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STRUCTURAL ORGANIZATION AND FUNCTION OF MOUSE PHOTORECEPTOR RIBBON SYNAPSES INVOLVE THE IMMUNOGLOBULIN ADHESION PROTEIN SYNCAM 1

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Abstract

Adhesive interactions in the retina instruct the developmental specification of inner retinal layers. However, potential roles of adhesion in the development and function of photoreceptor synapses remain incompletely understood. This contrasts with our understanding of synapse development in the central nervous system (CNS), which can be guided by select adhesion molecules such as the Synaptic Cell Adhesion Molecule 1 (SynCAM 1/CADM1/Nectin-like 2 protein). This immunoglobulin superfamily protein modulates the development and plasticity of classical excitatory synapses. We now show by immuno-electron microscopy and immunoblotting that SynCAM 1 is expressed on mouse rod photoreceptors and their terminals in the outer nuclear and plexiform layers (ONL and OPL) in a developmentally regulated manner. Expression of SynCAM 1 on rods is low in early postnatal stages (P3-P7), but increases after eye opening (P14). In support of functional roles in the photoreceptors, electroretinogram recordings demonstrate impaired responses to light stimulation in SynCAM 1 knockout (KO) mice. In addition, the structural integrity of synapses in the OPL requires SynCAM 1. Quantitative ultrastructural analysis of SynCAM 1 KO retina measured fewer fully assembled, triadic rod ribbon synapses. Further, rod synapse ribbons are shortened in KO mice and protein levels of Ribeye, a major structural component of ribbons, are reduced in SynCAM 1 KO retina. Together, our results implicate SynCAM 1 in the synaptic organization of the rod visual pathway and provide evidence for novel roles of synaptic adhesion in the structural and functional integrity of ribbon synapses.

Keywords

SynCAM; CADM; nectin-like protein; ribbon synapse; retina; rod; electroretinography

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: AR, MCC and TB. Acquisition of data: AR and XL. Analysis and interpretation of data: AR, XL, MCC and TB. Drafting of the manuscript: AR and TB. Critical revision of the manuscript for important intellectual content: XL and MCC. Statistical analysis: AR. Obtained funding: MCC and TB. Technical and material support: XL and MCC. Study supervision: TB.

INTRODUCTION

Precise synapse development is crucial for integration of neurons into functional networks (Fuerst and Burgess, 2009). Progress over recent years has implicated different classes of adhesion molecules in these processes (Fuerst and Burgess, 2009; Missler et al., 2012; Shapiro et al., 2007). Among these proteins, Cadherin and Immunoglobulin (Ig)-superfamily members, as well as Neurexins and Neuroligins, organize the development and maturation of synapses in the CNS. Specifically, the Ig protein SynCAM 1 that is enriched at central synapses of vertebrates, promotes the formation of hippocampal excitatory synapses *in vitro* and *in vivo* and contributes to their maintenance (Biederer et al., 2002; Fogel et al., 2007; Robbins et al., 2010).

SynCAM 1 mediates cellular adhesion in a variety of tissues and is most prominently expressed in the brain (Fogel et al., 2007; Fujita et al., 2007; Thomas et al., 2008; Watabe et al., 2003). SynCAM 1 is enriched at excitatory synapses in the forebrain and instructs their formation in developing hippocampal neurons via adhesive interactions across the synaptic cleft (Biederer et al., 2002; Fogel et al., 2007; Fogel et al., 2011; Robbins et al., 2010). It is then required in the maturing hippocampus to maintain the synapses it induced during development (Robbins et al., 2010). Roles of synaptic adhesion proteins such as SynCAM 1 in the assembly and function of neuronal circuitry, however, remain to be defined. Interestingly, SynCAM 1 transcripts are abundantly expressed in the retina of different species, including mice, zebrafish and chick (Fujita et al., 2005; Pietri et al., 2008; Wahlin et al., 2008). Roles of other cell adhesion molecules in retinal development, and particularly in the synaptic specification of inner retinal layers, are well described (Fuerst et al., 2009; Fuerst and Burgess, 2009; Lefebvre et al., 2008; Yamagata and Sanes, 2008). However, the localization and function of SynCAM 1 in this part of the CNS have not yet been investigated in detail.

We here address contributions of SynCAM 1 to retinal synaptic organization and function. Analyzing mice lacking SynCAM 1, our results demonstrate that this protein contributes to organizing the structure and molecular composition of photoreceptor synapses. Specifically, the ultrastructure of synaptic ribbons was altered and the content of the major ribbon protein Ribeye was reduced upon loss of SynCAM 1. Further, SynCAM 1 knock-out mice have fewer fully assembled, mature ribbon synapses. On a functional level, we show SynCAM 1 to be necessary for rod, but not cone, mediated visual transduction and SynCAM 1 knock-out mice have altered retinal network activity. These findings provide novel insights into the roles of synaptic adhesion in the organization of ribbon synapses and retinal circuits.

MATERIALS AND METHODS

Animals

Experiments were performed on C57BL6/J wild type mice (The Jackson Laboratory, Bar Harbor, ME), SynCAM 1 KO mice (Fujita et al., 2006) and their wild type littermates. SynCAM 1 KO mice had been backcrossed for at least 10 generations and were maintained on a C57BL6/J background. Animals of both sexes from postnatal day 3 (P3) to P50 were used for all experiments as indicated below and stated in the figure legends. SynCAM 1 KO and wild-type (WT) littermates were compared in all experiments and experimenters were blind to the genotype of animals used. Animals were kept on a 12/12 hour light/dark cycle with food and water ad libitum. All experiments were performed during the light phase (7 AM–7 PM). Animals were treated in accordance with the Yale Institutional Animal Care and Use Committee guidelines.

Characterization of antibodies and other reagents

Antibodies used, their properties and working concentrations are listed in Table 1. Antibody characterization for this study is described below.

Actin—Mouse anti-Actin monoclonal antibody was raised against amino-acids 18–40 of chicken gizzard Actin, which is a highly conserved region of the molecule (MP Biomedicals, Solon, OH; Catalogue number 69100; Clone C4). Immunoblots demonstrated specificity directed towards Actin across species (manufacturer's datasheet).

Calbindin—Rabbit anti-Calbindin D-28k polyclonal antibody was raised against recombinant rat Calbindin D-28k (Swant, Bellinzona, Switzerland; Catalogue number CB-38; Lot 9.03). Immunoblotting recognizes a single band of approximately 28 kDa and the antibody does not stain the brain of Calbindin D-28k KO mice (manufacturer's datasheet). The staining pattern of this antibody in our study is consistent with previously published results (Hirano et al., 2011).

mGluR6—Rabbit anti-mGluR6 polyclonal antibody was raised against rat mGluR6 C-terminal sequence AAPPQENADAK (Neuromics, Edina, MN; Cat. Number RA13105). Immunoblotting recognizes a band of approximately 97 kDa (manufacturer's datasheet). As a control, we used preincubation with immunizing peptide (Neuromics, Edina, MN; Cat. Number P13105), which abolished all signal in retinal sections of wild-type mice. Although this antibody in our hands did not result in punctate staining (tom Dieck et al., 2012), the staining pattern obtained was almost identical to previously published studies using different antibodies (Cooper et al., 2012).

Neurofilament—Mouse anti-Neurofilament monoclonal antibody was raised against semi-purified adult Wistar rat Neurofilaments (Developmental Studies Hybridoma Bank, University of Iowa; Clone RT97). The staining pattern of this antibody in our study is consistent with previously published studies using different antibodies (Haverkamp and Wässle, 2000).

PKC α —Rabbit anti-Protein kinase C α polyclonal antibody was raised against recombinant human PKC α (Cell Signaling Technology, Beverly, MA; Cat. Number 2056). Immunoblotting detects a single band at approximately 80 kDa and does not cross-react with other PKC isoforms (manufacturer's datasheet). The staining pattern of this antibody in our study is consistent with previously published studies using different antibodies against PKC α (Haverkamp and Wässle, 2000).

PNA—PNA-biotin conjugate (peanut lectin agglutinin, biotin conjugate; Sigma, St. Louis, MO; Cat. Number L6135) was used at a final concentration of 5 μ g/mL and recognized cone terminals in the OPL as previously published (Reim et al., 2009).

PSD-95—Rabbit anti-Postsynaptic density protein of 95 kDa (PSD-95) monoclonal antibody was raised against synthetic peptide corresponding to N-terminal human PSD-95 sequence CDTLEAPGYELQVNGTEGEMEY (Cell Signaling Technology, Beverly, MA; Cat. Number 3409). Immunoblotting recognizes a single band at approximately 95 kDa and the tissue staining is abolished in the presence of control peptide (manufacturer's datasheet). This antibody gave a staining pattern identical to previously published studies using different antibodies against PSD-95 (Yang et al., 2007).

Ribeye—Rabbit polyclonal antibody against Ribeye (kindly provided by Sreeganga Chandra, Yale University) used in immunoblotting was raised against glutathione S-

transferase (GST) fusion protein containing amino-acids 563–988 of rat Ribeye (U2656) as previously described (Schmitz et al., 2000). In our study, this antibody recognized a prominent band of approximately 120 kDa in retinal protein extracts and a weaker band corresponding to CtBP2 at around 50 kDa, as previously published (Schmitz et al., 2000). Because of high background this antibody gave in immunohistology, we used a commercially available mouse monoclonal antibody against Ribeye raised against amino acids 361–445 in Ribeye C-terminus (BD Biosciences, San Jose, CA; Cat. Number 612044). Staining with this antibody detected typical horse-shoe shaped ribbons in the outer plexiform layer (OPL) as described (Wahlin et al., 2010).

SynCAM 1—Anti-SynCAM 1 antibody was purified from hybridoma supernatant using anti-IgY affinity column (MBL Laboratories, Nagoya, Japan; CM004-3; Clone 3E1). This hybridoma was established by fusion of chicken B cell line MUH1 cell with chicken splenocyte immunized with recombinant extracellular domain of SynCAM 1 fused to Fc-fragment (manufacturer's datasheet). This antibody was extensively tested in our laboratory in various applications. In immunoblotting using retinal tissue homogenates, this antibody detected multiple bands around 100 kDa in wild type samples due to complex N-glycosylation of this protein (Figure 1) (Fogel et al., 2007). No bands were observed in SynCAM 1 KO samples (Figure 1). Additionally, this antibody stained SynCAM 1 throughout the wild-type retina, while almost no staining was observed in SynCAM 1 KO retinas (Figure 1).

VGlut1—Anti-Vesicular glutamate transporter 1 (VGluT1) mouse monoclonal antibody was produced against the cytoplasmic C-terminus of rat VGluT1 (amino acids 493–560; NeuroMab, UC Davis, CA; Cat. Number 75-066; Clone N28/9). Immunoblotting recognizes a single band of approximately 52 kDa using adult rat brain homogenates (manufacturer's data sheet). The labeling we detected with this antibody was identical to previously published studies (Sherry et al., 2003).

Secondary antibodies—For all immunostainings, secondary antibodies were applied in the absence of primary antibodies as a control. Following secondary antibodies were used for immunofluorescence: anti-chicken Alexa 488, anti-rabbit Alexa 488 (Invitrogen Life Technologies, Grand Island, NY), and anti-rabbit and anti-mouse Cy3 (1:1000; Jackson ImmunoResearch, West Grove, PA). PNA-biotin was detected with Streptavidin-Alexa 555 (1:1000; Invitrogen Life Technologies). For electron microscopy, secondary donkey anti-chicken 12 nm gold conjugate (Jackson ImmunoResearch) was used. For quantitative immunoblotting, secondary IRDye800 antibodies were used at 1:4000 (Rockland Immunochemicals, Inc., Gilbertsville, PA).

