). Instead, RBC dendrites terminated in the OPL, as in the WT retina. Calbindin staining (Fig. 6G–N) revealed a similar result for horizontal cells, with very little, if any, detectable outgrowth of processes into the ONL by P13 (Fig. 6G,M). However, by P15, and at all time points beyond, calbindin staining revealed long HC processes extending deep into the ONL (Fig. 6H–L,N). These processes were complex and branched and, by P15, reached the outer limiting membrane of the retina (Fig. 6H). In contrast, PKC α -labeled RBC processes were not evident in the ONL until P18, when tiny, hair-like processes were first seen invading the ONL (Fig. 6C). At time points beyond P18, PKC-labeled processes extended farther into the ONL, until, by P42, they were up to 30 μ m long (Fig. 6D–F). Aberrant RBC dendrites and HC processes therefore have different time courses and patterns of outgrowth into the ONL, with HC processes both preceding RBC dendrites and elaborating more rapidly growing and extensive processes.

We next examined the temporal and spatial formation of ectopic synaptic ribbons with respect to the outgrowth of RBC and HC processes. As in the adult *nob2* retina, RIBEYE labeling was more frequently associated with the tips of RBC dendrites than with the tips of HC processes. In support of this observation, the time course of the appearance of RIBEYE puncta progressively farther into the ONL closely matches that of the extension of RBC dendrites (compare Fig. 6A–F and G–L). At P18, calbindin-labeled processes extended across the full depth of the ONL but never had RIBEYE puncta at their tips, the RIBEYE being observed mainly in the first 20 μm of the ONL adjacent to the OPL (Fig. 6I). In contrast, when RBC processes grew out (see, e.g., Fig. 6D), each dendrite was capped by a cluster of RIBEYE puncta, although not all RIBEYE puncta were associated with a PKC α -labeled dendrite (see, e.g., Fig. 6E). Nonetheless, the continued localization of RIBEYE puncta to the tips of PKC α -labeled dendrites at all developmental stages suggests that RBC dendrites are the dominant postsynaptic players in the formation of ectopic contacts.

We also wanted to establish whether mGluR6 is consistently localized to the tips of outgrowing RBC dendrites at all developmental stages. mGluR6 is known to be present on rat ON-bipolar cell bodies at early developmental stages, becoming restricted to the dendritic tips only around the time of eye opening (P12; Nomura et al., 1994). We find the same pattern of localization in the *nob2* retina, with mGluR6 present on bipolar cell bodies at P7 and P9 (data not shown) and becoming strongly localized to the OPL only by P13 (Fig. 6O). However, at this stage, no mGluR6 labeling is evident beyond the OPL, in the ONL. Indeed, ectopic puncta of mGluR6 in the ONL only appear from P15/P18 onward (Fig. 6P) and are clearly associated with the tips of PKC-labeled RBC dendrites (data not shown) and apposed to presynaptic ribbon proteins (Fig. 6P). This situation persists until adult stages.

DISCUSSION

In this study, we characterize the ectopic synaptic contacts found in the ONL of the *nob2* mouse, which has a nonfunctional Ca $_v$ 1.4 L-type calcium channel α_1 subunit and, as such, is an animal model of the human visual disease CSNB2. The rod pathway is predominantly affected in *nob2*, with ectopic contacts occurring between rods and rod bipolar cells (RBCs). Cones are much less affected in *nob2*, and their presynaptic terminals remain in the OPL. This may be attributable to the expression of Ca $_v$ 1.3 in addition to Ca $_v$ 1.4 in cones (Taylor and Morgans, 1998; Morgans, 1999; Morgans et al., 2005), whereas mammalian rods have been shown to express only Ca $_v$ 1.4 (Morgans, 2001; Morgans et al., 2001, 2005; Berntson et al., 2003). We find that ectopic contacts contain numerous synaptic specializations, including the presynaptic ribbon proteins RIBEYE and Bassoon, synaptic vesicles, the postsynaptic metabotropic glutamate receptor mGluR6, and possible components of a transsynaptic dystrophin-dystroglycan complex. Furthermore, we find that the OPL forms normally during early postnatal development and that it is not until the days following eye opening that ectopic contacts begin to appear in the ONL. Ectopic contacts are consistently located between rods and RBCs, as assessed by the high proportion of aberrant RBC dendrites that terminate with ribeye puncta at their tips. Horizontal cell processes, which would normally invade the rod synaptic terminal, are flanked by ectopic presynaptic sites along their shafts but rarely terminate at them. Ultrastructurally, ectopic contacts are abnormal and variable in their morphology, yet their presence suggests that formation of rod

to RBC synapse-like structures can occur in the absence of voltage-gated calcium channel-mediated glutamate release.

