Immunocytochemical evidence for SNARE protein-dependent transmitter release from guinea pig horizontal cells

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Abstract

Horizontal cells are lateral interneurons that participate in visual processing in the outer retina but the cellular mechanisms underlying transmitter release from these cells are not fully understood. In non-mammalian horizontal cells, GABA release has been shown to occur by a non-vesicular mechanism. However, recent evidence in mammalian horizontal cells favors a vesicular mechanism as they lack plasmalemmal GABA transporters and some soluble NSF attachment protein receptor (SNARE) core proteins have been identified in rodent horizontal cells. Moreover, immunoreactivity for GABA and the molecular machinery to synthesize GABA have been found in guinea pig horizontal cells, suggesting that if components of the SNARE complex are expressed they could contribute to the vesicular release of GABA. In this study we investigated whether these vesicular and synaptic proteins are expressed by guinea pig horizontal cells using immunohistochemistry with well-characterized antibodies to evaluate their cellular distribution. Components of synaptic vesicles including vesicular GABA transporter, synapsin I and synaptic vesicle protein 2A were localized to horizontal cell processes and endings, along with the SNARE core complex proteins, syntaxin-1a, syntaxin-4 and synaptosomal-associated protein 25 (SNAP-25). Complexin I/II, a cytosolic protein that stabilizes the activated SNARE fusion core, strongly immunostained horizontal cell soma and processes. In addition, the vesicular Ca^{2+}-sensor, synaptotagmin-2, which is essential for Ca^{2+}-mediated vesicular release, was also localized to horizontal cell processes and somata. These morphological findings from guinea pig horizontal cells suggest that mammalian horizontal cells have the capacity to utilize a regulated Ca^{2+}-dependent vesicular pathway to release neurotransmitter, and that this mechanism may be shared among many mammalian species.

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Keywords

mammalian visual system; retina; synaptic proteins; synaptic vesicle

Introduction

Horizontal cells play an important, although not fully understood, role in visual information processing by interacting with photoreceptors and bipolar cells in the outer plexiform layer (OPL). There are two types of horizontal cells in the mammalian retina that separately serve the cone and rod pathways. The dendrites of B-type horizontal cells contact cones and their axon terminal system contacts rods, whereas A-type horizontal cells contact cones exclusively as they have no axon terminals (for review see Peichl *et al.*, 1998); both types are immunostained by antibodies to calbindin (Uesugi *et al.*, 1992; Peichl & González-Soriano, 1994; Raven & Reese, 2002; Hirano *et al.*, 2007). Physiological evidence demonstrates that horizontal cells contribute to center-surround properties, at least in part, through feedback onto photoreceptors in some species (Baylor *et al.*, 1971; O'Bryan, 1973; Burkhardt, 1977; Verweij *et al.*, 2003; Babai & Thoreson, 2009) and feedforward onto bipolar cells in other species (Dowling & Werblin, 1969; Yang & Wu, 1991; Billups & Attwell, 2002; Zhang & Wu, 2009). Small, clear-core vesicles in horizontal cell tips that invaginate the synaptic triad have been demonstrated at the ultrastructural level (Dowling *et al.*, 1966; Linberg & Fisher, 1988). The localization of the vesicular GABA transporter (VGAT) to horizontal cell endings in mammalian retinas (Haverkamp *et al.*, 2000; Cueva *et al.*, 2002; Jellali *et al.*, 2002; Guo *et al.*, 2009b) supports the view that horizontal cells can concentrate GABA into vesicles, as VGAT mediates the uptake and storage of GABA and glycine in neurons (Burger *et al.*, 1991; Liu & Edwards, 1997; McIntire *et al.*, 1997; Chaudhry *et al.*, 1998; Gasnier, 2004). Mammalian horizontal cells have also been found to express L-type (Ueda *et al.*, 1992; Löhrke & Hofmann, 1994; Rivera *et al.*, 2001) and N-type (Schubert *et al.*, 2006; Witkovsky *et al.*, 2006) calcium channels, suggesting the possibility of a Ca^{2+}-dependent vesicular release mechanism. Immunocytochemical studies demonstrate that $GABA_A$ or $GABA_C$ receptors or both (Vardi *et al.*, 1992, 1994; Greferath *et al.*, 1993, 1995; Grigorenko & Yeh, 1994; Enz *et al.*, 1996; Wässle *et al.*, 1998) are expressed in mammalian photoreceptors, bipolar cells and horizontal cells, suggesting that they may be potential targets of GABA released from horizontal cells.

Although a vesicular mechanism pertaining to the release of GABA from horizontal cells has not been established unequivocally, some protein components of the neuronal exocytotic machinery are expressed in mammalian horizontal cells. In central neurons, GABA release relies on Ca^{2+}-dependent vesicular mechanisms (Olsen & Tobin, 1990; Macdonald & Olsen, 1994; Poncer *et al.*, 1997). Moreover, new observations in guinea pig horizontal cells report the lack of plasmalemmal GABA transporter expression (Guo *et al.*, 2009b) but the presence of GABA and the biosynthetic machinery to synthesize GABA (Guo *et al.*, 2009a). Studies in some mammalian horizontal cells have identified soluble NSF attachment protein receptor (SNARE) proteins that are classically associated with synaptic vesicles and exocytosis, including synaptosomal-associated protein (SNAP-25) (Catsicas *et al.*, 1992; Ullrich & Südhof, 1994; Grabs *et al.*, 1996; von Kriegstein *et al.*, 1999; Greenlee *et al.*, 2001), syntaxin-1 (Nag & Wadhwa, 2001; Hirano *et al.*, 2005), syntaxin-4 (Sherry *et al.*, 2006; Hirano *et al.*, 2007) and complexin I/II (Hirano *et al.*, 2005). However, there have not been any studies evaluating these proteins comprehensively in a single animal model.