Tissue preparation for biochemistry and microscopy—Animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) in saline. For all experiments (except for Fig. 1) animals were dark adapted overnight and all procedures were performed under dim red light (scotopic conditions). For protein isolation (animals aged P3-P28 for Fig. 1, P35-P50 for Fig. 8), cornea was cut with a sterile blade and the lens was gently squeezed out of the eye with fine forceps. The retina was isolated by gently squeezing the eyecup with fine forceps after clearing out the vitreous fluid, and immediately frozen over dry ice, followed by sonication in 8 M urea. Protein concentrations were determined using the BCA method (Thermo-Fisher Scientific, Holtville, NY). For microscopy, animals (P3-P50, as indicated in figure legends) were transcardially perfused first with ice cold PBS and then with either 4% PFA (in PBS, pH 7.4) for tissues to be used in light microscopy or with Karnovsky's fixative (Karnovsky, 1964) for electron microscopy. For light microscopy, whole eyes were postfixed for 1 hour in 4% PFA and washed in PBS overnight at 4°C.

Isolated eyecups were cryoprotected in 30% sucrose in PBS prior to embedding in OCT (Tissue-Tek, Sakura Finetek Inc, Torrance, CA). Tissue was sectioned on a cryotome (Leica Nussloch, Germany) at a thickness of 15 μm and directly mounted on Superfrost Plus slides (EMS, Hatfield, PA). For all experiments, eyecups from all animals to be used were embedded together in a series in order to process them in the same way.

For electron microscopy, tissue was first postfixed in Karnovsky's fixative for 1 hour at 4°C and then washed in 0.1 M sodium-cacodylate. Central retina was dissected under a microscope before osmication in Palade's osmium (Palade, 1952). Tissue was then dehydrated through graded series of ethanol and propylene oxide, and embedded in EMBed-812 resin (EMS) before cutting on a Leica Ultracut UTC microtome at 70 nm thickness. The sections were then placed on 200 mesh formvar/carbon coated copper grids and stained with 2% aqueous uranyl-acetate and lead citrate.

For immunoelectron microscopy, samples were fixed after perfusion in 4% paraformaldehyde/0.1% glutaraldehyde in PBS for 15 minutes, and then with 4% PFA in PBS for 1 hour. Samples were cryo-protected in 2.3 M sucrose overnight at 4°C. These were then rapidly frozen onto aluminum pins in liquid nitrogen. The frozen block of tissue was trimmed on a Leica Cryo-EMUC6 UltraCut and 60 nm thick sections were collected using the Tokuyasu method (Tokuyasu, 1973). The frozen sections were thawed and placed on a nickel formvar/carbon coated grid floated in a dish of PBS ready for immunolabeling.

Immunolabeling of sections for electron microscopy—Grids were placed section side down on drops of 0.1 M ammonium chloride for 10 minutes to quench untreated aldehyde groups, then blocked for nonspecific binding in PBS buffer containing 1% BSA (Sigma) and 10% normal donkey serum (Jackson Immunoresearch) for 20 minutes. They were then incubated in primary chicken anti-SynCAM 1 (MBL; described above) for 30 minutes. Rinsed grids were placed in secondary donkey anti-chicken 12 nm gold conjugate (Jackson Immunoresearch) for 30 minutes, rinsed in PBS, fixed using 1% glutaraldehyde, rinsed in distilled water and transferred to a UA/methylcellulose drop for 10 minutes. Samples were viewed on FEI Tecnai Biotwin TEM (FEI, Hillsboro, OR) at 80 Kv. Images were taken using Morada CCD and iTEM (Olympus) software. Acquired images were imported in ImageJ (NIH) and immunogold distribution was analyzed using multi-point selection tool.

Retinal morphology and electron microscopy—For estimating the density of photoreceptor nuclei, we employed the disector method (Li and Cline, 2010; Sterio, 1984; Yen et al., 1993). Osmicated and dehydrated tissue from central retina (see above) was cut on a Leica Ultracut UTC microtome at 0.5 μm and stained with toluidine blue. Minimum 3 series of 5 pairs of adjacent sections (5 μm apart in order to avoid sampling the same nuclei) were imaged per animal (3 animals per group). Images were acquired using a Zeiss AxioImager Z2 microscope and AxioVision Software (Zeiss). Images were imported in ImageJ and scaled. Layer thickness was measured by drawing straight lines on random locations throughout the sections using the line tool in ImageJ. Photoreceptor count was performed on the same images manually using the Cell Counter plugin in ImageJ following rules of the disector method (in each chosen area, only nuclei that appeared in the reference section, but not in the look-up section, were counted) (Li and Cline, 2010; Sterio, 1984; Yen et al., 1993). All measurements were averaged per animal before performing statistical comparisons.

For electron microscopy, images were acquired using Zeiss 910 Electron Microscope. Series of 70 nm ultrathin sections (see above) were sampled for the disector analysis (Li and Cline, 2010; Sterio, 1984; Yen et al., 1993). Sections were 3 μm apart to avoid sampling the same

terminals in the OPL. 6 pairs of adjacent sections in a series (minimum 3 series per animal, 3 animals per group) were imaged for analysis at 16000x magnification using Kodak Electron Microscope film 4489. Films were then scanned at high resolution with Epson Perfection 4990 PHOTO scanner. To ensure equal sampling, all samples were treated in an identical manner. The majority of triad images acquired had arciform density present, as well as similar overall appearance of the presynaptic ribbon complex, confirming that all images were acquired from comparable planes of sectioning. Rods and cones were distinguished on the basis of previously published criteria (Carter-Dawson and LaVail, 1979). Digital images were imported and scaled in ImageJ and all analyses were performed with ImageJ. Terminal perimeter was measured only for terminals with entire cross-section profile in the acquired image. Synaptic vesicles were counted manually using multi-point selection in ImageJ. Terminal perimeter and perimeter of horizontal and bipolar cell processes were measured using the freehand tool in ImageJ. Synaptic ribbon height was measured using the segmented line tool in ImageJ. Triad and ribbon density, as well as terminal density, were counted manually using the disector method (Li and Cline, 2010; Sterio, 1984; Yen et al., 1993). All measurements were averaged per animal before performing statistical analysis. Representative micrographs were imported in CorelDraw X5 (Corel Inc., Mountain View, CA) and trace outlined in order to make relevant structures readily visible.

Immunohistochemistry and confocal microscopy—Primary antibodies used in double-labeling experiments were applied simultaneously and blocking steps were performed using normal serum of host species from which respective secondary antibodies were derived. Sections were encircled with Pap-Pen (Sigma, St. Louis, MO) and non-specific antibody binding sites were blocked with 3% normal serum and 0.03% Triton-X 100 (Sigma) in PBS for 1 h in a humid chamber. Primary and secondary antibodies were diluted in 3% normal serum and 0.03% Triton-X 100 in PBS and incubated either 1 hour at room temperature or overnight at 4°C. After the antibody incubation steps, sections were washed in PBS and in distilled water before coverslipping with mounting medium (Aqua-Polymount, Polysciences Inc., Warrington, PA, USA). Confocal microscopy was performed using a laser-scanning microscope (LSM 710 and LSM 510; Zeiss, Jena, Germany) with argon (488 nm) and helium/neon (543 nm) lasers. For image acquisition of SynCAM 1 and DAPI, FV10i (Olympus, Tokyo, Japan) with 60x silicone oil objective ($NA_{\text{objective}}=1.35$; $NA_{\text{oil}}=1.406$; Olympus) was used and settings were kept similar to LSM 510/710. Image acquisition for all images was performed in multiple-tracking mode. High magnification images of central retina (except as noted above) were obtained at a resolution of $1,024 \times 1,024$ or $2,048 \times 2,048$ pixels using a Zeiss EC Plan-Neofluar 40x air ($NA=0.75$; Fig. 1) or Zeiss Apochromat 63x oil objective ($NA=1.4$; all other experiments) and immersion oil (Immersionol, Zeiss; refractive index = 1.518). Pinholes for both lasers were kept such that all optical sections were of the same thickness (0.8 μm). For co-localization, regions of interest were acquired mainly as single optical sections. For developmental profiling of Calbindin immunoreactivity, acquisition settings were kept equal for all samples and were such to allow for detection of horizontal cell sprouting in the ONL. For Ribeye analysis, $50 \times 25 \mu\text{m}$ Z-stacks (at $2,048 \times 1,024$ pixels) were taken at random locations throughout the OPL at 0.43 μm intervals. They were later analyzed in ImageJ using the freehand and multipoint tools to label and measure their length and number. For length measurements, approximately 500 ribbons per animal were analyzed. For number measurements, we employed the optical disector technique (Jinno et al., 1998; West et al., 1991). For all experimental groups ($N=2-3$ animals per genotype per age), all imaging settings (laser power, gain and offset) were kept identical. Images were minimally corrected for brightness and contrast (in identical manner for all groups) using the Zen software program (Zeiss) and assembled in CorelDraw X5.

Quantitative immunoblotting—Proteins from retinal homogenates (30 µg, prepared as described above) were subjected to immunoblotting using standard procedures (Fogel et al., 2007) and scanned with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Quantification was performed using the gel analysis plugin in ImageJ, where actin served as loading control for all samples.

Electroretinogram (ERG) recordings—ERG recordings were performed as previously described (Vistamehr and Tian, 2004). Briefly, for scotopic responses animals (P35-P50) were dark-adapted overnight and anesthetized with ketamine and xylazine as described above. The pupils were dilated with 1% Atropine (Sigma, St. Louis, MO) and 2.5% Phenylephrine HCl (TCI, Portland, OR) and the corneal surface was protected with 0.5% methylcellulose (Bausch & Lomb, Rochester, NY). ERGs were evoked by LED-generated white-light flashes (less than 5 ms in duration; BigShot™ Ganzfeld, LKC Technologies Inc., Gaithersburg, MD) and recorded with circular corneal platinum custom-made electrodes. Signals were amplified and band-pass filtered between 0.3 Hz to 500 Hz. For rod responses, ERGs for each light intensity were averaged from 5 consecutive flashes with 30 s interstimulus recovery period. Light intensities delivered were in 5 dB steps ranging from −25 dB to 10 dB, where 0 dB=2.5 cd-s/m². Double-flash recordings were performed as previously described (Kim et al., 2005) and signals were averaged from 3 consecutive stimuli with 2 minutes of recovery period in between. For photopic ERGs, animals were light adapted and signals for each light intensity (−5 dB to 10 dB in 5 dB steps) were averaged from 10 consecutive flashes delivered at a frequency of 2 Hz. Traces were analyzed with EMWin (LKC Technologies Inc.). a-wave and b-wave amplitude and latency were measured after removing the oscillatory potentials, where a-wave was measured from baseline to the trough, and b-wave was measured from the trough of the a-wave to the peak of the b-wave. a- and b-wave time was measured from the time of the flash to a-wave trough and b-wave peak, respectively. Amplitude and latency of oscillatory potentials were measured using automatic finite impulse response high pass filtering function of EMWin with a corner frequency set at 75 Hz (LKC Technologies Inc.). Amplitude of oscillatory potential peaks was automatically analyzed with EMWin after filtering and is a sum of OP1-5 amplitudes. Latency was analyzed manually after filtering, where OP1 and OP5 were not evaluated because of their small amplitude (Vessey et al., 2012). For double-flash recordings, only a-wave was measured. For double-flash recordings, amplitude of the probe flash a-wave normalized to test flash a-wave was plotted in relation to the time between the two flashes and fit using second-order polynomial equation in GraphPad Prism 4.0.

Data analysis

All quantitated analyses were performed with the researchers blind to the condition. Statistical analyses were performed in GraphPad Prism 4.0 (GraphPad Inc., La Jolla, USA) using Student's t-test, Two-way ANOVA or Two-way RM ANOVA (as indicated in text and figure legends) unless stated otherwise. All data are reported as mean ± SEM.