The outgrowth of aberrant RBC and HC processes into the ONL, accompanied by the formation of ectopic contacts, is a feature of a class of mouse mutants with reduced neurotransmission at the photoreceptor synapse and a negative ERG phenotype. Mutations that directly affect presynaptic calcium channels, such as *Cacna1f* mutants (Mansergh et al., 2005; Chang et al., 2006), calcium channel β_2 -subunit mutants (Ball et al., 2002; Gregg et al., 2002), and mutants for the L-type calcium channel-associated protein CaBP4 (Haeseleer et al., 2004), show outgrowth of RBC dendrites and HC processes, misshapen synaptic ribbons, and puncta of the ribbon proteins Bassoon or RIBEYE at ectopic sites in the ONL. Other mutants showing second-order neuron outgrowth include those in which components of the presynaptic ribbon complex are affected. The Bassoon mutant, for example, shows extensive outgrowth of rod bipolar cells and horizontal cells and the formation of ectopic synapses in the ONL (Dick et al., 2003). Interestingly, the calcium current is reduced in hair cells lacking Bassoon (Khimich et al., 2005), and, in the retina, the ERG b-wave and distribution of α_1 calcium channel subunits are affected (Dick et al., 2003; tom Dieck et al., 2005). Thus it is likely that calcium entry into the rod terminal is affected in all of the mutants mentioned above.

Retinal detachment and some injury and degeneration models also bear similarities to the aberrant outgrowth and ectopic contact phenotype observed in *nob2*, raising the possibility that this phenotype is a direct consequence of impaired transmission at the photoreceptor synapse. After experimentally induced retinal detachment in cats, rod terminals rapidly retract from the OPL into the ONL, and RBCs concomitantly sprout, potentially maintaining contact with or at least growing toward the retracted rod terminal (Lewis et al., 1998; Fisher et al., 2005). The time course of this terminal withdrawal and reorganization (over 1–3 days), the progressive loss of invaginations into rod terminals, and the sprouting response of RBCs and horizontal cells (with B-type horizontal cells extending a combination of thick and thin processes into the ONL and subretinal space) indicate interesting parallels with the *nob2* phenotype. Similarly, in the RCS rat and models of retinal degeneration, second-order neuronal processes initially grow out into the ONL but are later eliminated as the degeneration advances (Claes et al., 2004; Cuenca et al., 2005). Retinal degeneration models also indicate that new patterns of connectivity may form as photoreceptors degenerate. For example, in the rhodopsin and cGMP phosphodiesterase β -subunit mutants, rod bipolar cells form ectopic synapses with cones in the absence of rods (Peng et al., 2000), and, in CNGA3/rhodopsin knockouts, cone bipolar cells form contacts with rods (Haverkamp et al., 2006). It would be interesting to assess whether a hijacking of the cone pathway by RBCs might play a role in the relative preservation of ganglion cell responses seen in the *nob2* retina (Chang et al., 2006).

In contrast to the presynaptic mutations described above, mutations affecting postsynaptic components of the photoreceptor synapse are not associated with morphological abnormalities in the outer retina, indicating that abrogation of transmission at this synapse is not sufficient to trigger dendrite outgrowth. A class of no-b-wave ERG mutants in which bipolar cells are unresponsive to glutamate does not show aberrant sprouting of second-

order retinal neurons. The *nob* (no-b-wave) mutant, null for the *nyx* gene (Ball et al., 2003; Gregg et al., 2003), the mGluR6 knockout mouse (Tagawa et al., 1999), and the G_{oα} knock-out mouse (Dhingra et al., 2000) all show normal outer retina morphology, including OPL ultrastructure. Neither is outgrowth into the ONL seen in all models of retinal degeneration, especially where photoreceptor death is rapid, although aberrant HC processes are often seen in the inner retina (Strettoi and Pignatelli, 2000; Strettoi et al., 2002; Marc et al., 2003; Jones and Marc, 2005). It seems, therefore, that the loss of neurotransmission at the photoreceptor synapse is necessary but not sufficient to trigger second-order neuronal outgrowth and that more likely the changes seen in *nob2*, and in other presynaptic mutants, are driven by altered calcium signaling or homeostasis in the presynaptic rod terminal.