The aim of the present study was to address the hypothesis that guinea pig horizontal cells could release GABA through a Ca^{2+}-dependent vesicular mechanism. The guinea pig is an emerging animal model for retina research and has been used to study the cellular

organization and function of the retina, including ganglion cells (Demb *et al.*, 1999), amacrine cells (Oh *et al.*, 1999; Fujieda *et al.*, 2000; Kao & Sterling, 2006) and Müller cells (Malgorzata Goczalik *et al.*, 2005; Rillich *et al.*, 2009), and is unique because it exhibits robust expression of proteins related to GABA neurotransmission (Guo *et al.*, 2009a). This is the first study to systematically evaluate vesicular and synaptic-related proteins in this species, which is necessary to validate the guinea pig for future studies evaluating retinal anatomy and synaptic function. Our findings in a mammalian species other than rodents provide a basis for understanding common mechanisms underlying transmitter release from mammalian horizontal cells.

Materials and methods

Animals

Adult Hartley guinea pigs (CRL 051) of either sex were purchased from Charles River Laboratories (Wilmington, MA, USA). All experiments were performed in accordance with the guidelines for the welfare of experimental animals issued by the UCLA Animal Research Committee and the U.S. Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Guinea pigs used for retinal tissue collection were killed by isoflurane inhalation anesthesia (Novaplus, Lake Forest, IL, USA) and decapitated.

Tissue preparation

Guinea pig eyes were enucleated, the cornea, lens and vitreous were removed, and the eyecups were immersion fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 15–30 min at 4°C. The fixed eyecups were subsequently transferred to a 30% sucrose solution overnight at 4°C for tissue cryoprotection. The eyecups were then briefly washed in 0.1 M PB, embedded in OCT compound (Sakura Finetek Inc., Torrance, CA, USA) and rapidly frozen with dry ice. Cryostat sections of 10–12 μm were made perpendicular to the vitreal surface and retinal sections were collected onto gelatin-coated slides. Sections were then air dried and stored at −20°C.

Immunohistochemistry

Immunohistochemical labeling was performed using an indirect immunofluorescence method (Hirano *et al.*, 2005, 2007). Retinal frozen sections were thawed for 15 min at 37°C on a tissue warming tray, then rinsed three times with 0.1 M PB (pH 7.4) for 10 min per rinse. Retinal sections were then incubated in a blocking solution of 10% normal goat serum, 1% bovine serum albumin and 0.5% Triton X-100 in 0.1 M PB for 1 h at room temperature (22°C). The blocking solution was removed and the primary antibody solution was immediately added to the sections. The sections were incubated with the primary antibody solution for 12–16 h at 4°C in a humidified chamber. Primary antibody solution contained 3% normal goat serum, 1% bovine serum albumin, 0.05% sodium azide and 0.5% Triton X-100 in 0.1 M PB, pH 7.4. Retinal sections were rinsed three times for 10 min per rinse with 0.1 M PB to remove excess primary antibody and then incubated in secondary antibodies conjugated with Alexa 568 or Alexa 488 (1 : 500; Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature in 0.1 M PB containing 0.5% Triton X-100. For labeling of cone photoreceptors, sections were incubated in fluorescein isothiocyanate-conjugated peanut agglutinin (1 : 500, Vector Labs, Burlingame, CA, USA) for 1 h, after primary antibody labeling. To remove the secondary antibody solution, sections were washed three times in 0.1 M PB for 10 min per rinse, air-dried and mounted using Aqua Poly/Mount (Polysciences, Inc., Warrington, PA, USA).

Antibodies

The optimal working dilution for each antibody was determined experimentally. Mouse monoclonal antibody against calbindin (1 : 2500; Sigma-Aldrich, St Louis, MO, USA; C9848 clone CB-955) and rabbit polyclonal antibody against calbindin (1 : 10 000; Swant, Bellinzona, Switzerland; CB38) were used as markers of type A and B horizontal cells in mammalian retina (Uesugi *et al.*, 1992; Peichl & González-Soriano, 1994; Raven & Reese, 2002; Hirano *et al.*, 2007). Antibodies used to identify synaptic vesicles were as follows: mouse monoclonal antibody against VGAT cytoplasmic domain (1 : 200; Synaptic Systems, Göttingen, Germany; 131 011 clone 117G4) to identify GABA-containing vesicles; mouse monoclonal antibody to synapsin I (1 : 100; Millipore, Billerica, MA, USA; MAB10137 clone 3C5) to identify conventional synapses; mouse monoclonal antibody to adult zebrafish hindbrain protein (Trevarrow *et al.*, 1990), which recognizes synaptotagmin-2 in mouse [1 : 200; Zebrafish International Resource Center, Eugene, OR, USA; Znp-1 (Fox & Sanes, 2007)]; and rabbit polyclonal antibody to synaptic vesicle protein 2 (SV2)A (1 : 500; Synaptic Systems, 119 002) to identify synaptic vesicles. SNARE complex and SNARE-related proteins were identified with the following antibodies: rabbit polyclonal antibodies against complexin I/II [1 : 15 000; Synaptic Systems; 122 102, which recognizes both complexin I and II (Reim *et al.*, 2001)]; rabbit monoclonal antibody to SNAP-25 (1 : 60 000; Sigma-Aldrich; S9684); mouse monoclonal antibody to syntaxin-1a (HPC-1) (1 : 1000; Sigma-Aldrich; S0664) and rabbit polyclonal antibody to syntaxin-4 (1 : 1000; Millipore; AB5330). Bipolar cells were identified with a rabbit polyclonal antibody to protein kinase C a (1 : 30 000; Sigma-Aldrich; P4334). Protein kinase C a is a widely used marker of retinal bipolar cells (Negishi *et al.*, 1988; Young *et al.*, 1988; Greferath *et al.*, 1990; Haverkamp *et al.*, 2000; Ghosh *et al.*, 2001). The characterization and evidence for the appropriate use of antibodies are summarized in Table 1; additional information about the antibodies used can be found in the Appendix S1 of the Supporting information.