RESULTS

Expression of retinal SynCAM 1 is developmentally regulated

We quantified the expression of SynCAM 1 protein during the main stages of mouse retinal development using a commercial antibody that specifically recognized SynCAM 1 in retinal homogenates and in immunohistochemistry (Fig. 1A) (Fogel et al., 2011). This antibody detected multiple bands of approximately 100 kDa in western blots of total retinal homogenates from wild type mice (Fig. 1A, left panel), likely corresponding to different glycosylation forms of SynCAM 1 (Fogel et al., 2007). No bands were detected in samples from SynCAM 1 KO mice (Fig. 1A, left panel). Additionally, SynCAM 1 protein was

specifically detected throughout the wild type adult mouse retina with immunohistochemistry (Fig. 1A, right panel).

The increase of SynCAM 1 protein levels in the retina closely followed the rates of synaptogenesis in this brain region (Fisher, 1979; Regus-Leidig et al., 2009; Sharma et al., 2003), being low early in postnatal development (P3), increasing at P7, and rising after eye opening (P14 and P28) (Fig. 1B, C). Similar expression level changes were observed by immunohistochemistry, which showed that SynCAM 1 was expressed at low amounts throughout the retina at P3 and steadily increased during later stages (P7-P28) (Fig. 1C). SynCAM 1 signal appeared particularly enriched in the outer nuclear layer (ONL) surrounding photoreceptor cell bodies, as well as in the outer plexiform layer (OPL) on the photoreceptor terminals (Fig. 1A and C; also Fig. 2A and D). Despite its abundance in the ONL and OPL, SynCAM 1 was almost undetectable in the photoreceptor inner and outer segments (IS and OS) (Fig. 1C and data not shown). SynCAM 1 was present in the inner retinal layers as well, but appeared not as abundant as in the ONL.

SynCAM 1 is localized to photoreceptor cell bodies and terminal membranes in the ONL and OPL

The membrane protein SynCAM 1 localizes to cellular adhesion sites in different systems (Fogel et al., 2007; Watabe et al., 2003). In agreement, immunostaining and cryo-immuno electron microscopy (EM) demonstrated the presence of SynCAM 1 at the contact points between photoreceptor cell bodies (Fig. 2A and B). Analysis of the distribution of gold particles showed that they predominantly localize to the photoreceptor plasma membrane in the ONL (80% of n=220 particles found on n=47 photoreceptor cell bodies) (Figure 2C), in agreement with our findings at the light microscopy level (Figure 2A). Gold particles were almost completely absent in SynCAM 1 knockout (KO) mice control samples (n=20 particles on n=42 photoreceptor cell bodies; data not shown).

SynCAM 1 in the brain is enriched at excitatory synapses and mediates their structural and functional development in the hippocampus (Fogel et al., 2011; Robbins et al., 2010; Shu et al., 2011). As our results in Fig. 1 demonstrated robust SynCAM 1 expression in the outer retinal synaptic layer, we examined if it is associated with photoreceptor synapses. Photoreceptors connect with their bipolar cell targets within the OPL through glutamatergic ribbon synapses, specialized for tonic release (Heidelberger et al., 2005; Sterling and Matthews, 2005; tom Dieck and Brandstätter, 2006). Photoreceptor terminals, bipolar cell dendrites, and horizontal cell processes form a triadic synapse in the OPL at whose interface is the ribbon, an electron-dense structure involved in vesicle release at the photoreceptor terminal (Fig. 2D; see also Fig. 7A) (Snellman et al., 2011; tom Dieck and Brandstätter, 2006). Photoreceptor terminals (both rod and cone) are specifically labeled with antibodies against VGlut1 (Johnson et al., 2003; Sherry et al., 2003) and were detected as dense clusters of VGlut1 immunoreactivity in the OPL (Fig. 2D). Interestingly, SynCAM 1 signal appeared to envelop the VGlut1 clusters (Fig. 2D; merged image), indicating its presence on the photoreceptor terminal membrane. We have not observed SynCAM 1 signal associated with Calbindin-labeled horizontal cell processes and PKC α -labeled bipolar cell processes in the OPL (data not shown). Immuno-EM analysis confirmed the presence of SynCAM 1 at plasma membrane areas along the rod terminal (Fig. 2E and F; 71% of n=303 particles found on 106 terminals), as well as at the contact sites between rods and horizontal cell processes (Fig. 2E and F; arrowhead; 9% of n=303 particles). SynCAM 1 was not detected at the synaptic ribbon complex, but we have occasionally observed gold particles at or near the membrane of horizontal cell processes (Fig. 2E and F). SynCAM 1 was never detected near the ribbons or close to bipolar cell dendrites, nor did we detect SynCAM 1 on Müller cell processes in the outer retina (data not shown). In addition, the few cone terminals we found in our sections (identified by criteria described in Materials and Methods) were

labeled very sparsely with SynCAM 1 antibody compared to rod terminals (data not shown). We also detected no SynCAM 1 signal in the photoreceptor IS and OS (data not shown). Specificity of labeling was confirmed in SynCAM 1 KO samples, where a low number of gold particles could be detected, but they were never present at plasma membrane areas along rod terminals (n=45 gold particles found on 61 terminals; data not shown).

Loss of SynCAM 1 affects photoreceptor function

The robust expression of SynCAM 1 in the outer retinal layers and its ultrastructural localization on rod terminals suggested a possible role in the function of photoreceptors. In order to investigate how the lack of SynCAM 1 affects photoreceptor function, we performed electroretinogram (ERG) recordings on SynCAM 1 KO mice. ERGs are a robust measure of photoreceptor integrity (a-wave), bipolar cell responses (b-wave), as well as the activity of inner retina (oscillatory potentials) (Kueng-Hitz et al., 1999; Pinto et al., 2007; Weymouth and Vingrys, 2008). ERG recordings were performed on dark-adapted mice in order to monitor rod activity. Scotopic (rod) response of SynCAM 1 KO mice showed all typical components (Fig. 3A). However, the a-wave of SynCAM 1 KO mice had significantly higher amplitude compared to WT mice at all light intensities (Fig. 3B). The a-wave time-to-peak appeared unaltered in KO (Fig. 3D, bottom). The a-wave in standard ERGs has substantial photoreceptor contribution (Brown and Watanabe, 1962a; b; Brown et al., 1965). Impairments in the a-wave amplitude such as observed in SynCAM 1 KO mice are hence considered to mainly stem from a dysfunction of the photoreceptors (Peachey and Ball, 2003).

The amplitude of bipolar cell response (b-wave) seemed intact in SynCAM 1 KO mice (Fig. 3C), but the time required for the b-wave to reach its peak was significantly prolonged in SynCAM 1 KO mice at almost all light intensities (Fig. 3D, top). Amplitude of oscillatory potentials was also normal in KO mice (Fig. 3E and F). We analyzed latency of oscillatory potentials and found no differences between WT and KO mice, even at light intensities where the b-wave had the most pronounced delay ($t_{OP3_{WT}}=45.6\pm1.2$ ms, $t_{OP3_{KO}}=44.5\pm0.88$ ms at 0.791 cd-s/m²; N=12 WT and 10 KO; and data not shown). This indicated intact gross inner retinal activity in SynCAM 1 KO mice.

In order to investigate if visual transduction pathway is impaired in SynCAM 1 KO photoreceptors, we performed double-flash ERG recordings (Lyubarsky and Pugh, 1996). This method monitors recovery of photoreceptor currents generated in the outer segments after a saturating flash of light, and a number of studies have shown defects in the kinetics of current recovery in mice carrying mutations in visual transduction pathway proteins (Howes et al., 2002; Song et al., 2011; Zhang et al., 2007). The normalized a-wave recovery amplitude in SynCAM 1 KO mice was not different from WT mice (Fig. 3G and H), indicating intact phototransduction in SynCAM 1 KO mice.

Although ERG recordings were obtained from dark-adapted mice in order to more selectively measure rod activity, responses are nonetheless mixed due to the overlap in wavelength sensitivity of mouse rods and cones (Abd-El-Barr et al., 2009; Ekesten et al., 1998). Therefore, we measured photopic responses in SynCAM 1 KO mice. The photopic ERG reflects mainly cone response, as the animals are light-adapted and all recordings are performed in the presence of rod-saturating background light (Duncan et al., 2006). The photopic b-wave of SynCAM 1 KO mice appeared intact at all light intensities (Fig. 4A), with no difference in amplitude (Fig. 4B) or time-to-peak (Fig. 4C) compared to WT mice. In addition, no differences were detected in the amplitude and timing of photopic a-wave (data not shown). Together, these results supported that SynCAM 1 contributes to the transduction of visual stimuli selectively in the retinal rod pathway.

Loss of SynCAM 1 causes distinct alterations of retinal structure

The temporal expression profile of SynCAM 1 in the retina (Fig. 1B and C) tracks the development of outer retinal layers (Fisher, 1979; Johnson et al., 2003; Sharma et al., 2003). Furthermore, SynCAM 1 KO mice show distinct functional deficits in the rod (scotopic) pathway (Fig. 3B, D). We therefore hypothesized that SynCAM 1 is involved in the organization of rod photoreceptors and their synapses in the OPL. To address this question, we studied the structure and morphology of the retina in adult SynCAM 1 KO mice. In order to control for potential variations in retinal morphology caused by light exposure (Balkema and Dräger, 1985), and as rod responses were specifically impaired in SynCAM 1 KO mice, all experiments were performed on dark-adapted animals (scotopic conditions).

Gross retinal morphology in adult SynCAM 1 KO mice appeared intact compared to their wild-type (WT) littermates, with all layers present and no obvious abnormalities (data not shown). However, upon closer examination, we found that SynCAM 1 KO mice have 12% and 20% thinner ONL and OPL, respectively, compared to WT mice ($KO_{OPL}=9.43\pm0.05\ \mu\text{m}$, $WT_{OPL}=10.73\pm0.34\ \mu\text{m}$, $N=3$, $p=0.019$; $KO_{ONL}=25.25\pm1.34\ \mu\text{m}$, $WT_{ONL}=31.42\pm0.26\ \mu\text{m}$, $N=3$, $p=0.01$; Student's *t*-test, $N=3/\text{group}$). The thickness of other layers was comparable in KO and WT (data not shown), as well as the overall width of the central retina ($WT=154\pm2\ \mu\text{m}$, $KO=157\pm12\ \mu\text{m}$; $N=3/\text{group}$). The density of nuclei in the ONL was not significantly different between KO and WT as measured in semi-thick sections of central retina ($WT=0.15\pm0.01\ \text{nuclei}/\mu\text{m}^3$; $KO=0.14\pm0.04\ \text{nuclei}/\mu\text{m}^3$; $N=3/\text{group}$), making it unlikely that the reduced ONL thickness in SynCAM 1 KO mice might be due to a decreased density of photoreceptors.