In the retinal-disease and -damage models mentioned above, the primary trigger for RBC dendritic outgrowth is likely to be the retraction of rod photoreceptor terminals. Retinal detachment, dissociation, and explanting/slicing, have all been shown to cause rapid retraction of rod terminals (Lewis et al., 1998; Nachman-Clewner et al., 1999; Jones and Marc, 2005), and the progressive appearance of presynaptic markers in the ONL of developing *nob2* retinæ suggests a similar sequence of events in *nob2*. Interestingly, Nachman-Clewner et al. (1999) reported that blocking L-type calcium channels with the dihydropyridine nifedipine inhibits rod terminal retraction, whereas our data suggest that the loss of calcium influx through Ca_v1.4 may cause rod retraction. It may be that any perturbation of calcium homeostasis in the rod terminal is sufficient to cause retraction.

If rod terminal retraction is the initial event in the development of the *nob2* phenotype, how does this then lead to second-order dendritic outgrowth? Two possible mechanisms would be: 1) rod photoreceptors retract and disengage from synapses (as proposed by Khodair et al., 2003), with the loss of contact acting as the stimulus for second-order neuron outgrowth, or 2) during rod retraction, pre- and postsynaptic specializations remain juxtaposed and are displaced as a unit to ectopic locations. Without live time-lapse imaging, it is impossible to track the movement of single synapses, but we favor the second mechanism for RBCs because apposition of mGluR6 and synaptic ribbon markers occurs at all developmental stages. If the tension exerted by rod retraction is what stimulates the outgrowth of RBC dendrites (Lamoureux et al., 2002; Fisher et al., 2005), then adhesion between the pre- and the postsynaptic components is essential, and we have shown that a component of the transsynaptic dystrophin/dystroglycan complex is present at ectopic contacts in *nob2*. It would be interesting to look more extensively into this question by establishing the presence at ectopic locations of further players in retinal synapse formation and maintenance, such as laminins (Libby et al., 1999), which have been shown to associate with L-type calcium channels (Nishimune et al., 2004), neurexins, and neuroligins (von Kriegstein and Schmitz, 2003).

If RBC dendrites are being “towed” into the ONL by retracting rod terminals, then what is stimulating the growth of HC processes? The difference in the time courses of RBC and HC dendritic outgrowth in the *nob2* retina suggests that HCs and RBCs respond to different cues with regard to synapse formation. Whereas the outgrowth of RBC dendrites appears to be dependent on contact with rod terminals, the outgrowth of HC processes occurs prior to rod terminal retraction. Possibly, the protrusion of HC dendrites into the invaginations of rod

terminals is necessary to cap outgrowth in the WT retina. Fewer invaginations form in *nob2* rod terminals, and the inability of HC dendrites to insert into rod terminals may lead to their continued extension into the ONL. Alternatively, glutamate release may be a more important cue for HCs than for RBCs. Detection of glutamate released from the photoreceptor terminals may provide a stop signal to HC dendrites in the WT retina, and in the absence of glutamate release in the *nob2* retina they continue to grow into the ONL. Clearly, HCs and RBCs respond differently to the loss of $\text{Ca}_v1.4$, with the outgrowth of HC processes preceding RBC processes and few HC processes terminating at sites of presynaptic specializations compared with RBC processes.

Our data are consistent with a model in which synaptic transmission at the rod ribbon synapse is necessary for the maintenance of morphologically normal and appropriately located rod synapses in the OPL. $\text{Ca}_v1.4$ activity, or the fulfillment of a structural role by this channel, may be required for formation of the invaginations into the pre-synaptic terminal and for the development and anchoring of a normal synaptic ribbon. In our model, synapses form “normally” before eye opening, but, after eye opening, and in the presumed absence of glutamate release from rod terminals, rod terminals retract into the ONL but still form contacts with RBC dendrites.

