Published in final edited form as:

Gene Expr Patterns. 2014 September ; 16(1): 36–50. doi:10.1016/j.gep.2014.07.003.

Developmental Localization of Adhesion and Scaffolding Proteins at the Cone Synapse

John S. Nuhn^a and Peter G. Fuerst^{b,c}

^a University of Idaho, Department of Psychology, Moscow, Idaho 83844, USA.

^b University of Idaho, Department of Biological Sciences, Moscow, Idaho 83844, USA.

^c University of Washington School of Medicine, WWAMI Medical Education Program, Moscow, Idaho 83844, USA.

Abstract

The cone synapse is a complex signaling hub composed of the cone photoreceptor terminal and the dendrites of bipolar and horizontal cells converging around multiple ribbon synapses. Factors that promote organization of this structure are largely unexplored. In this study we characterize the localization of adhesion and scaffolding proteins that are localized to the cone synapse, including alpha-n-catenin, beta-catenin, gamma-protocadherin, cadherin-8, MAGI2 and CASK. We describe the localization of these proteins during development of the mouse retina and in the adult macaque retina and find that these proteins are concentrated at the cone synapse. The localization of these proteins was then characterized at the cellular and subcellular levels. Alpha-ncatenin, gamma-protocadherin and cadherin-8 were concentrated in the dendrites of bipolar cells that project to the cone synapse but were not detected or stained very dimly in the dendrites of cells projecting to rod synapses. This study adds to our knowledge of cone synapse development by characterizing the developmental localization of these factors and identifies these factors as candidates for functional analysis of cone synapse formation.

Keywords

Differential Adhesion Hypothesis; Development; Adhesion; Pedicle; Connectome; Mosaic; Ctnna2; Ctnnb1; Cdh8; Pcdhg; Dscam

Introduction

Development of the nervous system requires the integration of a large number of cell types into functional neural circuits. The differential adhesion hypothesis posits that expression of

© 2014 Elsevier B.V. All rights reserved.

Corresponding Author: Peter G. Fuerst 145 Life Science South University of Idaho Moscow, Idaho 83844 fuerst@uidaho.edu 1 208 885 7512.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cell adhesion molecules that promote adhesion and repulsion underlies much of this connectivity. The cone synapse of the retina, the focus of this study, is the site of the first synapses in the photopic visual pathway. The cone synapse is a complex synapse between a single cone and the dendrites of multiple horizontal and bipolar cells, organized around a presynaptic ribbon, with the invaginating dendrites of ON bipolar cells and horizontal cells, referred to as a triad, and the dendrites of OFF bipolar cells making flat contacts at the base of each triad (Boycott and Hopkins, 1991; Hopkins and Boycott, 1992). Each cone synapse contains multiple triads; for example, in the primate retina each cone pedicle is estimated to contain approximately 30 triads (Ahnelt and Kolb, 1994). A further level of complexity is added in that a given bipolar or horizontal cell will sample multiple such synapses but will rarely contact the same cone twice (Reese, 2011).

Much work has successfully focused on understanding the organization and structure of the presynaptic ribbon. For example, bassoon is required for anchoring and maintenance of the presynaptic ribbon apposed to bipolar and horizontal cell neurites (Dick et al., 2003; Spiwoks-Becker et al., 2013). Additional studies have identified factors required for organization of the more simple rod synapse. MAGI and sidekick proteins were shown to promote development of the rod synapse and NGL-2 was shown to direct horizontal cell axons to the rod synapse (Soto et al., 2013; Yamagata and Sanes, 2010). Less work was devoted to understanding how the various types of bipolar cells and horizontal cells organize themselves into the cone synapse. In this study, the expression of adhesion and scaffolding proteins at the cone synapse was assayed. The developmental, cell type specific and sub-cellular localization of six such proteins is described. This work identifies these factors as candidates for further functions studies.

Results

The retina is composed of three cellular layers, the retinal ganglion cell layer, the inner nuclear layer and the outer nuclear layer, and two synaptic layers, the outer plexiform layer and the inner plexiform layer (Figure 1). To help address how the specificity of synaptic contacts is generated during development, a library of antibodies to adhesion and scaffolding proteins was screened to identify proteins that are localized preferentially or specifically to the mouse cone synapse. Of these, six were chosen for further investigation because of strong staining localized to the cone synapse, limited data about their localization in the retina and as representatives of classes of adhesion and scaffolding molecules. These included α -n-catenin and β -catenin, two scaffolding molecules that connect cadherins to the cytoskeleton, CASK, a PDZ protein, MAGI2, a PDZ scaffolding protein involved in organization of retinal circuits in the outer plexiform layer and two adhesion molecules: cadherin-8 and γ -protocadherin.

Adhesion and Scaffolding Proteins at the Cone Synapse

These proteins were all concentrated around the cone synapse, as visualized with antibodies to PSD95 and PNA (Figure 2). The localization of each of these proteins was also assayed with respect to the cone synapse in whole retina. α -n-catenin was localized on the postsynaptic face of the cone synapse and was nested within PNA staining, which labels the

dendritic tips of ON bipolar cells (Figure 3 A) (Koike et al., 2010). γ -protocadherin was also concentrated on the postsynaptic face of the cone synapse. γ -protocadherin was observed to both overlap with PNA staining and in between PNA positive processes (Figure 3 B). Cadherin-8 was observed along the cell body and dendrites of cells matching the morphology of bipolar cells. Cadherin-8 was also observed to both overlap with PNA staining and between PNA positive processes and in some sections dim staining was observed near the outer nuclear layer (Figure 3 C). β -catenin was localized around and within the cone synapse but did not overlap with PNA (Figure 3 D). Magi-2 was concentrated on the postsynaptic face of the cone pedicle, but was also observed within the rod spherule-containing portion of the outer plexiform layer (Figure 3 E). CASK overlapped with PNA staining at the cone pedicle and a single puncta of immunoreactivity was observed in each rod spherule (Figure 3 F).

Developmental Dynamics of Protein Localization

Each of these markers was assayed during development of the mouse cone synapse, at postnatal days 6, 8, 10 and 12, in the macaque retina, and in combination with markers that label Müller glia, horizontal cells and multiple types of bipolar cells.