We next examined general retinal morphology using previously described markers of different retinal cell types (Hoon et al., 2009). Photoreceptor terminals labeled with antibodies against VGlut1 and PSD-95 (Koulén et al., 1998) appeared normal in distribution and intensity in KO mice (Fig. 5A–D). Synaptic ribbons labeled with antibody against Ribeye exhibited their typical horse-shoe shape in both WT and KO mice, and had an apparently normal distribution and intensity in KO mice (Fig. 5E and F) (Regus-Leidig et al., 2010; Schmitz et al., 2000). As expected from the photopic ERG recordings, labeling with PNA showed a normal distribution of cone terminals in the OPL of SynCAM 1 KO mice (Fig. 5G and H) (Hoon et al., 2009). The morphology of rod bipolar cells analyzed with anti-PKC α antibody (Ruether et al., 2010) was indistinguishable between WT and SynCAM 1 KO mice, and neither were any differences observed for bipolar cell dendritic tips labeled with antibody against mGluR6 (Fig. 5I–L) (Cooper et al., 2012). However, horizontal cells exhibited morphological defects in SynCAM 1 KO mice (Fig. 5M–P). The horizontal cell marker Calbindin (Hirano et al., 2011) was detected in an ordered series of horizontal cell bodies just above the OPL in WT mice, while Neurofilament staining showed their processes extending through the OPL, but never crossing into the ONL (Fig. 5M) (Bayley and Morgans, 2007). In contrast, horizontal cell processes in SynCAM 1 KO mice sprouted into the ONL (Fig. 5N). Horizontal cell axons labeled with Neurofilament antibody exhibited ectopic growth, while Calbindin staining appeared patchy and disorganized compared to WT (Fig. 5N, arrowheads). Similar to Neurofilament labeling, Calbindin-positive processes also sprouted into the ONL of KO mice (Fig. 5N, arrowheads). We did not detect ectopic ribbons in the SynCAM 1 KO ONL with anti-Ribeye antibodies (Fig. 5P), nor did we detect an altered distribution of general inner nuclear, inner plexiform and ganglion cell layer (INL, IPL and GCL) markers (data not shown). In addition, we did not detect any abnormalities in GFAP and Vimentin staining in SynCAM 1 KO retinas, indicating absence of retinal degeneration (data not shown). SynCAM 1 KO mice hence exhibit impairments in retinal morphology that appeared limited to the ONL and OPL, as well as to horizontal cells.

Loss of SynCAM 1 impairs morphological development of horizontal cells

Thinning of retinal layers, as well as sprouting of bipolar and horizontal cells, has been commonly observed in aged mouse retina (Matsuoka et al., 2012; Samuel et al., 2011). As adult SynCAM 1 KO mice exhibited sprouting of horizontal cell processes (Fig. 5M–P), we proceeded to determine if this is due to premature aging or impairment in the development of horizontal cells. We compared Calbindin immunoreactivity between WT and SynCAM 1 KO retinas at different ages (Fig. 6A–H) (Matsuoka et al., 2012). At P4, Calbindin-positive cells, as well as the initial network of their lateral processes, could already be seen arranged in a single layer in both WT and SynCAM 1 KO mice (Fig. 6A and B) (Sharma et al., 2003). The density of the processes increased by P10, where OPL is already formed in both WT and SynCAM 1 KO mice (Fig. 6C and D). However, at both P4 and P10, horizontal cell processes could be seen extending outside of the OPL in SynCAM 1 KO retinas (Fig. 6B and D), unlike in WT retinas (Fig. 6A and C) (Sharma et al., 2003). Final stages of triad formation at P14 (Blanks et al., 1974b) revealed a rich plexus of horizontal cell processes in the WT retina (Fig. 6E). It is at this stage when the sprouting in SynCAM 1 KO retina became prominent (Fig. 6F; arrowheads). Calbindin-stained processes could be seen in the P14 KO retina extending throughout the entire ONL, similar to what we observed in adult KO retina (Fig. 5N and Fig. 6H). We have not observed alterations in the development and morphology of bipolar cells in SynCAM 1 KO retinas by PKC α staining at any developmental stage we examined (P10, P14 and adult/P40; data not shown). These results support that a likely cause for the presence of ectopic horizontal cell neurites in adult SynCAM 1 KO retina is a defect in horizontal cells development, and not premature retinal degeneration.

Ultrastructure and molecular composition of rod ribbon synapses are altered in SynCAM 1 KO mice

The distribution of synaptic markers in the OPL appeared normal in KO mice at a light microscopic level (Fig. 5A–H). However, SynCAM 1 KO mice display functional defects in the rod visual pathway (Fig. 3A–D). As loss of SynCAM 1 results in functional and ultrastructural abnormalities in classical excitatory synapses as represented by hippocampal synapses in CA1 (Robbins et al., 2010), we examined the ultrastructure of SynCAM 1 KO photoreceptors and their synapses in the OPL in order to detect fine structural abnormalities that might account for functional impairments seen in ERGs of SynCAM 1 KO mice.

We measured the ultrastructural parameters of ONL, IS and OS (number of nuclei, internuclear distance, cell body size, length and width of OS and IS, as well as the number and distribution of membranous discs in OS), but were unable to detect any abnormalities in the structure of photoreceptor cell bodies or their IS and OS (data not shown). We then studied ribbon synapses formed between rod photoreceptors, bipolar cells and horizontal cells. Ribbon synapses are characterized by an electron-dense, plate-like structure called the ribbon, anchored to the membrane of presynaptic photoreceptor terminals (Schmitz, 2009). It is decorated with synaptic vesicles and is involved in tonic vesicle release from photoreceptor terminals (Snellman et al., 2011). When viewed in cross-section, ribbons appear as elongated structures anchored to the photoreceptor plasma membrane and in direct apposition to bipolar cell dendrites in the OPL, with horizontal cell processes settled laterally (Fig. 7A–C) (tom Dieck and Brandstätter, 2006). All triad elements of ribbon synapses are present in SynCAM 1 KO mice (Fig. 7D and E). Synaptic vesicles fill the terminal and are anchored to the ribbon in both WT and KO mice (Fig. 7C and E). We used stereology to quantify a range of parameters of ribbon synapse ultrastructure (Table 2). As expected, we detected very few cone terminals in our sections (Carter-Dawson and LaVail, 1979), and they were excluded from this quantitative analysis. The results revealed significant differences in rod terminal structure between WT and KO mice. While the

density of rod terminals in sections of KO mouse retina was similar to WT mice, the number of ribbon-containing rod terminals was significantly lower in SynCAM 1 KO mice (Table 2). In addition, their ribbons were shorter by $27.6 \pm 5.8\%$ in KO mice. Rod terminal perimeter was also reduced by $12.2 \pm 1.5\%$ compared to WT mice. Furthermore, the fraction of terminals with triads in KO mice was almost half the value of their WT littermates ($KO = 56 \pm 5.5\%$ of WT). To assess ultrastructural properties of horizontal and bipolar cell processes, we measured their perimeter in rod terminals and found a significant reduction in the size of horizontal ($18.2 \pm 5.8\%$ compared to WT), but not bipolar, cell processes. A number of other parameters were unchanged in KO mice, such as the number of dyads, terminals with multiple ribbons and non-associated lateral elements (Table 2). We also found no difference between WT and KO mice in vesicle density (Table 2). To further determine the extent of changes in ribbons in SynCAM 1 KO mice, we measured their length and density throughout different developmental stages of the retina in high-resolution images obtained with confocal microscopy using an antibody against Ribeye (Fig. 8). Ribeye immunoreactivity in both WT and SynCAM 1 KO mice was in agreement with previously published studies (Regus-Leidig et al., 2009). Ribeye-stained spheres could be detected as early as P4 in both WT and KO mice (Fig. 8A and B), without any differences in their density between the genotypes (Fig. 8J). Horseshoe-shaped ribbons were detectable at P10 in both WT and KO (Fig. 8C and D). The length and the density of horseshoe-shaped ribbons increased at P14 in WT retina (Fig. 8E, F, I and J), but spheres were still detectable (Regus-Leidig et al., 2009). Ribbons of SynCAM 1 KO mice at P14 were of almost identical length to WT ribbons (Fig. 8I). By P40, all Ribeye-stained objects were horseshoe-shaped (Fig. 8G and H). In agreement with the ultrastructural measurements performed in adult retinas, we observed a significant reduction of ribbon length in P40 SynCAM 1 KO mice by $11 \pm 2.2\%$ compared to WT retina. Moreover, the density of ribbons in P40 KO retinas was decreased by $26.8 \pm 5.8\%$ (Fig. 8I and J).

We additionally performed quantitative western blotting of retinal homogenates from dark-adapted adult WT and SynCAM 1 KO retina (Fig. 8K and L). Ribeye protein is the major structural component of ribbons (Schmitz et al., 2000; Zenisek et al., 2004). Interestingly, we found that the level of Ribeye protein was reduced by $36 \pm 3.8\%$ in SynCAM 1 KO retina consistent with the reduction in ribbon density and size at P40 (Table 2 and Fig. 8I–L). Levels of other synaptic proteins (SNAP 25 and SV2) were not significantly changed (data not shown). These results revealed specific alterations in the molecular composition and ultrastructure of ribbon synapses in SynCAM 1 KO mice.

DISCUSSION

Synaptic adhesion molecules, as well as extracellular matrix and glial cell proteins, have critical roles in the induction of pre- and postsynaptic structures and their assembly into classical synapses (Eroglu and Barres, 2010; Missler et al., 2012). Adhesion molecules also contribute to the formation of classical synapses in the inner retina (Fuerst and Burgess, 2009), but their roles in the development of outer retinal layers remain incompletely understood. Analyzing the retina, we here report the select enrichment of SynCAM 1 in the outer retinal layers. The loss of SynCAM 1 in turn had significant effects on the structure and function of rod photoreceptors. Our study also points to a novel role for synaptic adhesion beyond classical synapses by providing evidence that SynCAM 1 functions in the structural modulation of photoreceptor ribbon synapses in the retina.

Distribution and subcellular localization of SynCAM 1 in the retina

Retinal expression of SynCAM 1 has been reported in different species, including mice (Fujita et al., 2005; Pietri et al., 2008; Wahlin et al., 2008). However, a detailed study on the expression and function of retinal SynCAM 1 has been lacking. This study shows for the

first time that SynCAM 1 is expressed throughout the developing and adult mouse retina, and that SynCAM 1 is particularly enriched on cell bodies and terminals of mature rod photoreceptors. This is also the first study to demonstrate in ultrastructural detail the specific localization of SynCAM 1 in the CNS. Antibodies used in a previous study (Biederer et al., 2002) recognize SynCAMs 1, 2, and 3 equally well (Fogel et al., 2007) and the precise localization of SynCAM 1 in the CNS remained to be determined (Fogel et al., 2011; Xi et al., 2007). Our immuno-EM study of photoreceptors detected endogenous SynCAM 1 at their synaptic terminal membranes (Fig. 2).

These findings significantly expand previous studies of SynCAM 1 localization at classical synapses by light microscopic analysis of hippocampus (Fogel et al., 2011; Xi et al., 2007) and the presynaptic localization of exogenously expressed SynCAM 1 detected by EM in cultured cortical neurons (Shu et al., 2011). As our light microscopy already suggested (Fig. 2), SynCAM 1 was not concentrated at synaptic sites in the OPL, but was evenly distributed on the terminal membrane, indicating roles in cell surface interactions throughout this synaptic region. In agreement with general roles in the organization of photoreceptor synaptic membranes, we additionally detected SynCAM 1 at the interface of photoreceptor terminals and horizontal cell processes. SynCAM 1 was also found in other retinal layers (Fig. 1), but its relative abundance in the photoreceptor layer agree with a role in the structural and functional integrity of photoreceptors and their synapses.

Modulation of retinal function by SynCAM 1

Our ERG recordings demonstrated that visual signal transmission in the scotopic (rod) pathway is impaired in SynCAM 1 KO mice. Normal double-flash ERG responses, the apparent lack of retinal degeneration indicated by normal appearance of glial markers (data not shown), as well as the overall normal ultrastructure of the ONL and OS (data not shown), suggest that these functional differences do not stem from altered photoreceptor number or the ultrastructure of their cell bodies and outer segments. The importance of gap junction adhesion proteins in synaptic transmission at the OPL is well known (Bloomfield and Volgyi, 2009; Kamermans et al., 2001; Kranz et al., 2012). Our study for the first time implicates a classical synaptic adhesion molecule (Missler et al., 2012) in the rod visual pathway.