Confocal microscopy

Retinal sections were examined and analyzed with an LSM 510 META laser scanning microscope (Zeiss, Thornwood, NY, USA) equipped with an argon laser for 488 nm excitation and two helium/neon lasers for 543 and 633 nm excitation, respectively, using a C-Apochromat 40 × 1.2 n.a. water objective. During acquisition of signals from double-labeled specimens, scans with each laser were performed sequentially to prevent spectral bleed-through. Specific band-pass filters were used to achieve proper separation of signals (single labeling, 488/505LP; double labeling, 488/505–530 and 543/560LP). To increase the signal-to-noise ratio, images were averaged online (e.g. $n = 4$) and the scan speed and photomultiplier detector gain were decreased. Digital images were acquired at a magnification zoom of 1.5 × and a resolution of 2048 × 2048 pixels. Confocal images were acquired at an optical thickness between 0.5 and 0.7 μm and approximately 1.0 Airy Units. The tortuous coursing of horizontal cell processes and spray of horizontal cell endings necessitated stacks through the OPL for clearer, more complete images of the localization of signals; however, images of individual scans of a single optical slice are available in supporting Figs S1–S8. For projections, 6–10 optical sections were acquired with a total thickness ranging from 2.5 to 6.3 μm and compressed for viewing. Digital confocal images were saved as Zeiss .LSM files and final publication quality images were exported in the .TIFF format at 300 dpi using LSM 510 META software version 4.2 (Zeiss). Images were adjusted for contrast and brightness, labeled and formatted using Photoshop CS3 (Adobe Systems, Inc., San Jose, CA, USA), and saved at 300 dpi at their final magnification.

Results

Vesicular GABA transporter expression in the outer retina

The VGAT immunoreactivity has been localized to horizontal cell processes and terminals in mouse, rat, monkey and human retinas (Haverkamp *et al.*, 2000; Cueva *et al.*, 2002; Jellali *et al.*, 2002; Guo *et al.*, 2009b). To determine if VGAT was also present in guinea pig horizontal cells, double labeling of guinea pig retinal sections with VGAT and calbindin D-28kD (CaBP) antibodies was performed. A- and B-type horizontal cells were identified by immunoreactivity for CaBP, which is an immunohistochemical marker of horizontal cell bodies, dendrites and axons in guinea pig retinas (Peichl & González-Soriano, 1994). CaBP densely labeled horizontal cell somata and processes but horizontal cell endings were generally labeled with less intensity. However, VGAT prominently labeled multiple, laterally running horizontal cell processes, as well as the horizontal cell endings in the OPL (Fig. 1). Individual VGAT-labeled puncta were in clusters near the proximal ONL, indicative of horizontal cell endings. These findings confirmed the presence of VGAT in horizontal cell processes and endings and also established VGAT as a useful marker of horizontal cell endings in guinea pig retina.

Synaptic vesicle proteins are expressed in mammalian horizontal cells

Synaptic vesicle protein 2A co-localizes with vesicular GABA transporter—
The SV2 is a ubiquitous integral membrane protein of synaptic vesicles that participates in Ca^{2+}-stimulated exocytosis and is present on all synaptic and secretory vesicles (Buckley & Kelly, 1985). There are three known isoforms of this protein, SV2A, SV2B and SV2C (Bajjalieh *et al.*, 1994; Janz & Südhof, 1999), each of which may have synapse-specific functions. A previous study has shown differential expression of these isoforms in the mouse retina, with SV2A broadly expressed at conventional synapses and prevalent in cone terminals and developing horizontal cells in the OPL (Wang *et al.*, 2003). To assess whether SV2A is present in guinea pig horizontal cell processes or putative horizontal cell release sites, double-labeling experiments with VGAT and SV2A antibodies were performed. These experiments revealed co-localized immunoreactivity in horizontal cell endings in the OPL producing a punctate pattern (Fig. 2, arrow), with numerous labeled puncta identifying horizontal cell endings. SV2A labeling of photoreceptor terminals was also seen, which showed no co-localization with VGAT.

Synaptotagmin-2 localizes to horizontal cell processes and endings—
Synaptotagmin-2 is an important synaptic protein that has been shown to function as a trigger for fast, Ca^{2+}-mediated vesicular exocytosis in central and neuromuscular synapses (Pang *et al.*, 2006) and is required for synaptic exocytosis. Because synaptotagmin has been shown to be required for tightly regulated and synchronous synaptic exocytosis, which is characteristic of neurotransmission, we examined the expression of this protein in guinea pig horizontal cells. We used the Znp-1 monoclonal mouse antibody, which has been shown to recognize synaptotamin-2 through western blot and immunoprecipitation analysis (Fox & Sanes, 2007). In guinea pig retina, synaptotagmin-2 and CaBP immunostaining were co-localized to horizontal cell processes and the endings emerging from these processes (Fig. 3). These findings parallel the results of a previous report demonstrating a differential distribution of synaptotagmin-1 and synaptotagmin-2 in mouse retinas, with synaptotagmin-2 as the prevailing isoform present in mouse horizontal cells (Fox & Sanes, 2007).