α -n-catenin was widely localized in the developing retina but became concentrated apposed to the presynaptic marker PSD95 at the cone synapse by postnatal day 10, consistent with a postsynaptic localization (Figure 4 A-E). α -n-catenin was also concentrated apposed to PSD95 staining in sections of macaque retina, as well as around cells in the macaque inner and outer plexiform layers (Figure 4 F). α -n-catenin was observed around horizontal cells and overlapped with markers of bipolar cell subtypes (Figure 4 G-I and Table 1 and 2).

Limited γ -protocadherin staining was observed in the developing outer plexiform layer until post natal day 8, after which it was concentrated apposed to PSD95 staining at the cone synapse (Figure 5 A-E). A similar staining pattern was observed in mouse and macaque retina (Figure 5 F). Overlap between calbindin, a marker of horizontal cells, was not observed opposite of the cone synapse, but was observed in the portion of the outer plexiform layer facing the outer nuclear layer (Figure 5 G). Likewise overlap between GS (glutamine synthetase), a marker of Müller glia, and γ -protocadherin was not observed at the cone synapse but was observed in processes in between cone synapses (Figure 5 H). Expression of γ -protocadherin in both of these cell types was previously described (Lefebvre et al., 2008). Overlap was also detected between γ -protocadherin and all assayed bipolar cell types (Figure 5 I and Tables 1 and 2).

Cadherin-8 was first observed at postnatal day 10 and was observed dimly in cell bodies and apposed to PSD95 staining at the cone synapse (Figure 6 A-E). Cadherin-8 staining was not observed at the macaque cone pedicle (Figure 6 F). Cadherin-8 did not overlap with calbindin or GS, a marker of Müller glia but was observed in a limited subset of bipolar cells consistent with it being expressed by type 2 OFF bipolar cells and some ON bipolar cells (Figure 6 G-I, Figure 8 and Tables 1 and 2). All type 2 bipolar cells colocalized with cadherin-8 and the majority (81.8%) of cadherin-8 positive cells expressed Syt2 (Figure 6 I).

β -catenin was widely distributed in the mouse retina, as previously described (Fu et al., 2006). β -catenin became concentrated at the cone synapse as this structure developed, around P10 (Figure 7 A-E). A similar pattern of distribution was observed in mouse and in macaque (Figure 7 F). β -catenin was observed around the cell bodies of all labeled cell types and was especially abundant in Müller glia and in and around the tips of rod bipolar cells (Figure 7 G-I and Tables 1 and 2).

MAGI2 was observed in the developing outer plexiform layer at the first assayed time point, postnatal day 6 (Figure 8 A). MAGI2 protein was observed apposed to PSD95 staining at the cone synapse, but this was not observed at the cone synapse during development (Figure 8 B-E). A similar pattern of MAGI2 localization was observed in the macaque retina compared to the mouse retina (Figure 8 F). Colocalization of MAGI2 in the outer plexiform layer overlapped with GS, a marker of Müller glia, and with bipolar cell markers (Figure 8 G-I).

CASK was observed in the developing outer plexiform layer as early as post natal day 6 (Figure 9 A-D). CASK staining overlapped with PNA staining, and was centered in the rod spherule, as outlined by PSD95 (Figure 9 E). A similar pattern of CASK localization was observed in the macaque retina with respect to what was observed in the mouse (Figure 9 F). CASK was localized adjacent to the tips of calbindin-positive horizontal cell dendrites at the rod spherule (Figure 9 G). CASK did not overlap with GS and was localized adjacent to the tips of rod bipolar cells (Figure 9 H and I).

Protein Expression on Green, Blue and Green/Blue Cones

Cones were labeled with cone arrestin and blue cone opsin to determine if any of these proteins could underlie reported differences in the synaptic organization of blue versus green cones. Each of the six antigens was localized to both blue and green cone terminals (Figure 10).

Combinatorial Protein Expression

Finally, the overlap of these six proteins was assayed as allowed by antibody type. Significant overlap between α -n-catenin and γ -protocadherin, β -catenin, cadherin-8 and MAGI2 was observed on the postsynaptic face of the cone synapse (Figure 11). Areas of cadherin-8 and γ -protocadherin staining that did not overlap with α -n-catenin overlapped PNA staining (Figure 11 A and B). All α -n-catenin staining overlapped with β -catenin staining (Figure 11 E). Limited overlap between MAGI2 and β -catenin, cadherin-8 and γ -protocadherin was observed. MAGI2 immuno-reactivity was observed adjacent to CASK staining at the rod spherule (Figure 11 I).

Discussion

Defects in synaptic organization underlie many human neurological disorders such as autism, and identifying the factors that facilitate organization of the nervous system is therefore a central goal of neuroscience. To that end, in this study we assay localization of adhesion and scaffolding molecules at the cone synapse in the mouse retina to identify proteins that guide the organization of the cell types that make up this particular synapse.

A number of factors that promote organization of cells in the retinal inner plexiform layer have been identified. Surprisingly, many of these factors appear to function through repulsion, that is by preventing interactions, rather than promoting them. This list would include DSCAM and sidekick proteins, MEGF proteins, the γ -protocadherin, semaphorins and plexins (de Andrade et al., 2014; Fuerst et al., 2009; Fuerst et al., 2008; Kay et al., 2012; Lefebvre et al., 2012; Matsuoka et al., 2011; Yamagata and Sanes, 2008). While neurexins and neuroligins are required for synaptic pairing, the limited diversity of neuroligins in the mouse retina suggests that other factors are playing a role in promoting adhesion (Ichtchenko et al., 1995). This study explores the localization of cadherin anchoring proteins, the catenins, γ -protocadherin, cadherin-8 and two scaffolding proteins, CASK and MAGI2, at the cone synapse.