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Fig. 1.

A,B: Immunolabeling of WT (A) and *nob2* (B) retinal sections with an antibody to PKC α reveals that rod bipolar cell dendrites terminate in the OPL of the WT retina but extend aberrantly into the *nob2* ONL. **C,D:** Immunolabeling of WT (C) and *nob2* (D) retina with an antibody to calbindin D28K reveals that horizontal cell processes terminate in the OPL of the WT retina but extend aberrantly into the *nob2* ONL. **E:** Labeling both rod bipolar cells (PKC α , green) and horizontal cells (Calbindin, magenta) in *nob2* retina reveals that the aberrant processes from these two cell types frequently exit the OPL at the same points and navigate together through the ONL (arrowheads). **F:** Double labeling of *nob2* retina with antibodies to PKC α (rod bipolar cells, green) and G α_o (all ON-bipolar cells, magenta) indicates that, despite clear labeling of cone ON-bipolar cell bodies (arrow-heads), there are no aberrant dendrites labeled exclusively by G α_o in the ONL. GCL, ganglion cell layer; IPL,

inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bars = 20 μm in A (applies to A,B); 20 μm in C (applies to C,D); 10 μm in E,F.

Fig. 2.

A,B: Immunolabeling for mGluR6 (magenta) is localized to the tips of PKC α -labeled rod bipolar cell (RBC) dendrites (green) in both WT (A) and *nob2* (B) retinas, despite the aberrant extension of RBC dendrites into the *nob2* ONL (arrowheads in B). **C,D:** Puncta of mGluR6 (magenta; postsynaptic) and RIBEYE (green; presynaptic) lie apposed to each other in the WT OPL (C) and also at ectopic locations in the *nob2* ONL (arrowheads in D). **E,F:** Puncta of RIBEYE (magenta) lie presynaptic to PKC α -labeled RBC dendrites (green) in the WT OPL (E) and are frequently located at the tips of aberrant rod bipolar cell dendrites in the *nob2* ONL (arrowheads in F). **G,H:** Puncta of RIBEYE (magenta) lie presynaptic to calbindin-labeled horizontal cell (HC) processes (green) in the WT OPL (G) and are frequently located along the shafts of HC processes (arrowhead in H), but rarely at their tips, in the *nob2* ONL (H). **I:** Graph summarizing data showing the percentage apposition of RIBEYE puncta with aberrant RBC dendrites and HC processes in the *nob2* OPL. X-axis categories are percentages (mean $\pm$ SEM) of 1) RBC/HC processes terminating in one or more RIBEYE puncta; 2) RBC/HC processes not terminating in RIBEYE; RIBEYE puncta associated with the tips (3) and shafts (4) of RBC/HC processes; and 5) RIBEYE puncta not associated with RBC/HC processes. * $P < 0.05$, as assessed by a two-way Student's *t*-test; $n = 3$. **J,K:** Largely coincident immunolabeling for the vesicular glutamate transporter VGLUT1 (green) with RIBEYE (magenta) indicates that glutamate-filled synaptic vesicles are present in both WT (J) and *nob2* (K) photoreceptor terminals in the OPL and additionally in ectopic photoreceptor terminals in the *nob2* ONL (K). OPL,

outer plexiform layer; ONL, outer nuclear layer. Scale bars = 20 μm in A (applies to A–H); 20 μm in J (applies to J,K).

Fig. 3.

A: Transmission electron microscopy showing a cluster of WT rod terminals in the OPL. Arrows indicate synaptic ribbons. **B:** In the *nob2* retina, some rod photoreceptor terminals occupy an ectopic location in the ONL. The terminal-like structure magnified in **B'** contains pale, membrane-enclosed vesicles and an electron-dense aggregate that may comprise ribbon protein (arrow). Normal invaginations of postsynaptic processes into the terminal are absent. **C:** A second ectopically located terminal in the *nob2* ONL, magnified in **C'**, contains vesicles and a possible invagination with postsynaptic processes (asterisks). OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bars = 1 μm in A; 4 μm in B; 400 nm in B', C'; 2 μm in C.