Synapsin I labels horizontal cells in a punctate pattern—Synapsin I is a synaptic vesicle-associated membrane protein (VAMP) that has been shown to be absent in ribbon synapses but is a marker of conventional synapses (Mandell *et al.*, 1990). The expression of

synapsin I in horizontal cells was examined because they are thought to mediate trafficking of synaptic vesicles to their target membrane through interactions with the cell cytoskeleton (Hirokawa *et al.*, 1989; Bennett *et al.*, 1991). Double-labeling experiments with synapsin I and CaBP antibodies showed co-localization at the horizontal cell processes and endings (Fig. 4A–C). Synapsin I antibodies also labeled several cell bodies in the inner nuclear layer. Double-labeling experiments with synapsin I and protein kinase C α (a rod bipolar cell marker) antibodies (Fig. 4D–F) revealed that synapsin I also labels rod dendrites, as has been reported in the rabbit retina (Hirano *et al.*, 2005).

SNARE proteins, the mediators of vesicular fusion, in mammalian horizontal cells

Central to the regulation of vesicular fusion are the SNARE proteins as they not only provide the energy to drive bilayer fusion but they also confer a degree of specificity to the fusion process (Jahn & Scheller, 2006). The synaptic proteins syntaxin, SNAP-25 and synaptobrevin or VAMP form the essential protein core complex for catalyzing synaptic vesicle fusion in the conventional model of exocytosis (Weber *et al.*, 1998) and are probably necessary to carry out vesicular transmitter release in horizontal cells. Our immunohistochemical studies have evaluated the expression of SNARE proteins in the guinea pig retina by focusing on syntaxin-1a, syntaxin-4, SNAP-25 and complexin I/II.

Syntaxin-1a labels horizontal cell endings in the outer plexiform layer—There are multiple syntaxin isoforms, at least four to date (isoforms 1–4), which participate in vesicle fusion by targeting the plasma membrane (Chen & Scheller, 2001; Brandie *et al.*, 2008; Aran *et al.*, 2009). Syntaxin-1a is an integral membrane protein classically involved in Ca^{2+}-regulated secretion in neurons and neuroendocrine cells (for reviews see Jahn & Südhof, 1999; Jahn & Scheller, 2006; Lang & Jahn, 2008). In the retina, syntaxin-1a is restricted to conventional synapses and is notably absent in photoreceptor and ribbon synapses (Brandstätter *et al.*, 1996a; Hirano *et al.*, 2005; Sherry *et al.*, 2006; Curtis *et al.*, 2008). To examine the distribution of this protein in the guinea pig retina, we performed double-labeling experiments with syntaxin-1a and CaBP antibodies, which showed immunolabeling of amacrine cells as well as within horizontal cell processes and endings (Fig. 5). These results agree with previous reports in rat (Barnstable *et al.*, 1985; Inoue *et al.*, 1992; Morgans *et al.*, 1996) and rabbit (Hirano *et al.*, 2005) retina.

Syntaxin-4 co-localizes with vesicular GABA transporter in horizontal cells—A recent study has demonstrated robust syntaxin-4 immunoreactivity in horizontal cell processes and tips in mouse, rat and rabbit retinas (Hirano *et al.*, 2007). To examine the distribution of syntaxin-4 in guinea pig retina, double-labeling experiments with syntaxin-4 and VGAT antibodies were performed, which confirmed a similar staining pattern between these antibodies (Fig. 6A–C). Co-localization of VGAT and syntaxin-4 occurred along horizontal cell processes and endings, whereas the cell bodies were only faintly labeled. Similar to what has been observed in other mammalian species (Hirano *et al.*, 2007), syntaxin-4 densities were seen at regular intervals along the OPL, which correspond to clusters of horizontal cell endings abutting the cone pedicle, which were labeled with peanut agglutinin (Fig. 6D–F). The arrow in Fig. 6D points to a horizontal cell terminating at a rod spherule, which is more distal to horizontal cells than cone pedicles, demarcated in Fig. 6E by an asterisk.

SNAP-25 strongly labels horizontal cell processes and endings—SNAP-25, a critical component of the neural SNARE complex that facilitates membrane fusion between synaptic vesicles and the presynaptic plasma membrane, has been reported in multiple retinal cell types, including horizontal cells (Catsicas *et al.*, 1992). Recently, it was shown that SNAP-25 not only subserves cholinergic and glutamatergic neurotransmission but is

also critical for evoked GABA release and is expressed by mature GABAergic neurons (Tafoya *et al.*, 2006). We tested whether horizontal cells in guinea pig retina express SNAP-25 with double-labeling experiments with both CaBP and SNAP-25 antibodies. In guinea pig, immunoreactivity using the SNAP-25 anti-rabbit antibody was robust in horizontal cell endings, identified by co-labeling with CaBP antibody (Fig. 7). Immunostaining was very weak or absent in horizontal cell somata (arrow) but the horizontal cell terminals and lateral processes along the OPL beneath photoreceptor terminals were intensely labeled. SNAP-25 also labeled the bipolar cell bodies (asterisk), dendrites and axons, and has been reported to occur in other retinal cell types (Galli *et al.*, 1995; Brandstätter *et al.*, 1996a; von Kriegstein *et al.*, 1999; Morgans & Brandstätter, 2000; von Kriegstein & Schmitz, 2003).