Catenins at the Cone Synapse

Both α -n-catenin and β -catenin loss of function was assayed in the mouse nervous system. β -catenin is widely expressed in the retina and functional studies indicate that it is required for large-scale organization of retinal lamination (Fu et al., 2006). Loss of α -n-catenin results in cell migration, patterning defects and lengthening of dendritic spines, but has not been functionally studied in the mouse retina (Cook et al., 1997; Togashi et al., 2002). In this study we report that α -n-catenin is localized to the mouse cone synapse and is expressed by at least some populations of OFF bipolar cells, with overlap not observed between the α -n-catenin and PNA positive ON bipolar cell processes. OFF bipolar cells make a series of flat contacts at the base of the invaginating processes of ON bipolar cells and horizontal cells. Differential expression of factors such as α -n-catenin by ON and OFF bipolar cells would help to explain how the ON and OFF bipolar cell pathways make different types of connections in different species, for example OFF bipolar cells in the fish retina make invaginating contacts, whereas they make flat contacts in the mouse retina (Li et al., 2012). The localization of α -n-cadherin and its expression by OFF bipolar cells at the cone synapse suggests that it is a good candidate as an organizer of OFF bipolar cells at the cone synapse, although its widespread early expression throughout the retina may complicate loss of function analysis.

Cadherins at the Cone Synapse

The catenins function as adaptor and signaling molecules that connect transmembrane cadherins to the cytoskeleton. Cadherin localization and function during retinal development is currently best studied in zebrafish and a role for cadherins 2 and 4 in lamination of retinal layers was demonstrated, although synapse formation appeared to be intact, despite otherwise dramatic dysgenesis of the retina (Babb et al., 2005; Erdmann et al., 2003; Liu et al., 2009; Liu et al., 2006; Liu et al., 2002; Malicki et al., 2003; Masai et al., 2003). Localization and function of some cadherin molecules in the mouse retina has also been reported (De la Huerta et al., 2012; Faulkner-Jones et al., 1999). We focused on cadherin-8 in this study because of its localization at the cone synapse and because detailed localization in the retina has not been reported beyond its expression in bipolar cells (Honjo et al., 2000). We found that cadherin-8 is concentrated in the soma, axons and dendrites of type 2 OFF bipolar cells that project to the cone. Identifying different classes of bipolar cells and antigen and transgenic markers of bipolar cells was the focus of considerable efforts (Haverkamp et

al., 2003; Haverkamp et al., 2008; Wassle et al., 2009). We observed cadherin-8 immunoreactivity in all type 2 OFF bipolar cells and in a small number of other bipolar cells, most likely type 1 OFF bipolar cells based on costaining with NK3R, a marker of both type 1 and type 2 bipolar cells in the mouse retina.

PDZ Scaffolding at the Cone Synapse

In addition to cadherins and the catenins anchoring them to the cytoskeleton, PDZ domain containing scaffolding molecules and the PDZ interacting proteins they anchor help to organize neural circuits. CASK and MAGI2 are PDZ domain containing scaffolding protein that binds to members of the immunoglobulin superfamily such as the Down syndrome cell adhesion molecule (Dscam) and sidekick proteins (Yamagata and Sanes, 2010). Functional studies of MAGI proteins with respect to the rod synapse suggest they facilitate pairing pre and postsynaptic cells, and the localization we observe at the rod synapse is consistent with this. At the cone synapse, MAGI2 localization was observed overlapping with postsynaptic cell type markers suggesting the PDZ proteins it anchors may facilitate interactions between OFF bipolar cells at the cone synapse, whereas CASK was observed to overlap with presynaptic markers at the rod and cone synapses, although detailed examination by immuno-electron microscopy will be required to confirm this apparent localization.

Protocadherins at the Cone Synapse

PDZ domain proteins serve as scaffolding for several types of cell adhesion molecules. We have previously characterized the role of the DSCAM at the cone synapse and found that it facilitates organization of several types of bipolar cells (de Andrade et al., 2014). Mammalian Dscams lack the splice diversity of fly Dscams and the role of providing this diversity may be subsumed by the protocadherins in the vertebrate (Sanes and Zipursky, 2010). The splice diversity of γ -protocadherin makes it a good candidate to promote organization of the complicated cone synapse. In the inner plexiform layer, γ -protocadherin provides isoneuronal avoidance cues within cell types and are also widely expressed in different populations of retinal neurons (Lefebvre et al., 2012; Lefebvre et al., 2008). In this study we observed developmental concentration of γ -protocadherin on the postsynaptic face as the cone synapse developed and also observed protein overlapping with PSD95 on the presynaptic face. The splice diversity allows like cells to overlap with each other and a similar mechanism might be anticipated to facilitate projection of bipolar cell dendrite branches to distinct cones.

OFF Bipolar Cell Organization

A largely unexplored aspect of cone synapse development is the placement of OFF bipolar cell synapses making flat contacts at the base of the triad. In this study we observed concentration of two factors, α -n-catenin and cadherin-8, apposed to PSD95 staining consistent with localization on the postsynaptic face of the cone synapse. The factors were expression in populations of OFF bipolar cells with minimal overlap with PNA-positive ON bipolar cell processes. When viewed in sections these factors do not overlap with PNA. In projections of whole retina; however, cadherin-8 overlaps with PNA, suggesting it is localized directly at the base of the triad, while α -n-catenin is displaced to the side. Colocalization of both antigens largely overlaps, although some areas of cadherin-8 closer to

PNA staining and α -n-catenin staining more distal to PNA was observed (Figure 11). Based on the types of OFF bipolar cells these antigens are expressed in, these results suggest type 2 OFF bipolar cells may be displaced from the base of the triad with respect to other populations of OFF bipolar cells or that the distal tips of OFF bipolar cells differ in the adhesion molecules they express, for example if α -n-catenin is restricted from the distal most tip of the bipolar cell dendrite. Further investigation of this localization pattern by immuno-electron microscopy will help to answer this question.

This study characterizes the developmental localization of six adhesion and scaffolding proteins at the mouse cone pedicle. Identification of adhesion molecules that are specifically localized to the cone synapse is a first step in better understanding how the complex architecture of these synapses develops. The cellular and subcellular localization of proteins reported here identifies them as excellent candidates for functional studies to determine how the cone synapse forms in their absence.