The increased scotopic a-wave we measured in SynCAM 1 KO mice is extremely rare in animal models of retinal dysfunction (Brockerhoff et al., 1997; Kameya et al., 1997; Kranz et al., 2012) and could be a cause of delay in b-wave time-to-peak in SynCAM 1 KO mice. What may cause this enhanced amplitude of rod responses to light? This effect of SynCAM 1 loss might be due to abnormal phototransduction in the outer segments (Knop et al., 2008; Liu et al., 2005; Seeliger et al., 2011). This is usually assessed with double-flash ERG, which monitors recovery of photoreceptor responses (Lyubarsky and Pugh, 1996). However, recovery was not impaired in SynCAM 1 KO mice. We therefore consider it unlikely that the increased a-wave in SynCAM 1 KO mice stems from a dysfunction of the rod outer segments. Alternatively, the functional block of channels that localize near the photoreceptor terminal membrane can affect the a-wave properties (Chang et al., 2006; Kranz et al., 2012; Mansergh et al., 2005). Adhesion molecules organize and cluster different classes of channels on the neuronal membranes (Gu and Gu, 2011; Leterrier et al., 2011; Missler et al., 2003). Hence, a cause of defective a-wave seen in SynCAM 1 KO mice may be impaired clustering of ion channels at the photoreceptor terminal membranes. As SynCAM 1 organizes synapses in hippocampal neurons (Cheadle and Biederer, 2012; Fogel et al., 2011) and is present at rod terminal membranes in the OPL, this possibility can now be tested.

Recent studies have also highlighted a role of negative feedback from horizontal cells to photoreceptors (Liu et al., 2013). Abnormal development of horizontal cells in SynCAM 1

KO mice, as well as a reduction in the surface area of their processes within ribbon synapses in adult mice, may cause an impairment in negative feedback to rods and hence contribute to the defect in rod response upon light stimulation. The localization of SynCAM 1 near the horizontal cell processes supports this notion. A recent study has suggested such impaired feedback from horizontal cells to rod photoreceptors as a cause of increased a-wave amplitude in mice lacking Pannexin1, a membrane protein related to the gap junction-forming Connexins (Kranz et al., 2012). Scotopic a-wave also has post-receptoral contributions, mainly from the OFF-pathway (Dang et al., 2011; Robson and Frishman, 1998). Additionally, Müller cells can contribute to photoreceptor responses (Witkovsky et al., 1975). However, we did not detect SynCAM 1 on Müller glia and we did not observe any abnormalities in their morphology or the morphology of bipolar cells (data not shown), which makes their involvement in SynCAM 1 functions unlikely. Due to the mixed nature of the ERG response (Robson and Frishman, 1998), the precise origin of impairments in scotopic ERG of SynCAM 1 KO mice remains to be defined.

Altered structural and molecular organization of photoreceptor ribbon synapses in the absence of SynCAM 1

The temporal and spatial expression profile of SynCAM 1 closely follows the structural and functional development of outer retinal layers and photoreceptor synapses. A key finding in support of a role for SynCAM 1 in the formation of these synapses is the decrease in density of terminals with ribbons in SynCAM 1 KO mice by 28.4 ± 9.3 . Perhaps even more importantly, KO mice have a significant $56 \pm 5.5\%$ reduction in the number of synaptic triads formed between rod terminals, bipolar cell dendrites and horizontal cell processes. No other structural abnormalities were observed, such as free-floating or club-shaped ribbons seen in other mouse models with retinal defects (Dick et al., 2003; Reim et al., 2009). The number of other structures in retinal sections (dyads between rod spherules and horizontal cells, for example) was surprisingly unaffected in KO mice, contrasting with other mutants where a reduction in triad density is closely correlated with an increase in dyad density (Blanks et al., 1974a; Bramblett et al., 2004; Sato et al., 2008). The significant reduction in Ribeye protein expression in adult SynCAM 1 KO retina provides biochemical support for these structural results in rod photoreceptor terminals. Ribeye is the core structural component of ribbons (Zanazzi and Matthews, 2009), both in the OPL and in the IPL (Johnson et al., 2003). While a decrease in its expression in IPL synapses of SynCAM 1 KO mice may contribute to its observed overall reduction in their retina, Ribeye is enriched in the OPL, as compared to IPL (Schmitz et al., 2000). The Ribeye reduction in SynCAM 1 KO mice therefore likely reflects changes in the OPL. The reduced Ribeye level in adult KO retina further agrees with the reduction of ribbon density and length observed here.

SynCAM 1 in the hippocampus contributes to both early stages of synapse induction and their later maintenance (Robbins et al., 2010). The developmental expression profile of SynCAM 1 in the retina would agree with synaptogenic functions as the formation of synapses in the OPL starts around P5 (Sharma et al., 2003). Results of our study demonstrated that ribbons in SynCAM 1 KO mice develop normally until P14 and that their number and length are reduced only in adult animals. Final stages of triad formation occur at P14 (Blanks et al., 1974a) and SynCAM 1 expression in the retina continues to increase after P14. Together, this points to roles of SynCAM 1 in the final steps of the assembly of rod ribbon synapses and their maintenance. An unexpected finding of this study related to the development of outer retina is that SynCAM 1 may guide proper development and targeting of horizontal cell (HC) processes in the OPL (Sharma et al., 2003). Ectopic horizontal cell processes can be seen as early as P4 in SynCAM 1 KO mice, and their development appeared abnormal at all later stages examined. No abnormalities were detected in the morphology of bipolar cells in SynCAM 1 KO mice (data not shown), which differs for the

coupled horizontal and bipolar cell aberrations e.g. in mice carrying mutations in ribbon synapse proteins (Dick et al., 2003; Haeseleer et al., 2004; Strettoi et al., 2002). The select effect of SynCAM 1 loss on horizontal cell differentiation is reminiscent of mice deficient in plexin/semaphorin signaling, as well as mice lacking adhesive interactions by the protein NGL-2 (Matsuoka et al., 2012; Soto et al., 2013). SynCAM 1-mediated cell surface interactions may therefore contribute to the proper targeting of horizontal cell neurites during development, perhaps reminiscent of the roles of this protein in shaping axonal growth cones (Stagi et al., 2010).

SynCAM 1 in classical versus ribbon synapses: evidence for general contributions to synapse organization and function

Roles of SynCAM 1 at classical synapses are well described (Fogel et al., 2007; Robbins et al., 2010). Interestingly, the reduced triad numbers and shortened ribbons in the KO retina are consistent with the decreased synapse density and shortened active zones in the hippocampus (Robbins et al., 2010). Unlike Neuroligins (Hoon et al., 2009; Hoon et al., 2011) or other immunoglobulin-family proteins, such as DSCAMs (Fuerst et al., 2009), that contribute to the development and function of the IPL, SynCAM 1 in the retina appears to predominantly provide for the structural and functional integrity of photoreceptor synapses in the OPL. To the best of our knowledge, SynCAM 1 is the first synaptic adhesion molecule with complementary roles at different types of synapses, including ribbon synapses.

The results we obtained in the outer retina extend the well-defined effects of adhesion molecules on cell- and synapse- specification of the inner retina (Yamagata and Sanes, 2012). Further, these findings indicate a general role for SynCAM 1-mediated adhesion in the organization of excitatory synapses and neuronal circuits, such as reported here. Our results warrant studies of visual signal processing in KO mice, especially considering that they show sufficient vision in gross behavioral vision assays (Robbins et al., 2010).

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References

- Abd-El-Barr MM, Pennesi ME, Saszik SM, Barrow AJ, Lem J, Bramblett DE, Paul DL, Frishman LJ, Wu SM. Genetic dissection of rod and cone pathways in the dark-adapted mouse retina. *Journal of Neurophysiology*. 2009; 102(3):1945–1955. [PubMed: 19587322]
- Balkema GW, Dräger UC. Light-dependent antibody labelling of photoreceptors. *Nature*. 1985; 316(6029):630–633. [PubMed: 2412124]
- Bayley PR, Morgans CW. Rod bipolar cells and horizontal cells form displaced synaptic contacts with rods in the outer nuclear layer of the nob2 retina. *The Journal of Comparative Neurology*. 2007; 500(2):286–298. [PubMed: 17111373]
- Biederer T, Sara Y, Mozhayeva M, Atasoy D, Liu X, Kavalali ET, Südhof TC. SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science*. 2002; 297(5586):1525–1531. [PubMed: 12202822]

- Blanks JC, Adinolfi AM, Lolley RN. Photoreceptor degeneration and synaptogenesis in retinal-degenerative (rd) mice. *The Journal of Comparative Neurology*. 1974a; 156(1):95–106. [PubMed: 4836657]
- Blanks JC, Adinolfi AM, Lolley RN. Synaptogenesis in the photoreceptor terminal of the mouse retina. *The Journal of Comparative Neurology*. 1974b; 156(1):81–93. [PubMed: 4836656]
- Bloomfield SA, Volgyi B. The diverse functional roles and regulation of neuronal gap junctions in the retina. *Nature Reviews Neuroscience*. 2009; 10(7):495–506.
- Bramblett DE, Pennesi ME, Wu SM, Tsai MJ. The transcription factor Bhlhb4 is required for rod bipolar cell maturation. *Neuron*. 2004; 43(6):779–793. [PubMed: 15363390]
- Brockherhoff SE, Hurley JB, Niemi GA, Dowling JE. A new form of inherited red-blindness identified in zebrafish. *The Journal of Neuroscience*. 1997; 17(11):4236–4242. [PubMed: 9151740]
- Brown KT, Watanabe K. Isolation and identification of a receptor potential from the pure cone fovea of the monkey retina. *Nature*. 1962a; 193:958–960. [PubMed: 13873685]
- Brown KT, Watanabe K. Rod receptor potential from the retina of the night monkey. *Nature*. 1962b; 196:547–550. [PubMed: 14016006]
- Brown KT, Watanabe K, Murakami M. The early and late receptor potentials of monkey cones and rods. *Cold Spring Harbor Symposia on Quantitative Biology*. 1965; 30:457–482.
- Carter-Dawson LD, LaVail MM. Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. *The Journal of Comparative Neurology*. 1979; 188(2):245–262. [PubMed: 500858]
- Chang B, Heckenlively JR, Bayley PR, Brecha NC, Davisson MT, Hawes NL, Hirano AA, Hurd RE, Ikeda A, Johnson BA, McCall MA, Morgans CW, Nusinowitz S, Peachey NS, Rice DS, Vessey KA, Gregg RG. The nob2 mouse, a null mutation in *Cacna1f*: anatomical and functional abnormalities in the outer retina and their consequences on ganglion cell visual responses. *Visual Neuroscience*. 2006; 23(1):11–24. [PubMed: 16597347]
- Cheadle L, Biederer T. The novel synaptogenic protein Farp1 links postsynaptic cytoskeletal dynamics and transsynaptic organization. *The Journal of Cell Biology*. 2012; 199(6):985–1001. [PubMed: 23209303]
- Cooper B, Hemmerlein M, Ammermüller J, Imig C, Reim K, Lipstein N, Kalla S, Kawabe H, Brose N, Brandstätter JH, Varoqueaux F. Munc13-independent vesicle priming at mouse photoreceptor ribbon synapses. *The Journal of Neuroscience*. 2012; 32(23):8040–8052. [PubMed: 22674279]
- Dang TM, Tsai TI, Vingrys AJ, Bui BV. Post-receptor contributions to the rat scotopic electroretinogram a-wave. *Documenta Ophthalmologica Advances in Ophthalmology*. 2011; 122(3):149–156. [PubMed: 21465307]
- Dick O, tom Dieck S, Altrock WD, Ammermüller J, Weiler R, Garner CC, Gundelfinger ED, Brandstätter JH. The presynaptic active zone protein bassoon is essential for photoreceptor ribbon synapse formation in the retina. *Neuron*. 2003; 37(5):775–786. [PubMed: 12628168]
- Duncan JL, Yang H, Doan T, Silverstein RS, Murphy GJ, Nune G, Liu X, Copenhagen D, Tempel BL, Rieke F, Krizaj D. Scotopic visual signaling in the mouse retina is modulated by high-affinity plasma membrane calcium extrusion. *The Journal of Neuroscience*. 2006; 26(27):7201–7211. [PubMed: 16822977]
- Ekesten B, Gouras P, Moschos M. Cone properties of the light-adapted murine ERG. *Documenta Ophthalmologica Advances in Ophthalmology*. 1998; 97(1):23–31. [PubMed: 10710239]
- Eroglu C, Barres BA. Regulation of synaptic connectivity by glia. *Nature*. 2010; 468(7321):223–231. [PubMed: 21068831]
- Fisher LJ. Development of synaptic arrays in the inner plexiform layer of neonatal mouse retina. *The Journal of Comparative Neurology*. 1979; 187(2):359–372. [PubMed: 489784]
- Fogel AI, Akins MR, Krupp AJ, Stagi M, Stein V, Biederer T. SynCAMs organize synapses through heterophilic adhesion. *The Journal of Neuroscience*. 2007; 27(46):12516–12530. [PubMed: 18003830]
- Fogel AI, Stagi M, Perez de Arce K, Biederer T. Lateral assembly of the immunoglobulin protein SynCAM 1 controls its adhesive function and instructs synapse formation. *The EMBO Journal*. 2011; 30(23):4728–4738. [PubMed: 21926970]