Complexin I/II labels horizontal cell soma, processes and endings—Fast Ca^{2+}-triggered fusion requires a host of proteins, including complexins. These are soluble SNARE complex-binding proteins that have been shown to have an essential role in synaptic fusion by regulating a late step in Ca^{2+}-dependent neurotransmitter release (Reim *et al.*, 2001; Tang *et al.*, 2006). They control the force transfer from SNARE complexes to membranes in fusion (Maximov *et al.*, 2009) by serving as 'grappling proteins' to hold the SNARE complex into an activated but frozen state (Rizo & Rosenmund, 2008; Südhof & Rothman, 2009). Given that these are essential proteins involved in Ca^{2+}-dependent vesicular fusion, we tested whether they are expressed in guinea pig horizontal cells. Double-labeling experiments with complexin I/II and CaBP showed that complexin I/II immunoreactivity was localized to all parts of the guinea pig horizontal cells, including the soma, processes and endings (Fig. 8). These results agree with earlier findings in mouse and rabbit retina, which revealed complexin I/II immunoreactivity in the entire horizontal cell, including the endings, at both the light and electron microscopy level (Hirano *et al.*, 2005; Reim *et al.*, 2005). Amacrine cells are also labeled with the complexin I/II antibody (Fig. 8, asterisk).

Discussion

This study provides novel insights and morphological evidence for the mechanism of transmitter release from horizontal cells. It has been shown previously that both GABA and the biosynthetic machinery to synthesize GABA are present in guinea pig horizontal cells (Guo *et al.*, 2009a). This study extends these findings to reveal key protein components involved in Ca^{2+}-dependent and SNARE protein-dependent exocytosis. Most notably, the expression of VGAT, synaptotagmin-2, SNAP-25 and syntaxin-1a and syntaxin-4 at horizontal cell tips and processes argues strongly in favor of a regulated exocytotic vesicular pathway for GABA release from guinea pig horizontal cells. However, the nature and trafficking course of the vesicles storing and releasing GABA in guinea pig horizontal cells are currently unknown. Furthermore, the existence of Ca^{2+}-regulated vesicular exocytosis in mammalian horizontal cells is still debated. Based on our findings, GABA probably utilizes a vesicular-regulated secretory pathway in mammalian horizontal cells.

Synaptic vesicle proteins in mammalian horizontal cells

Generally, synaptic vesicle fusion with the plasma membrane involves the SNARE proteins syntaxin-1a, SNAP-25 and VAMP-1 for transmitter release (Südhof & Jahn, 1991; Südhof, 2004; Takamori *et al.*, 2006) but there are many examples of heterogeneity in SNARE core complex combinations within the central nervous system. For instance, astroglial precursor cells, oligodendrocytes and microglia, which undergo vesicular release of glutamate and aspartate, express SNAP-23, an analog of SNAP-25, VAMP-3 and syntaxin-1a (Parpura *et al.*, 1995; Hepp *et al.*, 1999; Maienschein *et al.*, 1999; Montana *et al.*, 2004). In this instance, the interaction of SNAP-23, VAMP-3 and syntaxin-1a forms the core SNARE complex,

mediating general membrane insertion mechanisms including secretion, phagocytosis and myelinogenesis (Hepp *et al.*, 1999; Ni *et al.*, 2007). Within the retina, ribbon synapses in photoreceptors express syntaxin-3 (Ullrich & Südhof, 1994; Morgans *et al.*, 1996; Sherry *et al.*, 2006; Curtis *et al.*, 2008), VAMP-1 and VAMP-2 (Sherry *et al.*, 2003; Morgans *et al.*, 2009), and SNAP-25 (Ullrich & Südhof, 1994; Brandstätter *et al.*, 1996b; Greenlee *et al.*, 2001; Mazelova *et al.*, 2009). The syntaxins also show heterogeneity in the retina, with syntaxin-1 and syntaxin-2 in conventional amacrine cell synapses in a non-overlapping fashion, syntaxin-3b in glutamatergic ribbon synapses of photoreceptors and bipolar cells, and syntaxin-4 in horizontal cells (Sherry *et al.*, 2006; Hirano *et al.*, 2007; Curtis *et al.*, 2008). Thus, although the paradigm of SNARE-mediated fusion may be universal, the substrates mediating this action may involve greater isoform variability, which may be the case for proteins involved in SNARE-mediated vesicular release from mammalian horizontal cells.