Material and Methods

Animal Care and Ethics

All procedures were performed in accordance with the University of Idaho Institutional Animal Care and Use Committee. Mice were fed ad libitum under a 12-hour light/dark cycle. Mice taken for study were deeply anesthetized with tribromoethanol (500 mg/kg). Cardiac perfusion was performed with phosphate buffered saline (PBS) to flush blood out of vessels before tissue collection. Mice used in this study were from either of a C57Bl/6J inbred background, including HTR2a-GFP mice, which were generously provided by Dr. Lane Brown, or from an inbred C3H/HeJ background in which the defective allele of *Pde6b* (*rd1*) was replaced with a wild type allele (Costa et al., 2010). HTR2a-GFP mice express GFP in type 4 OFF bipolar cells (Lu et al., 2009). Macaque retinas were obtained from the University of California Primate Center and were fixed in 4% PFA for one hour on ice.

Retina Dissection and Staining

Eyes were carefully enucleated following cardiac perfusion and hemisected. The posterior half of the eye was incubated in 4% paraformaldehyde for thirty minutes at room temperature followed by three washes in large volumes of PBS. For sectioning, retinas were isolated from the posterior half of the eye, equilibrated in 30% sucrose for one hour, and then frozen in optical cutting technology freezing medium (OCT). Sections were cut with a cryostat at 10 μ m thickness onto super frost charged slides. Frozen sections were blocked in 7.5% normal donkey serum and 0.1% triton X-100 in PBS (block) for fifteen minutes. Primary antibodies were diluted in block solution and 100 μ l mixed solution was applied to slides for incubation overnight at 4° C. Sections were washed twice in PBS for ten minutes after primary antibody incubation. Secondary antibodies were diluted in block and 500 μ l was applied over a given slide and incubated at room temperature for two hours. Slides were then washed for fifteen minutes in PBS, stained with 100 μ l 1:50,000 DAPI in PBS solution for fifteen minutes, and then washed again for fifteen minutes in PBS. Whole retinas were stained in a similar fashion except the block contained 0.4% triton and incubation of primary

antibodies occurred over four days at 4° C, while secondary antibodies were incubated at 4° C for three days.

Antibodies/Lectins

The following antibodies and stains were used in this study: Dapi (Cell Signaling Technology, 1:50,000), PNA-488 and PNA-647 (Invitrogen, 1:1000), mouse anti-PSD95 (Neuromabs, 1:400), rabbit anti-HCN4 (Alomone labs, 1:500), rabbit anti-NK3R (Novus, 1:2,000), rabbit anti-recoverin (Millipore, 1:500), mouse anti-PKARII β (BD transduction laboratories, 1:500), mouse anti-Syt2 (ZNP1) (ZFIN, 1:200), mouse anti-PKC α (SCBT, 1:500), rabbit anti-PKC α (SCBT, 1:500), rabbit anti-calbindin (Swant, 1:200), mouse anti- β -catenin (BD transduction laboratories, 1:500), rat anti- α -n-catenin (DSHB, 1:200), rabbit anti-Magi 2 (Sigma, 1:500), goat anti-blue cone opsin (Santa Cruz Biotechnology 1:500), mouse anti- γ -protocadherin (Neuromabs, 1:400), rabbit anti-GS (BD transduction labs, 1:1,000), mouse anti-cadherin-8 (DSHB, 1:100), mouse anti-CASK (Neuromabs, 1:400), rat anti-r-cadherin (DSHB, 1:50). Secondary antibodies used in this study were purchased from Jackson Immuno Research, conjugated to alexa 488, cy3 or dylight 647 and used at a concentration of 1:500.

Antibody Specificity

α -n-catenin: This monoclonal antibody recognizes a band by Western blot analysis (WBA) of the expected size of 102 kDA and did not coreact with related molecules (Hirano et al., 1992). Specificity was further confirmed by electron microscopy studies of the protein's localization at the expected cell junctions (Uchida et al., 1996). **γ -protocadherin:** The specificity of the antibody to γ -protocadherin was characterized by WBA, in which it recognized a band of approximately 100 kDA in lysates of mouse brain. This band was absent in mice in which the γ -protocadherin gene was knocked out. A lack of immunohistochemical staining was also observed in knock out mice, compared to wild type mice in which the protein is abundantly observed in p0 spinal cord (Lobas et al., 2012).

Cadherin-8: The specificity of the cadherin-8 antibody used in this study was demonstrated by WBA of spinal cord lysate from mice of wild type and cadherin-8 knockout backgrounds. No band was detected in lysates made from cadherin-8 knockout mice (Suzuki et al., 2007).

β -catenin: The β -catenin antibody used in this study detects a band of 92 kDA in lysates of HeLa cells and the protein localizes to cell boundaries (manufacturer's website). Consistent staining patterns with other β -catenin antibodies in spinal cord was also reported (Alfaro-Cervello et al., 2012). **MAGI2:** The MAGI2 antibody used in this study recognizes a bands of 180, 160 and 105 kDA and does not cross react with related molecules. Depletion of *Magi2* transcript in chick retina results in elimination of most MAGI2 staining (Yamagata and Sanes, 2010).

CASK: WBA of mouse brain lysate recognizes a band of 100 kDA. Antibody specificity was further confirmed by WBA of lysates produced from the brains of knockout mice (Neuromabs: manufacturer's website).

Imaging

Sections and whole retina were imaged using an Olympus Fluoview confocal microscope. Images were cropped and rotated using Adobe Photoshop software. Any changes to brightness or contrast were made across entire images.

Acknowledgments

This research was supported by the National Eye Institute Grant EY020857. Imaging support was provided by NIH Grant Nos. P20 RR016454, P30 GM103324-01 and P20 GM103408. Aaron Simmons, Shuai Li and Joshua Sukeena assisted with immunohistochemistry.