- Fuerst PG, Bruce F, Tian M, Wei W, Elstrott J, Feller MB, Erskine L, Singer JH, Burgess RW. DSCAM and DSCAML1 function in self-avoidance in multiple cell types in the developing mouse retina. *Neuron*. 2009; 64(4):484–497. [PubMed: 19945391]
- Fuerst PG, Burgess RW. Adhesion molecules in establishing retinal circuitry. *Current Opinion in Neurobiology*. 2009; 19(4):389–394. [PubMed: 19660931]
- Fujita E, Kouroku Y, Ozeki S, Tanabe Y, Toyama Y, Maekawa M, Kojima N, Senoo H, Toshimori K, Momoi T. Oligo-astheno-teratozoospermia in mice lacking RA175/TSLC1/SynCAM/IGSF4A, a cell adhesion molecule in the immunoglobulin superfamily. *Molecular and Cellular Biology*. 2006; 26(2):718–726. [PubMed: 16382161]
- Fujita E, Tanabe Y, Hirose T, Aurrand-Lions M, Kasahara T, Imhof BA, Ohno S, Momoi T. Loss of partitioning-defective-3/isotype-specific interacting protein (par-3/ASIP) in the elongating spermatid of RA175 (IGSF4A/SynCAM)-deficient mice. *The American Journal of Pathology*. 2007; 171(6):1800–1810. [PubMed: 18055550]
- Fujita E, Urase K, Soyama A, Kouroku Y, Momoi T. Distribution of RA175/TSLC1/SynCAM, a member of the immunoglobulin superfamily, in the developing nervous system. *Brain Research Developmental Brain Research*. 2005; 154(2):199–209. [PubMed: 15707673]
- Gu C, Gu Y. Clustering and activity tuning of Kv1 channels in myelinated hippocampal axons. *The Journal of Biological Chemistry*. 2011; 286(29):25835–25847. [PubMed: 21602278]
- Haeseleer F, Imanishi Y, Maeda T, Possin DE, Maeda A, Lee A, Rieke F, Palczewski K. Essential role of Ca²⁺-binding protein 4, a Cav1.4 channel regulator, in photoreceptor synaptic function. *Nature Neuroscience*. 2004; 7(10):1079–1087.
- Haverkamp S, Wässle H. Immunocytochemical analysis of the mouse retina. *The Journal of Comparative Neurology*. 2000; 424(1):1–23. [PubMed: 10888735]
- Heidelberger R, Thoreson WB, Witkovsky P. Synaptic transmission at retinal ribbon synapses. *Progress in Retinal and Eye Research*. 2005; 24(6):682–720. [PubMed: 16027025]
- Hirano AA, Brandstätter JH, Morgans CW, Brecha NC. SNAP25 expression in mammalian retinal horizontal cells. *The Journal of Comparative Neurology*. 2011; 519(5):972–988. [PubMed: 21280047]
- Hoon M, Bauer G, Fritschy JM, Moser T, Falkenburger BH, Varoqueaux F. Neuroligin 2 controls the maturation of GABAergic synapses and information processing in the retina. *The Journal of Neuroscience*. 2009; 29(25):8039–8050. [PubMed: 19553444]
- Hoon M, Soykan T, Falkenburger B, Hammer M, Patrizi A, Schmidt KF, Sassoe-Pognetto M, Lowel S, Moser T, Taschenberger H, Brose N, Varoqueaux F. Neuroligin-4 is localized to glycinergic postsynapses and regulates inhibition in the retina. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108(7):3053–3058. [PubMed: 21282647]
- Howes KA, Pennesi ME, Sokal I, Church-Kopish J, Schmidt B, Margolis D, Frederick JM, Rieke F, Palczewski K, Wu SM, Detwiler PB, Baehr W. GCAP1 rescues rod photoreceptor response in GCAP1/GCAP2 knockout mice. *The EMBO Journal*. 2002; 21(7):1545–1554. [PubMed: 11927539]
- Jinno S, Aika Y, Fukuda T, Kosaka T. Quantitative analysis of GABAergic neurons in the mouse hippocampus, with optical disector using confocal laser scanning microscope. *Brain research*. 1998; 814(1–2):55–70. [PubMed: 9838044]
- Johnson J, Tian N, Caywood MS, Reimer RJ, Edwards RH, Copenhagen DR. Vesicular neurotransmitter transporter expression in developing postnatal rodent retina: GABA and glycine precede glutamate. *The Journal of Neuroscience*. 2003; 23(2):518–529. [PubMed: 12533612]
- Kamermans M, Fahrenfort I, Schultz K, Janssen-Bienhold U, Sjoerdsma T, Weiler R. Hemichannel-mediated inhibition in the outer retina. *Science*. 2001; 292(5519):1178–1180. [PubMed: 11349152]
- Kameya S, Araki E, Katsuki M, Mizota A, Adachi E, Nakahara K, Nonaka I, Sakuragi S, Takeda S, Nabeshima Y. Dp260 disrupted mice revealed prolonged implicit time of the b-wave in ERG and loss of accumulation of beta-dystroglycan in the outer plexiform layer of the retina. *Human Molecular Genetics*. 1997; 6(13):2195–2203. [PubMed: 9361023]

- Kim TS, Maeda A, Maeda T, Heinlein C, Kedishvili N, Palczewski K, Nelson PS. Delayed dark adaptation in 11-cis-retinol dehydrogenase-deficient mice: a role of RDH11 in visual processes in vivo. *The Journal of Biological Chemistry*. 2005; 280(10):8694–8704. [PubMed: 15634683]
- Knop GC, Seeliger MW, Thiel F, Mataruga A, Kaupp UB, Friedburg C, Tanimoto N, Muller F. Light responses in the mouse retina are prolonged upon targeted deletion of the HCN1 channel gene. *The European Journal of Neuroscience*. 2008; 28(11):2221–2230. [PubMed: 19019198]
- Koulen P, Fletcher EL, Craven SE, Brecht DS, Wässle H. Immunocytochemical localization of the postsynaptic density protein PSD-95 in the mammalian retina. *The Journal of Neuroscience*. 1998; 18(23):10136–10149. [PubMed: 9822767]
- Kranz K, Dorgau B, Pottel M, Herrling R, Schultz K, Bolte P, Monyer H, Penuela S, Laird DW, Dedek K, Weiler R, Janssen-Bienhold U. Expression of Pannexin1 in the outer plexiform layer of the mouse retina and physiological impact of its knock-out. *The Journal of Comparative Neurology*. 2012
- Kueng-Hitz N, Rol P, Niemeyer G. The electroretinogram (ERG) of the mouse: normal values, optimal stimulation and recording. *Klinische Monatsblätter für Augenheilkunde*. 1999; 214(5):288–290. [PubMed: 10420369]
- Lefebvre JL, Zhang Y, Meister M, Wang X, Sanes JR. gamma-Protocadherins regulate neuronal survival but are dispensable for circuit formation in retina. *Development*. 2008; 135(24):4141–4151. [PubMed: 19029044]
- Leterrier C, Brachet A, Dargent B, Vacher H. Determinants of voltage-gated sodium channel clustering in neurons. *Seminars in Cell & Developmental Biology*. 2011; 22(2):171–177. [PubMed: 20934527]
- Li J, Cline HT. Visual deprivation increases accumulation of dense core vesicles in developing optic tectal synapses in *Xenopus laevis*. *The Journal of comparative neurology*. 2010; 518(12):2365–2381. [PubMed: 20437533]
- Liu J, Timmers AM, Lewin AS, Hauswirth WW. Ribozyme knockdown of the gamma-subunit of rod cGMP phosphodiesterase alters the ERG and retinal morphology in wild-type mice. *Investigative Ophthalmology & Visual Science*. 2005; 46(10):3836–3844. [PubMed: 16186371]
- Liu X, Hirano AA, Sun X, Brecha NC, Barnes S. Calcium channels in rat horizontal cells regulate feedback inhibition of photoreceptors through an unconventional GABA- and pH-sensitive mechanism. *The Journal of Physiology*. 2013
- Lyubarsky AL, Pugh EN Jr. Recovery phase of the murine rod photoresponse reconstructed from electrophoretographic recordings. *The Journal of Neuroscience*. 1996; 16(2):563–571. [PubMed: 8551340]
- Mansergh F, Orton NC, Vessey JP, Lalonde MR, Stell WK, Tremblay F, Barnes S, Rancourt DE, Bech-Hansen NT. Mutation of the calcium channel gene *Cacna1f* disrupts calcium signaling, synaptic transmission and cellular organization in mouse retina. *Human Molecular Genetics*. 2005; 14(20):3035–3046. [PubMed: 16155113]
- Matsuoka RL, Jiang Z, Samuels IS, Nguyen-Ba-Charvet KT, Sun LO, Peachey NS, Chedotal A, Yau KW, Kolodkin AL. Guidance-cue control of horizontal cell morphology, lamination, and synapse formation in the mammalian outer retina. *The Journal of Neuroscience*. 2012; 32(20):6859–6868. [PubMed: 22593055]
- Missler, M.; Südhof, TC.; Biederer, T. Cold Spring Harbor Perspectives in Biology. 2012. Synaptic Cell Adhesion.
- Missler M, Zhang W, Rohlmann A, Kattenstroth G, Hammer RE, Gottmann K, Südhof TC. Alpha-neurexins couple Ca²⁺ channels to synaptic vesicle exocytosis. *Nature*. 2003; 423(6943):939–948. [PubMed: 12827191]
- Peachey NS, Ball SL. Electrophysiological analysis of visual function in mutant mice. *Documenta Ophthalmologica Advances in Ophthalmology*. 2003; 107(1):13–36. [PubMed: 12906119]
- Pietri T, Easley-Neal C, Wilson C, Washbourne P. Six *cadm/SynCAM* genes are expressed in the nervous system of developing zebrafish. *Developmental Dynamics*. 2008; 237(1):233–246. [PubMed: 18095341]