Mechanism of transmitter release in mammalian horizontal cells

Studies in non-mammalian retinas have argued for GABA uptake and release occurring via a Na^+-dependent and Ca^{2+}-independent transport process in these horizontal cells due to the presence of a plasmalemmal GABA transporter (Schwartz, 1982, 1987; Yazulla *et al.*, 1985; Ayoub & Lam, 1987; O'Malley & Masland, 1989; Connaughton *et al.*, 2008; Nelson *et al.*, 2008). In non-mammalian retinas GABA is synthesized and accumulates in cone-driven horizontal cells (Yazulla & Brecha, 1981; Zucker *et al.*, 1984; Yazulla *et al.*, 1989; Marc, 1992; Connaughton *et al.*, 1999), and is released when horizontal cells are depolarized (Ayoub & Lam, 1984; Yang & Wu, 1989, 1993; Kamermans & Werblin, 1992). Evidence for mammalian plasmalemmal transmitter release is absent in mammals, which prompted the hypothesis that mammalian horizontal cells might utilize an alternative mechanism of transmitter release. High-affinity transport of GABA or GABA analogs has not been reported in any adult mammalian horizontal cells (Goodchild & Neal, 1973; Ehinger, 1977; Agardh & Ehinger, 1982, 1983; Agardh *et al.*, 1986; Brecha & Weigmann, 1994) and studies in the developing and adult mouse retina found that GABA transporter (GAT-1 and GAT-3) transcripts and proteins were limited to Müller cell processes (Brecha & Weigmann, 1994; Durkin *et al.*, 1995; Honda *et al.*, 1995; Johnson *et al.*, 1996; Hu *et al.*, 1999; Casini *et al.*, 2006; Guo *et al.*, 2009b). However, L-glutamic acid decarboxylase, the synthesizing enzyme for GABA, is known to be present in horizontal cells particularly in early development (Schnitzer & Rusoff, 1984). Glutamate, the synthetic precursor of GABA, and the glutamate transporter, excitatory amino acid carrier 1, have been localized to the somata of rat and cat horizontal cells (Rauen *et al.*, 1996; Fyk-Kolodziej *et al.*, 2004). The non-synaptic location of this transporter has been implicated in the synthesis and release of GABA in the hippocampus (Coco *et al.*, 1997; Sepkuty *et al.*, 2002), supporting the notion that horizontal cells may also utilize excitatory amino acid carrier 1 to accumulate glutamate for the subsequent synthesis of GABA.

Ultrastructural studies demonstrate the presence of small, spherical, clear-core agranular vesicles classically associated with synaptic vesicles containing fast neurotransmitters such as glutamate and GABA (for review see De Camilli & Jahn, 1990; Torrealba & Carrasco, 2004) within rabbit, mouse, rat, cat, monkey and human horizontal cells (Dowling & Boycott, 1966; Linberg & Fisher, 1988; Spiwoks-Becker *et al.*, 2001). Reports of vesicles clustered at membrane specializations in horizontal cell processes (Dowling *et al.*, 1966; Raviola & Gilula, 1975; Linberg & Fisher, 1988) adjacent to photoreceptor terminals are small in number and the vesicles do not aggregate preferentially at the cell membrane (Dowling & Boycott, 1966; Spiwoks-Becker *et al.*, 2001). Although a previous study by Loeliger & Rees (2005) speculated that only one type of horizontal cell contains GABA in the adult guinea pig retina, GABA release probably occurs from both A- and B-type

horizontal cells in the guinea pig, as all calbindin-immunoreactive horizontal cell bodies were shown to contain GABA as well as glutamic acid decarboxylase 65 (GAD_{65}) immunoreactivity (Guo *et al.*, 2009a).

Although these findings support the hypothesis that horizontal cells release GABA in the outer retina, they do not exclude the possibility that there may be other sources of GABA. Interplexiform cells, which have been reported in all vertebrate retinas (Boycott *et al.*, 1975; Savy *et al.*, 1991; Nguyen-Legros *et al.*, 1997), are a subtype of amacrine cells whose processes ramify in both plexiform layers and to the inner nuclear layer and contain dopamine (Dowling & Ehinger, 1975), GABA (Nakamura *et al.*, 1980; Witkovsky *et al.*, 2008), L-glutamic acid decarboxylase and VGAT (Witkovsky *et al.*, 2008) in varicosities along the interplexiform cell processes. GABAergic interplexiform cells co-localized with SV2A, indicating that GABA may also be released from these cells at a conventional synapse (Witkovsky *et al.*, 2008). A second possible source of GABA in the outer retina is the indoleamine-accumulating type 3 cells (Sandell & Masland, 1989) located at the outer edge of the inner nuclear layer (Sandell & Masland, 1989; Massey *et al.*, 1992). These cells arborize widely in the OPL and specifically take up ^{3}H-GABA (Sandell & Masland, 1989) or the GABA analog ^{3}H-muscimol (Massey *et al.*, 1992). However, these cells are likely to have a minor influence on GABA levels in the OPL overall as they are concentrated in the ventral retina and they only ramify in some retinal regions, making it more probable that these cells represent developmental anomalies (Sandell & Masland, 1989). In guinea pigs, horizontal cells are probably the predominant cellular source of GABA in the outer retina (Guo *et al.*, 2009a) as tyrosine hydroxylase-immunoreactive interplexiform cell processes are not present in the inner nuclear layer or OPL of guinea pig retina (Oh *et al.*, 1999; Fujieda *et al.*, 2000; Loeliger & Rees, 2005). Furthermore, there was no evidence of tyrosine hydroxylase-immunolabeled amacrine cell processes in the guinea pig ramifying in the OPL (Oh *et al.*, 1999).