References

- Ahnelt P, Kolb H. Horizontal cells and cone photoreceptors in human retina: a Golgi-electron microscopic study of spectral connectivity. *J Comp Neurol*. 1994; 343(3):406–427. [PubMed: 8027450]
- Alfaro-Cervello C, Soriano-Navarro M, Mirzadeh Z, Alvarez-Buylla A, Garcia-Verdugo JM. Bicyclicated ependymal cell proliferation contributes to spinal cord growth. *J Comp Neurol*. 2012; 520(15):3528–3552. [PubMed: 22434575]
- Babb SG, Kotradi SM, Shah B, Chiappini-Williamson C, Bell LN, Schmeiser G, Chen E, Liu Q, Marrs JA. Zebrafish R-cadherin (Cdh4) controls visual system development and differentiation. *Dev Dyn*. 2005; 233(3):930–945. [PubMed: 15918170]
- Boycott BB, Hopkins JM. Cone bipolar cells and cone synapses in the primate retina. *Vis Neurosci*. 1991; 7(1-2):49–60. [PubMed: 1931800]
- Cook SA, Bronson RT, Donahue LR, Ben-Arie N, Davisson MT. Cerebellar deficient folia (cdf): a new mutation on mouse chromosome 6. *Mamm Genome*. 1997; 8(2):108–112. [PubMed: 9060409]
- Costa AC, Stasko MR, Schmidt C, Davisson MT. Behavioral validation of the Ts65Dn mouse model for Down syndrome of a genetic background free of the retinal degeneration mutation Pde6b(rd1). *Behav Brain Res*. 2010; 206(1):52–62. [PubMed: 19720087]
- de Andrade GB, Long SS, Fleming H, Li W, Fuerst PG. DSCAM localization and function at the mouse cone synapse. *J Comp Neurol*. 2014
- De la Huerta I, Kim IJ, Voinescu PE, Sanes JR. Direction-selective retinal ganglion cells arise from molecularly specified multipotential progenitors. *Proc Natl Acad Sci U S A*. 2012
- Dick O, tom Dieck S, Altrock WD, Ammermuller J, Weiler R, Garner CC, Gundelfinger ED, Brandstatter 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]
- Erdmann B, Kirsch FP, Rathjen FG, More MI. N-cadherin is essential for retinal lamination in the zebrafish. *Dev Dyn*. 2003; 226(3):570–577. [PubMed: 12619142]
- Faulkner-Jones BE, Godinho LN, Tan SS. Multiple cadherin mRNA expression and developmental regulation of a novel cadherin in the developing mouse eye. *Exp Neurol*. 1999; 156(2):316–325. [PubMed: 10328938]
- Fu X, Sun H, Klein WH, Mu X. Beta-catenin is essential for lamination but not neurogenesis in mouse retinal development. *Dev Biol*. 2006; 299(2):424–437. [PubMed: 16959241]
- 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, Koizumi A, Masland RH, Burgess RW. Neurite arborization and mosaic spacing in the mouse retina require DSCAM. *Nature*. 2008; 451(7177):470–474. [PubMed: 18216855]
- Haverkamp S, Ghosh KK, Hirano AA, Wassle H. Immunocytochemical description of five bipolar cell types of the mouse retina. *J Comp Neurol*. 2003; 455(4):463–476. [PubMed: 12508320]
- Haverkamp S, Specht D, Majumdar S, Zaidi NF, Brandstatter JH, Wasco W, Wassle H, Tom Dieck S. Type 4 OFF cone bipolar cells of the mouse retina express calnenilin and contact cones as well as rods. *J Comp Neurol*. 2008; 507(1):1087–1101. [PubMed: 18095322]

- Hirano S, Kimoto N, Shimoyama Y, Hirohashi S, Takeichi M. Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization. *Cell*. 1992; 70(2):293–301. [PubMed: 1638632]
- Honjo M, Tanihara H, Suzuki S, Tanaka T, Honda Y, Takeichi M. Differential expression of cadherin adhesion receptors in neural retina of the postnatal mouse. *Invest Ophthalmol Vis Sci*. 2000; 41(2): 546–551. [PubMed: 10670487]
- Hopkins JM, Boycott BB. Synaptic contacts of a two-cone flat bipolar cell in a primate retina. *Vis Neurosci*. 1992; 8(4):379–384. [PubMed: 1562572]
- Ichchenko K, Hata Y, Nguyen T, Ullrich B, Missler M, Moomaw C, Sudhof TC. Neuroligin 1: a splice site-specific ligand for beta-neurexins. *Cell*. 1995; 81(3):435–443. [PubMed: 7736595]
- Kay JN, Chu MW, Sanes JR. MEGF10 and MEGF11 mediate homotypic interactions required for mosaic spacing of retinal neurons. *Nature*. 2012; 483(7390):465–469. [PubMed: 22407321]
- Koike C, Obara T, Uriu Y, Numata T, Sanuki R, Miyata K, Koyasu T, Ueno S, Funabiki K, Tani A, Ueda H, Kondo M, Mori Y, Tachibana M, Furukawa T. TRPM1 is a component of the retinal ON bipolar cell transduction channel in the mGluR6 cascade. *Proc Natl Acad Sci U S A*. 2010; 107(1): 332–337. [PubMed: 19966281]
- Lefebvre JL, Kostadinov D, Chen WV, Maniatis T, Sanes JR. Protocadherins mediate dendritic self-avoidance in the mammalian nervous system. *Nature*. 2012; 488(7412):517–521. [PubMed: 22842903]
- 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]
- Li YN, Tsujimura T, Kawamura S, Dowling JE. Bipolar cell-photoreceptor connectivity in the zebrafish (*Danio rerio*) retina. *J Comp Neurol*. 2012; 520(16):3786–3802. [PubMed: 22907678]
- Liu Q, Chen Y, Pan JJ, Murakami T. Expression of protocadherin-9 and protocadherin-17 in the nervous system of the embryonic zebrafish. *Gene Expr Patterns*. 2009; 9(7):490–496. [PubMed: 19616130]
- Liu Q, Duff RJ, Liu B, Wilson AL, Babb-Clendenon SG, Franc J, Marrs JA. Expression of cadherin10, a type II classic cadherin gene, in the nervous system of the embryonic zebrafish. *Gene Expr Patterns*. 2006; 6(7):703–710. [PubMed: 16488669]
- Liu Q, Lonsdale RL, Azodi E, Babb SG, Chiappini-Williamson C, Marrs JA, Raymond PA. Up-regulation of cadherin-2 and cadherin-4 in regenerating visual structures of adult zebrafish. *Exp Neurol*. 2002; 177(2):396–406. [PubMed: 12429186]
- Lobas MA, Helsper L, Vernon CG, Schreiner D, Zhang Y, Holtzman MJ, Thedens DR, Weiner JA. Molecular heterogeneity in the choroid plexus epithelium: the 22-member gamma-protocadherin family is differentially expressed, apically localized, and implicated in CSF regulation. *J Neurochem*. 2012; 120(6):913–927. [PubMed: 22092001]
- Lu Q, Ivanova E, Pan ZH. Characterization of green fluorescent protein-expressing retinal cone bipolar cells in a 5-hydroxytryptamine receptor 2a transgenic mouse line. *Neuroscience*. 2009; 163(2): 662–668. [PubMed: 19589372]
- Malicki J, Jo H, Pujic Z. Zebrafish N-cadherin, encoded by the glass onion locus, plays an essential role in retinal patterning. *Dev Biol*. 2003; 259(1):95–108. [PubMed: 12812791]
- Masai I, Lele Z, Yamaguchi M, Komori A, Nakata A, Nishiwaki Y, Wada H, Tanaka H, Nojima Y, Hammerschmidt M, Wilson SW, Okamoto H. N-cadherin mediates retinal lamination, maintenance of forebrain compartments and patterning of retinal neurites. *Development*. 2003; 130(11):2479–2494. [PubMed: 12702661]
- Matsuoka RL, Nguyen-Ba-Charvet KT, Parray A, Badea TC, Chedotal A, Kolodkin AL. Transmembrane semaphorin signalling controls laminar stratification in the mammalian retina. *Nature*. 2011; 470(7333):259–263. [PubMed: 21270798]
- Reese BE. Development of the retina and optic pathway. *Vision Res*. 2011; 51(7):613–632. [PubMed: 20647017]
- Sanes JR, Zipursky SL. Design principles of insect and vertebrate visual systems. *Neuron*. 2010; 66(1): 15–36. [PubMed: 20399726]