Fig. 4.

A: Cone photoreceptor terminals, labeled by a fluorescent-conjugated peanut agglutinin, are evident in the WT OPL. **B:** In the *nob2* retina, cone terminals are also seen exclusively in the OPL, and never in the ONL. OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar = 20 μ m.

Fig. 5.

Immunolabeling for mGluR6 (magenta; **A,D**) and dystrophin (green; **B,E**) reveals a high degree of overlap of these markers in the WT OPL, indicating that a transynaptic complex involving dystrophin may be present at the photoreceptor to ON-bipolar cell synapse. **C,F**: In the *nob2* retina, mGluR6 and dystrophin are additionally localized to ectopic contacts in the ONL (arrowheads). OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar = 20 μ m.

Fig. 6.

Projections of stacks of confocal sections from *nob2* retinæ at the developmental stages shown at top right (P13, postnatal day 13). **A–F:** Rod bipolar cells (labeled by anti-PKC α ; green) do not extend aberrant dendrites into the ONL until P18 (C) but at all stages show clusters of RIBEYE puncta (magenta) at their dendritic tips (arrowhead in D at P21). However, not all RIBEYE puncta are associated with rod bipolar cells (arrowhead in E at P33). **G–L:** Calbindin-labeled horizontal cells (green) do not send aberrant processes into the ONL at P13 (G), but such processes are extensive by P15 (H) and at all time points beyond (I–L). Aberrant horizontal cell processes rarely terminate with RIBEYE puncta (magenta) at their tips at any developmental stage, the puncta rather being associated with their shafts, especially at later developmental stages (K,L). **M,N:** At the time of eye opening, P13, neither rod bipolar cells (labeled by anti-PKC α ; magenta) nor horizontal cells (labeled by calbindin; green) extend aberrant processes into the ONL (M); however, by P33, long horizontal cell processes and shorter rod bipolar cell dendrites are seen in the ONL (N). **O,P:** mGluR6 (magenta) and RIBEYE (green) are both localized to the OPL at P13, the time of eye opening (O). However, by P15, puncta of RIBEYE and mGluR6 are beginning to appear in the ONL directly adjacent to the OPL (P). OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar = 20 μ m.

TABLE 1

Details of the Primary Antibodies Used¹

Antibody	Manufacturer	Catalog No.	Lot No.	Animal	Dilution	Antigen	Controls
Calbindin D28K	Sigma, St. Louis, MO	C-8666	027H4820	Mouse	1:1,000	Purified chicken calbindin D	28-kDa band in WB
Calbindin D28K	Chemicon Temecula, CA	AB1778	25020096	Rabbit	1:1,000	Recombinant calbindin	Recognizes calbindin on WB. Specifically labels cerebellar Purkinje cells
CBP2/RIBEYE	BD Biosciences, San Jose, CA	612044	19649	Mouse	1:2,000	Amino acids 361-445 (C-terminus) of mouse CBP2	48-kDa band in WB
G _{oα}	Biomol, Plymouth Meeting, PA	SA-280	L1425	Mouse	1:1,000	Bovine brain G _{oα} subunit protein	40-kDa band in WB
MANDRA1	Nguyen et al., 1992			Mouse	1:500	Amino acids 3,558-3,684 of human dystrophin	427-kDa band in WB of mouse muscle extract
mGluR6	Morgans et al., 2006			Sheep	1:100	C-terminal 19 amino acids of rat mGluR6	Peptide block of immunolabeling
PKCα	Sigma, St. Louis, MO	P4334	094K4810	Rabbit	1:20,000	C-terminal V5 region of rat PKCα; amino acids 659-672	80-kDa band in WB of rat brain extract; peptide block
PKCα	Novus, Littleton, CO	NB600-201	255	Mouse	1:1,000	Purified PKC from bovine brain	79-kDa band in WB of rat C6 glial and human Ramos cell lines
vGluT1	Chemicon, Temecula, CA	AB5905	24080852	Guinea pig	1:1,000	Peptide GATHSTVQP PRPPPPVRDY	Peptide block of immunolabeling

¹WB, Western blot.