Several studies support the notion that all cells in the photoreceptor triad are end targets of GABA released from horizontal cells. Mammalian horizontal cells exhibit GABA-induced currents (Feigenspan & Weiler, 2004), consistent with $GABA_A$ receptor expression on horizontal cells (Greferath *et al.*, 1994, 1995; Blanco *et al.*, 1996). These findings suggest that GABA acts as an auto-receptor. $GABA_{A,C}$ receptors have also been detected on bipolar cell dendrites (Greferath *et al.*, 1994; Enz *et al.*, 1996; Vardi *et al.*, 1998; Pattnaik *et al.*, 2000; Delgado *et al.*, 2009), which provides evidence in support of horizontal cells mediating feedforward action onto bipolar cells. ON-bipolar cells require that GABAergic input be depolarizing to provide them with the corrective horizontal cell input analogous to that received by OFF-bipolar cells. Indeed, Duebel *et al.* (2006) reports that ON-bipolar cells employ a somatodendritic $[Cl^-]_{(i)}$ gradient to invert GABAergic horizontal cell input, thereby depolarizing ON-bipolar cells with a high dendritic $[Cl^-]_{(i)}$. There are several studies showing the presence of $GABA_{A,C}$ receptor immunoreactivity at photoreceptor terminals (Greferath *et al.*, 1995; Picaud *et al.*, 1998; Haverkamp & Wässle, 2000; Pattnaik *et al.*, 2000). However, GABA's action at photoreceptor terminals remains controversial. GABA-evoked currents are reported for mouse and pig cones (Picaud *et al.*, 1998; Pattnaik *et al.*, 2000) but the predominant finding is a lack of GABA-evoked currents in mammalian cones (Verweij *et al.*, 2003).

There are still many questions that must be answered regarding the mechanism underlying transmitter release from horizontal cells. The results of this study argue for regulated vesicular-mediated exocytosis as the underlying mechanism of release and suggest that the guinea pig retina is uniquely suited for functional studies of mammalian horizontal cells. Understanding the mechanism of release will contribute to understanding how these cells function and communicate within the OPL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

CaBP	calbindin D-28kD
OPL	outer plexiform layer
PB	phosphate buffer
SNAP-25	synaptosomal-associated protein
SNARE	soluble NSF attachment protein receptor
SV2	synaptic vesicle protein 2
VAMP	vesicle-associated membrane protein
VGAT	vesicular GABA transporter