- 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. *J Neurosci.* 2013; 33(29):11949–11959. [PubMed: 23864682]
- Spiwoeks-Becker I, Lamberti R, Tom Dieck S, Spessert R. Evidence for synergistic and complementary roles of Bassoon and darkness in organizing the ribbon synapse. *Neuroscience.* 2013; 236:149–159. [PubMed: 23295987]
- Suzuki SC, Furue H, Koga K, Jiang N, Nohmi M, Shimazaki Y, Katoh-Fukui Y, Yokoyama M, Yoshimura M, Takeichi M. Cadherin-8 is required for the first relay synapses to receive functional inputs from primary sensory afferents for cold sensation. *J Neurosci.* 2007; 27(13):3466–3476. [PubMed: 17392463]
- Togashi H, Abe K, Mizoguchi A, Takaoka K, Chisaka O, Takeichi M. Cadherin regulates dendritic spine morphogenesis. *Neuron.* 2002; 35(1):77–89. [PubMed: 12123610]
- Uchida N, Honjo Y, Johnson KR, Wheelock MJ, Takeichi M. The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones. *J Cell Biol.* 1996; 135(3):767–779. [PubMed: 8909549]
- Wassle H, Puller C, Muller F, Haverkamp S. Cone contacts, mosaics, and territories of bipolar cells in the mouse retina. *J Neurosci.* 2009; 29(1):106–117. [PubMed: 19129389]
- 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. Synaptic localization and function of Sidekick recognition molecules require MAGI scaffolding proteins. *J Neurosci.* 2010; 30(10):3579–3588. [PubMed: 20219992]

Highlights

Cone synapse localization of antigen markers was assayed in mouse and macaque

α -n-catenin, γ -protocadherin and cadherin 8 are concentrated at the cone synapse

β -catenin, MAGI2 and CASK are localized at rod and cone synapses

Assayed antigens do not differentiate blue from blue/green or green cones Graphical Abstract

Figure 1. Overview of the mouse retina

Sections of mouse retina stained with H&E or with antibodies to cone arrestin, blue cone opsin and neurofascin. The retina is organized in three cellular layers and two synaptic layers. The outer nuclear layer (ONL) contains the cell bodies of rods and cones. The inner nuclear layer (INL) contains the soma of bipolar cells, horizontal cells, amacrine cells and Müller glia. The retinal ganglion cell layer (RGL) contains the soma of retinal ganglion cells and amacrine cells. The outer plexiform layer (OPL) contains the synapses between photoreceptors and bipolar and horizontal cells, while the inner plexiform layer (IPL) contains the synapses of bipolar cells, amacrine cells and retinal ganglion cells. The focus of this study is on synapses localized in the outer plexiform layer (dashed box). The scale bar in (B) is equivalent to 106.5 μm .

Figure 2. Localization of adhesion and matrix proteins in the outer plexiform layer of the retina Sections of adult retina (P35-60, N>3) were stained with antibodies to PSD95, PNA and antibodies to α -n-catenin (A), γ -protocadherin (B), cadherin-8 (C), β -catenin (D), MAGI2 (E) or CASK (F). **A**, α -n-catenin immunostaining was concentrated on the bipolar cell facing half of the cone synapse. Staining was proximal to but not observed to overlap with PNA or PSD95. **B**, γ -protocadherin immunoreactivity was concentrated in puncta on the bipolar cell facing half of the cone synapse. Some puncta were observed to overlap with PNA staining, but not with PSD95 staining. **C**, Cadherin-8 immunoreactivity was observed on the bipolar cell facing half of the cone synapse. **D**, β -catenin staining was observed in the ONL, INL and OPL. **E**, MAGI2 staining was observed on the bipolar cell facing side of the cone synapse and within the area of the outer plexiform layer containing rod synapses. While some staining was observed to overlap with PNA, most MAGI2 staining around the cone synapse did not overlap with PNA. **F**, CASK staining was observed overlapping with PNA and PSD95 immunoreactivity at the cone synapse and as bright distinct puncta within the rod spherules. The scale bar in (F) is equivalent to 32 μ m in A-F (insets=4 μ m).