- Pinto LH, Invergo B, Shimomura K, Takahashi JS, Troy JB. Interpretation of the mouse electroretinogram. *Documenta Ophthalmologica Advances in Ophthalmology*. 2007; 115(3):127–136. [PubMed: 17636411]
- Regus-Leidig H, tom Dieck S, Brandstätter JH. Absence of functional active zone protein Bassoon affects assembly and transport of ribbon precursors during early steps of photoreceptor synaptogenesis. *European Journal of Cell Biology*. 2010; 89(6):468–475. [PubMed: 20188438]
- Regus-Leidig H, Tom Dieck S, Specht D, Meyer L, Brandstätter JH. Early steps in the assembly of photoreceptor ribbon synapses in the mouse retina: the involvement of precursor spheres. *The Journal of Comparative Neurology*. 2009; 512(6):814–824. [PubMed: 19067356]
- Reim K, Regus-Leidig H, Ammermüller J, El-Kordi A, Radyushkin K, Ehrenreich H, Brandstätter JH, Brose N. Aberrant function and structure of retinal ribbon synapses in the absence of complexin 3 and complexin 4. *Journal of Cell Science*. 2009; 122(Pt 9):1352–1361. [PubMed: 19386896]
- Robbins EM, Krupp AJ, Perez de Arce K, Ghosh AK, Fogel AI, Boucard A, Südhof TC, Stein V, Biederer T. SynCAM 1 adhesion dynamically regulates synapse number and impacts plasticity and learning. *Neuron*. 2010; 68(5):894–906. [PubMed: 21145003]
- Robson JG, Frishman LJ. Dissecting the dark-adapted electroretinogram. *Documenta ophthalmologica Advances in Ophthalmology*. 1998; 95(3–4):187–215. [PubMed: 10532405]
- Ruether K, Feigenspan A, Pirngruber J, Leitges M, Baehr W, Strauss O. PKC{alpha} is essential for the proper activation and termination of rod bipolar cell response. *Investigative Ophthalmology & Visual Science*. 2010; 51(11):6051–6058. [PubMed: 20554612]
- Samuel MA, Zhang Y, Meister M, Sanes JR. Age-related alterations in neurons of the mouse retina. *The Journal of Neuroscience*. 2011; 31(44):16033–16044. [PubMed: 22049445]
- Sato S, Omori Y, Katoh K, Kondo M, Kanagawa M, Miyata K, Funabiki K, Koyasu T, Kajimura N, Miyoshi T, Sawai H, Kobayashi K, Tani A, Toda T, Usukura J, Tano Y, Fujikado T, Furukawa T. Pikachurin, a dystroglycan ligand, is essential for photoreceptor ribbon synapse formation. *Nature Neuroscience*. 2008; 11(8):923–931.
- Schmitz F. The making of synaptic ribbons: how they are built and what they do. *The Neuroscientist*. 2009; 15(6):611–624. [PubMed: 19700740]
- Schmitz F, Königstorfer A, Südhof TC. RIBEYE, a component of synaptic ribbons: a protein's journey through evolution provides insight into synaptic ribbon function. *Neuron*. 2000; 28(3):857–872. [PubMed: 11163272]
- Seeliger MW, Brombas A, Weiler R, Humphries P, Knop G, Tanimoto N, Müller F. Modulation of rod photoreceptor output by HCN1 channels is essential for regular mesopic cone vision. *Nature Communications*. 2011; 2:532.
- Shapiro L, Love J, Colman DR. Adhesion molecules in the nervous system: structural insights into function and diversity. *Annual Review of Neuroscience*. 2007; 30:451–474.
- Sharma RK, O'Leary TE, Fields CM, Johnson DA. Development of the outer retina in the mouse. *Brain Research Developmental Brain Research*. 2003; 145(1):93–105. [PubMed: 14519497]
- Sherry DM, Wang MM, Bates J, Frishman LJ. Expression of vesicular glutamate transporter 1 in the mouse retina reveals temporal ordering in development of rod vs. cone and ON vs. OFF circuits. *The Journal of Comparative Neurology*. 2003; 465(4):480–498. [PubMed: 12975811]
- Shu X, Lev-Ram V, Deerinck TJ, Qi Y, Ramko EB, Davidson MW, Jin Y, Ellisman MH, Tsien RY. A genetically encoded tag for correlated light and electron microscopy of intact cells, tissues, and organisms. *PLoS Biology*. 2011; 9(4):e1001041. [PubMed: 21483721]
- Snellman J, Mehta B, Babai N, Bartoletti TM, Akmentin W, Francis A, Matthews G, Thoreson W, Zenisek D. Acute destruction of the synaptic ribbon reveals a role for the ribbon in vesicle priming. *Nature Neuroscience*. 2011; 14(9):1135–1141.
- Song X, Vishnivetskiy SA, Seo J, Chen J, Gurevich EV, Gurevich VV. Arrestin-1 expression level in rods: balancing functional performance and photoreceptor health. *Neuroscience*. 2011; 174:37–49. [PubMed: 21075174]
- Soto F, Watkins KL, Johnson RE, Schottler F, Kerschensteiner D. NGL-2 regulates pathway-specific neurite growth and lamination, synapse formation, and signal transmission in the retina. *The Journal of Neuroscience*. 2013; 33(29):11949–11959. [PubMed: 23864682]

- Stagi M, Fogel AI, Biederer T. SynCAM 1 participates in axo-dendritic contact assembly and shapes neuronal growth cones. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107(16):7568–7573. [PubMed: 20368431]
- Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. *Journal of microscopy*. 1984; 134(Pt 2):127–136. [PubMed: 6737468]
- Sterling P, Matthews G. Structure and function of ribbon synapses. *Trends in Neurosciences*. 2005; 28(1):20–29. [PubMed: 15626493]
- Strettoi E, Porciatti V, Falsini B, Pignatelli V, Rossi C. Morphological and functional abnormalities in the inner retina of the rd/rd mouse. *The Journal of Neuroscience*. 2002; 22(13):5492–5504. [PubMed: 12097501]
- Thomas LA, Akins MR, Biederer T. Expression and adhesion profiles of SynCAM molecules indicate distinct neuronal functions. *The Journal of Comparative Neurology*. 2008; 510(1):47–67. [PubMed: 18615557]
- tom Dieck S, Brandstätter JH. Ribbon synapses of the retina. *Cell and Tissue Research*. 2006; 326(2):339–346. [PubMed: 16775698]
- tom Dieck S, Specht D, Strenzke N, Hida Y, Krishnamoorthy V, Schmidt KF, Inoue E, Ishizaki H, Tanaka-Okamoto M, Miyoshi J, Hagiwara A, Brandstätter JH, Lowel S, Gollisch T, Ohtsuka T, Moser T. Deletion of the presynaptic scaffold CAST reduces active zone size in rod photoreceptors and impairs visual processing. *The Journal of Neuroscience*. 2012; 32(35):12192–12203. [PubMed: 22933801]
- Vessey KA, Greferath U, Jobling AI, Phipps JA, Ho T, Waugh M, Fletcher EL. Ccl2/Cx3cr1 knockout mice have inner retinal dysfunction but are not an accelerated model of AMD. *Investigative Ophthalmology & Visual Science*. 2012; 53(12):7833–7846. [PubMed: 23074204]
- Vistamehr S, Tian N. Light deprivation suppresses the light response of inner retina in both young and adult mouse. *Visual Neuroscience*. 2004; 21(1):23–37. [PubMed: 15137579]
- Wahlin KJ, Hackler L Jr, Adler R, Zack DJ. Alternative splicing of neuroligin and its protein distribution in the outer plexiform layer of the chicken retina. *The Journal of Comparative Neurology*. 2010; 518(24):4938–4962. [PubMed: 21031560]
- Wahlin KJ, Moreira EF, Huang H, Yu N, Adler R. Molecular dynamics of photoreceptor synapse formation in the developing chick retina. *The Journal of Comparative Neurology*. 2008; 506(5):822–837. [PubMed: 18076030]
- Watabe K, Ito A, Koma YI, Kitamura Y. IGSF4: a new intercellular adhesion molecule that is called by three names, TSLC1, SgIGSF and SynCAM, by virtue of its diverse function. *Histol Histopathol*. 2003; 18(4):1321–1329. [PubMed: 12973698]
- West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *The Anatomical record*. 1991; 231(4):482–497. [PubMed: 1793176]
- Weymouth AE, Vingrys AJ. Rodent electroretinography: methods for extraction and interpretation of rod and cone responses. *Progress in retinal and eye research*. 2008; 27(1):1–44. [PubMed: 18042420]
- Witkovsky P, Dudek FE, Ripps H. Slow PIII component of the carp electroretinogram. *The Journal of General Physiology*. 1975; 65(2):119–134. [PubMed: 1117278]
- Xi Q, Pauer GJ, Ball SL, Rayborn M, Hollyfield JG, Peachey NS, Crabb JW, Hagstrom SA. Interaction between the photoreceptor-specific tubby-like protein 1 and the neuronal-specific GTPase dynamin-1. *Investigative Ophthalmology & Visual Science*. 2007; 48(6):2837–2844. [PubMed: 17525220]
- Yamagata M, Sanes JR. Dscam and Sidekick proteins direct lamina-specific synaptic connections in vertebrate retina. *Nature*. 2008; 451(7177):465–469. [PubMed: 18216854]
- Yamagata M, Sanes JR. Expanding the Ig superfamily code for laminar specificity in retina: expression and role of contactins. *The Journal of Neuroscience*. 2012; 32(41):14402–14414. [PubMed: 23055510]
- Yang J, Pawlyk B, Wen XH, Adamian M, Soloviev M, Michaud N, Zhao Y, Sandberg MA, Makino CL, Li T. Mpp4 is required for proper localization of plasma membrane calcium ATPases and

maintenance of calcium homeostasis at the rod photoreceptor synaptic terminals. *Human Molecular Genetics*. 2007; 16(9):1017–1029. [PubMed: 17341488]

- Yen LH, Sibley JT, Constantine-Paton M. Fine-structural alterations and clustering of developing synapses after chronic treatments with low levels of NMDA. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 1993; 13(11):4949–4960. [PubMed: 8229207]
- Zenisek D, Horst NK, Merrifield C, Sterling P, Matthews G. Visualizing synaptic ribbons in the living cell. *The Journal of Neuroscience*. 2004; 24(44):9752–9759. [PubMed: 15525760]
- Zhang H, Li S, Doan T, Rieke F, Detwiler PB, Frederick JM, Baehr W. Deletion of PrBP/delta impedes transport of GRK1 and PDE6 catalytic subunits to photoreceptor outer segments. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104(21): 8857–8862. [PubMed: 17496142]

Figure 1. Expression of SynCAM 1 in the retina is developmentally regulated

A. Left, SynCAM 1 is detected as multiple bands around 100 kDa in the retina, indicating a similar extent of glycan modifications as in hippocampus (Fogel et al., 2007). SynCAM 1 KO retina served as specificity control for the antibody. Actin was a loading control. Molecular weights are indicated in kDa.

Right, maximum intensity projection of SynCAM 1 labeling throughout the adult mouse retina (P40) measured its prominent expression in outer nuclear and plexiform layers. Only low background staining was detected in SynCAM 1 KO. Scale bar, 30 μ m.

B. Top, expression of SynCAM 1 progressively increased during retinal development as detected by quantitative immunoblotting, starting to be detectable at P3 and steadily rising through P28. Bottom, quantification of immunoblots shows a steady increase in SynCAM 1 expression during development of retina. Data are represented as mean $\pm$ SEM of three independent experiments. N=2 animals/age point.

C. Gradual increase of SynCAM 1 expression was evident in immunohistochemistry. SynCAM 1 is initially expressed at low levels in all developing layers (P3). At P7, expression starts to be more detectable in the ONL. SynCAM 1 already appears enriched in ONL and OPL at P14, with a high expression level at P28. All images are maximum intensity projections of Z-stacks through central retina. Scale bar for all images, 50 μ m. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OS, outer segments; NBL, neuroblast layer; P, postnatal day.

Figure 2. SynCAM 1 is localized on photoreceptor cell bodies and surrounds terminal membranes

A. SynCAM 1 (green) in the ONL envelops DAPI-labeled photoreceptor nuclei (arrowheads in merged channel) in P40 mouse retina. Scale bar, 10 μ m.

B. Immunoelectron microscopy demonstrated even distribution of SynCAM 1 at the photoreceptor cell body membrane (magenta circles) in P42 mouse retina. Inset depicts enlarged SynCAM 1-immunogold localization from the boxed area (magenta rectangle). Scale bar, 500 nm.

C. Distribution of SynCAM 1-immunogold label (percentage) in different subregions of photoreceptor cell bodies in the ONL (total of 220 gold particles counted on 47 photoreceptor cell bodies; see text for details).