Figure 3. Localization of adhesion and scaffolding proteins at the cone synapse

Sections of adult retina (P35-60, N>3) were stained with antibodies to PSD95 α -n-catenin (A), γ -protocadherin (B), cadherin-8 (C), β -catenin (D), MAGI2 (E) or CASK (F). **A**, α -n-catenin immunoreactivity was localized within PNA staining with no overlap observed. **B**, γ -protocadherin immunoreactivity was observed within and partially overlapping with PNA staining. While staining was concentrated within the PNA staining, γ -protocadherin was also observed outside the cone synapse. **C**, Cadherin-8 immunoreactivity was observed around the soma of what appear to be bipolar cells, based on location and morphology, and along the dendrites of these cells, which were observed to project towards PNA staining. Cadherin-8 staining was localized within and overlapping with PNA staining. Very dim puncta were observed closer to the ONL (arrows). **D**, β -catenin staining was observed throughout the outer plexiform layer, although an absence of staining was observed overlapping with PNA staining. **E**, MAGI2 staining was concentrated INL-proximal to PNA staining. MAGI2 immuno-reactive puncta were also observed spread throughout the OPL. **F**, CASK immunoreactivity was observed overlapping with PNA staining, proximal to the cone terminal (left inset), and with PNA more distal to the cone terminal and in distinct puncta in the rod synapse containing ONL-proximal portion of the OPL. The scale bar in (**F**) is equivalent to 53.25 μ m (insets are 23.7 μ m).

Figure 4. Localization of α -N-catenin

A-E, Sections of retina were labeled with PNA and antibodies to PSD95 and α -n-catenin ($N=3$ at each age). The panel below the color images in A-F is the channel containing α -n-catenin staining in the OPL. **A**, At postnatal day six α -n-catenin overlaps with PSD95 immunoreactivity and can be observed outlining cells in both the INL and ONL. **B**, By postnatal day 8 α -n-catenin is only partially colocalized with PSD95. Staining within the developing ONL is limited and concentrations of staining can be observed in the developing OPL. **C-E**, By postnatal day 10, α -n-catenin is concentrated INL-proximal to PNA staining and no longer overlaps with PSD95. **F**, α -n-catenin in macaque retina was observed localized throughout the INL and ONL and was concentrated near the cone synapse ($N=2$ retinas). **G**, α -n-catenin staining overlapped with calbindin staining, a marker of horizontal cells. **H**, α -n-catenin staining did not overlap with GS staining, a marker of Müller glia. **I**, α -n-catenin staining overlapped with bipolar cell type specific markers, such as PKARII β , which is expressed by type 3b OFF bipolar cells. The scale bar in (**I**) is equivalent to 31.9 μ m in **A-F** (insets are 8.5 μ m) and 21.3 μ m in **G-I**.

Figure 5. Localization of γ -protocadherin

A-E, Sections of retina were labeled with PNA and antibodies to PSD95 and γ -protocadherin ($N=3$ at each age). **A**, Diffuse γ -protocadherin staining is observed in the developing OPL at postnatal day 6. **B**, By postnatal day 8, puncta of γ -protocadherin immunoreactivity that do not overlap with PSD95 were observed in the developing OPL. **C**, At postnatal day 10, concentrations of γ -protocadherin puncta were observed INL-proximal to PNA staining. Some γ -protocadherin staining was also observed in the soma of cells within the INL. **D** and **E**, After postnatal day 10, γ -protocadherin staining was observed concentrated INL-proximal to PNA staining, a pattern that persists in the adult retina. **F**, γ -protocadherin staining in the macaque retina was similar to what was observed in mouse, with a concentration of γ -protocadherin puncta INL-proximal to and overlapping with PNA staining. **G**, Some overlap between γ -protocadherin staining and calbindin was observed. **H**, γ -protocadherin staining proximal to PNA staining did not overlap with GS staining. **I**, γ -protocadherin staining overlapped with bipolar type specific markers, such as HCN4, which is expressed in type 3a OFF cone bipolar cells. The scale bar in **(I)** is equivalent to 31.9 μm in **A-F** (insets are 8.5 μm) and 21.3 μm in **G-I**.

Figure 6. Localization of cadherin-8

A-E, Sections of retina were labeled with PNA and antibodies to PSD95 and cadherin-8 (N=3 at each age). **A**, cadherin-8 staining was not observed in the developing OPL at postnatal day 6. **B**, Minimal cadherin-8 immunoreactivity was observed in the developing OPL at postnatal day 8. **C**, At postnatal day 10 cadherin-8 immunoreactivity was observed in inner nuclear layer cells that had a bipolar cell type morphology. **D**, By postnatal day 10 cadherin-8 staining was observed in the soma of cells in the INL, proximal to the OPL, and began to be concentrated INL-proximal to PNA staining. **E**, By postnatal day 35, cadherin-8 staining is concentrated INL-proximal to PNA staining. **F**, cadherin-8 staining was not observed INL-proximal to the cone synapse in the macaque retina, but rather was localized within the rod synapse containing portion of the macaque retina. **G**, cadherin-8 staining did not overlap with calbindin staining. **H**, cadherin-8 staining proximal to PNA staining did not overlap with GS staining. **I**, cadherin-8 staining overlapped with bipolar type specific markers, such as Syt2, which is expressed in type 2 OFF and type 6 ON cone bipolar cells (it only labels the axon terminals of the latter). Of note, all Syt2 positive bipolar cells contained cadherin-8, which was localized to Syt2-positive processes adjacent to cone synapses but not adjacent to horizontal cell processes projecting to rod synapses. The scale bar in **(I)** is equivalent to 31.9 μm in **A-F** (insets are 8.5 μm) and 21.3 μm in **G-I**.