D. SynCAM 1 (green) is expressed in OPL, where it encircles VGlut1-positive photoreceptor terminals (magenta) in P40 mouse retina (arrowheads in merged image). Scale bar, 3 μ m.

E. Immunoelectron microscopy demonstrated presence of SynCAM 1 at the membrane of photoreceptor terminals (magenta circles), as well as at the interface of photoreceptor terminal with a horizontal cell process (arrowhead) in P42 mouse retina. Inset depicts enlarged SynCAM 1-immunogold localization from the boxed area (magenta rectangle). Scale bar, 500 nm.

F. Distribution of SynCAM 1-immunogold label in different subregions of rod ribbon complex (total of 303 gold particles counted on 106 rod terminals in the OPL; see text for details). Abbreviations: ONL, outer nuclear layer; n, nucleus; OPL, outer plexiform layer; sph, spherule; rb, ribbon; hc, horizontal cell; bc, bipolar cell.

Figure 3. Transmission of visual signals is impaired in SynCAM 1 KO retina

A. Representative original scotopic ERG traces from SynCAM 1 KO and WT mice (5–7 weeks old) recorded at the highest light intensity used (25 cds/m²).

B. Rod response was significantly increased in KO mice upon light stimulation. Differences between groups were analyzed by Two-way RM ANOVA with post-hoc Holm-Sidak comparison. No interaction was found between light intensity and genotype ($F_{(5,100)}=0.78$, $p=0.56$). Effects of genotype on amplitude of light responses were significantly altered: $F_{(1,100)}=6.90$; *, $p=0.016$. N=12 WT and 10 KO, age: P40-P50.

C. Bipolar cell responses are normal in amplitude in KO mice across all light intensities. Differences between groups were analyzed by Two-way RM ANOVA: ns, not significant. N=12 WT and 10 KO.

D. Top: b-wave time-to-peak is mildly, but significantly delayed in KO mice. No interaction was found between light intensity and genotype ($F_{(7,140)}=1.34$, $p=0.23$). Effects of genotype on time-to-peak of light responses were significantly altered ($F_{(1,140)}=5.25$; *, $p=0.033$). Bottom: a-wave time-to-peak is not significantly changed in KO mice. Differences between groups were analyzed by Two-way RM ANOVA. N=12 WT and 10 KO.

E. Representative oscillatory potentials extracted from ERGs of SynCAM 1 KO and WT mice recorded at the highest light intensity used (25 cds/m²).

F. Amplitude of oscillatory potentials is intact in SynCAM 1 KO mice. Differences between groups were analyzed by Two-way RM ANOVA: ns, not significant. N=12 WT and 10 KO.

G. Representative original traces of double-flash ERG recordings obtained from WT and KO mice at light intensities indicated. Interstimulus interval was 1600 ms.

H. Recovery of a-wave amplitude as a function of interstimulus interval. Data were fitted using non-linear regression, as described in Material and Methods, where r^2 was 0.907 for WT and 0.909 for KO. N=6 WT and 8 KO, age: P40-P50.

Figure 4. Cone pathway is not affected in SynCAM 1 KO mice

A. Representative original photopic ERG traces from SynCAM 1 KO and WT mice (5–7 weeks old) at highest light intensity used (25 cds/m2).

B. Amplitude of photopic b-wave is normal in SynCAM 1 KO mice.

C. Time-to-peak of photopic b-wave is unaffected in SynCAM 1 KO mice. Differences between groups were analyzed by Two-way RM ANOVA: ns, not significant. N=9 WT and 12 KO, age: P40-P50.

Figure 5. SynCAM 1 KO mice display morphological defects of horizontal cell processes in the outer retina

A–D. Photoreceptor terminals labeled with PSD-95 (A) and VGLUT1 (C) had normal appearance in SynCAM 1 KO mice (B and D).

E, F. Synaptic ribbons labeled with anti-Ribeye also displayed apparently normal distribution at a macroscopic level (E and F) (but see Figure 7 and Table 2 for structural aberrations).

G, H. Cone terminals labeled with PNA also showed normal intensity and distribution in SynCAM 1 KO animals (G and H).

I, J. Rod bipolar cells displayed normal morphology in KO mice shown by immunostaining with antibodies against PKCα.

K, L. Bipolar cell dendritic tips labeled with mGluR6 also displayed normal morphology.

M, N. Antibodies against Calbindin (green) and Neurofilament (magenta) demonstrated a regular arrangement of horizontal cell bodies and their processes in WT mice (M). In contrast, KO mice (N) showed sprouting of horizontal cell processes into the ONL (arrowheads). Ectopic cell processes in SynCAM 1 KO did not have ribbons apposing them in the ONL (O and P). Scale bars: A–H, 5 μm; I–L, 15 μm; M and N, 15 μm; O and P, 20

μm. All images are representative maximum intensity projections of Z-stacks through central retina in N=3 mice/group (5–7 weeks old). Abbreviations: Cb, Calbindin; NF, Neurofilament.

Figure 6. Horizontal cell development is impaired in SynCAM 1 KO mice

A, C, E and G. Calbindin immunostaining of WT retinas at different ages depicts gradual development of the horizontal cell plexus in the OPL. Horizontal cell bodies are seen arranged just above the OPL with their processes never crossing into the ONL.

B, D, F and H. In contrast, horizontal cell processes are seen crossing into ONL at all ages examined in SynCAM 1 KO mice. Ectopic growth is very prominent at P14 and in the adult SynCAM 1 KO retina. Scale bar: 30 μ m.

Figure 7. SynCAM 1 KO mice have defects in the ultrastructure of ribbon synapses

A. Schematic depiction of a rod terminal and its ribbon synapse. A synaptic ribbon (sr, magenta) is shown as an elongated structure opposed to bipolar cell dendrites (bc, blue), with horizontal cell processes settled laterally (hc, green).

B., C. Trace outline (B) of a representative WT (P40-P45) rod ribbon synapse electron micrograph (C). Relevant structures are labeled and color-coded for easier identification and comparison to the schematic structure (A).

D., E. Trace outline (D) of a representative SynCAM 1 KO (P40-P45) rod ribbon synapse (E). All elements of the triad are present. For quantification of ultrastructural measurements, see Table 2. Scale bar for C and E (in E): 1 μ m. Abbreviations: mit, mitochondria; n, nucleus; sr, synaptic ribbon; hc, horizontal cell processes; bc, bipolar cell dendrites.

Figure 8. Ribbons are sparse and shortened and Ribeye levels are reduced in adult SynCAM 1 KO mice

A–H. Representative images of Ribeye staining in WT and SynCAM 1 KO mice at different ages. Inset in H shows an outline of ribbon length measurement. Statistical analysis performed with Two-way ANOVA revealed no interaction between the age and genotype for both length and density of ribbons, as well as no effects of genotype on either ribbon length or their density (length: $F_{(1,10)}=0.0949$, $p=0.353$; density: $F_{(1,13)}=1.169$, $p=0.299$). **I.** Ribbon length in KO animals was significantly reduced only in adult (P40) animals as shown by post-hoc Holm-Sidak test (*, $p=0.016$; WT= $1.53\pm0.007\ \mu\text{m}$, KO= $1.37\pm0.03\ \mu\text{m}$). **J.** Post-hoc Holm-Sidak test revealed that ribbon density was reduced only in adult KO animals (J; *, $p=0.02$; WT= $1.14\pm0.08/\mu\text{m}^3$, KO= $0.84\pm0.07/\mu\text{m}^3$; N=3/group). N=3 WT and 2 KO for P4-P14, N=3 WT and 3 KO for P40. Scale bar, 20 μm .

K., L. SynCAM 1 KO mice show a significant decrease ($p=0.012$) in the abundance of Ribeye as determined by quantitative immunoblotting of P40 dark adapted retinas (KO= $64\pm3.8\%$ of WT; N=3/group, Student's *t*-test).

Table 1

List of antibodies.

Antibody name	Immunogen	Manufacturer and catalogue number	Host species and clonality	Dilution and application
Actin	Amino-acids 18–40 of chicken gizzard actin	MP Biomedicals, Solon, OH; 08691002; Clone C4	Mouse Monoclonal	1:8000 (WB)
Calbindin	Recombinant rat calbindin D-28k	Swant (Bellinzona, Switzerland); CB-38	Rabbit Polyclonal	1:2000 (IHC)
mGluR6	C-terminus of rat mGluR6 (AAPPQENADAK)	Neuromics, Edina, MN; RA13105	Rabbit Polyclonal	1:300 (IHC)
Neurofilament	Semi-purified adult Wistar rat neurofilaments	Developmental Studies Hybridoma Bank, University of Iowa; Clone RT97	Mouse Monoclonal	1:10 (IHC)
PKC α	Recombinant human PKC α	Cell Signaling Technology, Beverly, MA; 2056	Rabbit Polyclonal	1:200 (IHC)
PSD95	N-terminus of human PSD-95 (CDTLEAPGYELQV NGTEGEMEY)	Cell Signaling Technology, Beverly, MA; 3409	Rabbit Monoclonal	1:200 (IHC)
Ribeye	Glutathione S- transferase (GST) fusion protein containing aa 563–988 of rat ribeye (U2656)	Dr. Thomas Südhof; Stanford University School of Medicine, CA (Kindly provided by Dr. Sreeganga Chandra, Yale University, CT)	Rabbit Polyclonal	1:1000 (WB)
Ribeye	Mouse ribeye C-terminus, aa 361–445	BD Biosciences (San Jose, CA); 612044	Mouse Monoclonal	1:500 (IHC)
SynCAM 1	Extracellular domain of SynCAM 1 fused to Fc fragment	MBL Laboratories, Nagoya, Japan; CM004-3; Clone 3E1	Chicken Monoclonal	1:1000(WB, IHC); 1:20 (Cryo-EM)
VGlut1	C-terminus of 493–560 of rat VGlut1 (aa 493–560)	NeuroMab, University of California Davis, CA; 75-066; Clone N28/9	Mouse Monoclonal	1:200 (IHC)

Table 2

Synaptic parameters quantified in SynCAM 1 KO mice.

Parameter	WT	SynCAM 1 KO
Rod terminal number/ μm^3	0.26 ± 0.04	0.27 ± 0.01
Rod terminal perimeter (μm)	18.68 ± 0.71 (60 terminals from N=3 mice)	16.4 ± 0.28 * (61 terminals from N=3 mice)
Rod ribbon height (nm)	509 ± 27 (105 ribbons from N=3 mice)	369 ± 29 * (68 ribbons from N=3 mice)
Terminals with ribbon (% of total terminal number)	74 ± 3.6 of 64 terminals from N=3 mice	49 ± 6.4 * of 67 terminals from N=3 mice
Terminals with triads/ μm^3	0.055 ± 0.006	0.024 ± 0.003 *
Terminals with dyads/ μm^3	0.02 ± 0.003	0.02 ± 0.005
Terminals with multiple ribbons/ μm^3	0.005 ± 0.0002	0.005 ± 0.003
Terminals with non-associated ribbons/ μm^3	0.007 ± 0.004	0.017 ± 0.007
Terminals with non-associated lateral elements/ μm^3	0.006 ± 0.001	0.008 ± 0.003
Number of synaptic vesicles/terminal profile area	470 ± 53	380 ± 27
Perimeter of invaginating HC processes (μm)	4.012 ± 0.08 (65 lateral elements from N=3 mice)	3.28 ± 0.23 * (44 lateral elements from N=3 mice)
Perimeter of invaginating BC processes (μm)	2.18 ± 0.05 (100 lateral elements from N=3 mice)	2.36 ± 0.08 (67 lateral elements from N=3 mice)

Significant differences between littermate groups as determined with Student's t-test:

* $P < 0.05$;** $P < 0.01$. All values were averaged per animal before performing the statistical tests. N=3 WT and 3 KO.