Figure 7. Localization of β -catenin

A-E, Sections of retina were labeled with PNA and antibodies to PSD95 and β -catenin ($N=3$ at each age). **A and B**, β -catenin staining is observed in the developing OPL and in the membranes of cells in both the INL and ONL at postnatal days 6 and 8. **C**, At postnatal day 10 concentrations of β -catenin were observed in the OPL, as well as in cell bodies and neurites in the INL and ONL. **D and E**, By postnatal day 12 and in the adult retina, β -catenin staining is observed concentrated INL-proximal to PNA staining, throughout the IPL and in the soma and neurites of cells in both the INL and ONL. **F**, β -catenin staining in the macaque retina was similar to what was observed in mouse. **G**, β -catenin staining did not overlap specifically with calbindin staining. **H**, β -catenin staining overlapped with GS staining, the latter a marker of Müller glia. While this accounted for most β -catenin staining, overlap between GS and β -catenin was not observed in the concentrations of β -catenin INL-proximal to PNA staining. **I**, β -catenin staining overlapped with bipolar type specific markers, such as PKC α , which is a marker of rod bipolar cells. The scale bar in (**I**) is equivalent to 31.9 μm in **A-F** (insets are 8.5 μm) and 21.3 μm in **G-I**.

Figure 8. Localization of MAGI2

A-E, Sections of retina were labeled with PNA and antibodies to PSD95 and MAGI2 (N=3 at each age). **A-D**, Punctate MAGI2 staining overlapping with PSD95 staining was observed in the developing OPL between postnatal day 6 and 12. **E**, By postnatal day 35, MAGI2 staining was also apparent INL-proximal to PNA staining. **F**, MAGI2 staining was similar in mouse and macaque retina. Clear puncta of MAGI2 protein were observed INL-proximal to PNA staining. **G**, MAGI2 staining did not overlap with SMI32 staining, which labels the proximal neurites of horizontal cells. **H**, MAGI2 staining did not overlap with GS staining. **I**, MAGI2 staining overlapped with bipolar type specific markers, such as r-cadherin. The scale bar in **(I)** is equivalent to 31.9 μm in **A-F** (insets are 8.5 μm) and 21.3 μm in **G-I**.

Figure 9. Localization of CASK

A-E, Sections of retina were labeled with PNA and antibodies to PSD95 and CASK (N=3 at each age). **A** and **B**, Punctate CASK staining overlapping with PSD95 staining was observed in the developing OPL between postnatal days 6 and 8. **C** and **D**, A band of CASK staining INL-proximal to PSD95 staining becomes apparent at postnatal day 10 and persists through postnatal day 12. At both ages CASK overlaps with PNA staining. **E**, After postnatal day 12, CASK staining overlaps with PNA staining and is also concentrated in a single puncta within the rod synapses outlined by PSD95 staining. **F**, CASK staining was similar in mouse and macaque retina. **G**, CASK staining is localized adjacent to the tips of horizontal cells. **H**, CASK staining did not overlap with GS staining. **I**, CASK staining is localized to the tips of rod bipolar cell dendrites. The scale bar in **(I)** is equivalent to 31.9 μm in **A-F** (insets are 8.5 μm) and 21.3 μm in **G-I**.

Figure 10. Localization of antigens at blue and green cone terminals

A-D and **F**, Retina sections were stained with antibodies to one of the six markers, blue cone opsin and cone arrestin. **E**, Retina section stained with antibodies to MAGI2, PNA and blue cone opsin. All six antigens were localized at both blue and green cone terminals. The scale bar in (**F**) is equivalent to 106.5 μ m.

Figure 11. Pair wise analysis of markers described in this study at the cone synapse

Adult retinas were stained with antibody pairs and PNA (N=3). **A**, Overlap between α -n-catenin and cadherin-8 was observed. Cadherin-8 staining largely overlapped with α -n-catenin while some α -n-catenin staining, especially that more proximal to the inner nuclear layer, did not overlap with cadherin-8. **B**, Overlap between α -n-catenin and γ -protocadherin was observed. γ -protocadherin that did not overlap with PNA staining overlapped with α -n-catenin staining, while some α -n-catenin staining did not overlap with γ -protocadherin staining. **C**, MAGI2 staining proximal to PNA staining overlapped with α -n-catenin, while MAGI2 staining in the rod synapse-containing portion of the OPL did not. **D**, Overlap between CASK and α -n-catenin was not observed. **E**, Overlap between α -n and β -catenin was observed INL-proximal to PNA staining. **F**, Overlap between MAGI2 and β -catenin was observed INL-proximal to PNA staining. **G**, Overlap between MAGI2 and cadherin-8 was occasionally observed, although both proteins also occurred independent of each other. **H**, Overlap between MAGI2 and γ -protocadherin was observed, although both proteins occurred independently of each other as well. **I**, Minimal overlap between CASK and MAGI2 was observed proximal to PNA staining. MAGI2 puncta in the outer plexiform layer were localized adjacent to CASK puncta. The scale bar in **(I)** is equivalent to 31.9 μ m.

Table 1

Antigen colocalization by cell type adjacent to cone synapse

Antigen: Cell types:	<u>Calbindin</u> Horizontal Cells	<u>NK3R</u> Type 1 and 2 CBCs	<u>Recoverin</u> Type 2 CBCs	<u>HCN4</u> Type 3A CBCs	<u>Calsenilin</u> Type 4 CBCs	<u>ZNP1</u> Type 2 CBCs, HC tips	<u>GS</u> Müller Glia	<u>PKCα</u> RodBipolarcells
α -n-catenin	Yes	N.A. ¹	N.A.	Yes	Yes	Yes	No	No
γ -protocaherin	Yes	N.A.	N.A.	Yes	Yes	Yes	Yes	Yes
Cadherin-8	No	Yes	Yes	No	No	Yes	No	No
β -catenin	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

¹ N.A. Non-applicable as the markers do not label overlapping cellular compartments

Table 2

Subcellular Localization

	Axon	Soma	Pedicle	Spherule	PNA
α -n-catenin	Yes	Weak	Yes	Not Detected	No
γ -protocaderin	Yes	Not Detected	Yes	Weak	Yes
Cadherin-8	Yes	Yes	Yes	Weak	Yes
β -catenin	Yes	Yes	Yes	Yes	No
MAGI2	Yes	Not Detected	Yes	Yes	No
CASK	Yes	Not Detected	Yes	Yes	Yes