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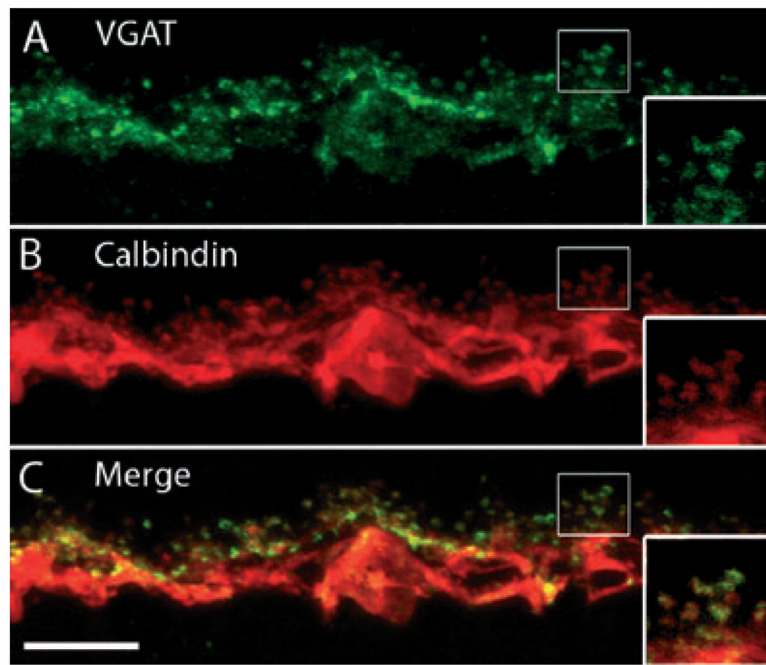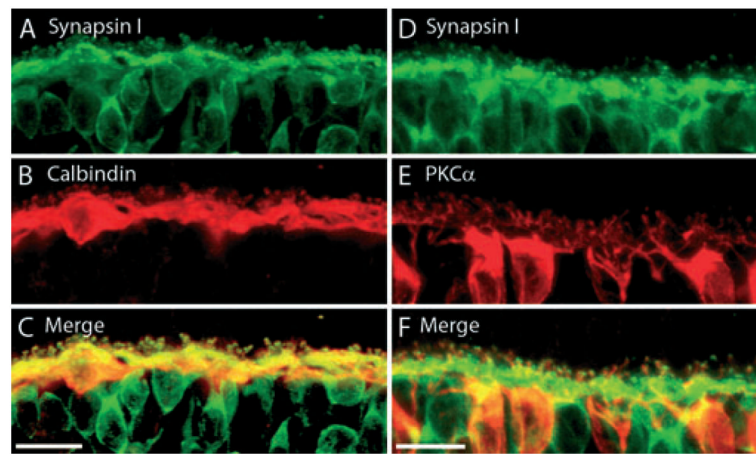
Fig. 1.
VGAT immunoreactivity is localized to horizontal cell processes and endings in the OPL. A vertical section of guinea pig retina was double labeled with antibodies to CaBP and VGAT. (A) VGAT immunostaining revealed horizontal cell processes and terminals and a punctate-like pattern of labeling in the OPL. Horizontal cell somata were also faintly labeled. (B) CaBP immunoreactivity was strong throughout the horizontal cell somata, processes and terminals. (C) Merged images reveal the co-localization of VGAT and CaBP to horizontal cells, especially in the processes and endings. Insets reveal a digital magnification of the boxed region, showing the bulb-like endings emerging from laterally coursing horizontal cell processes. Confocal images were scanned at 0.5 μm intervals and a total of 10 optical images were obtained and compressed for viewing. Scale bar: 10 μm.
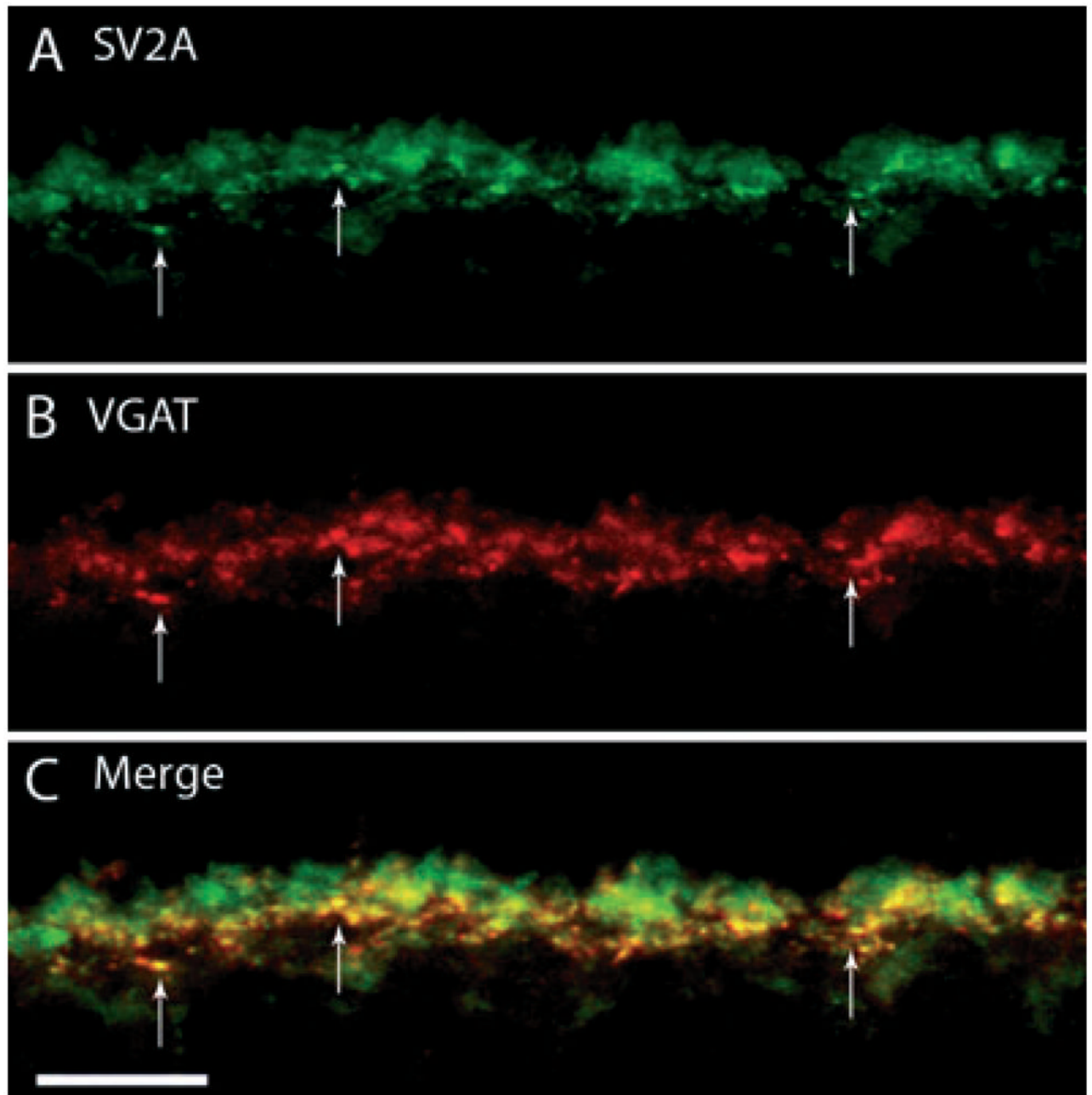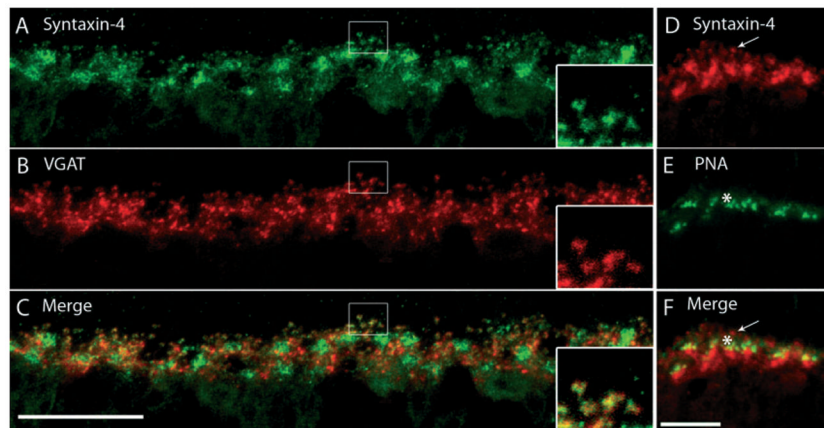
Fig. 2.
SV2A, a synaptic vesicle protein, labels horizontal cell terminals. A vertical section of guinea pig retina was double labeled with antibodies to SV2A and VGAT. (A) SV2A immunolabeling was localized to horizontal cell endings in the OPL, as well as photoreceptor terminals. (B) VGAT antibodies labeled horizontal cell terminals in the outer retina. (C) Merged images reveal the co-localization of SV2A labeling with that of VGAT. Arrows point to co-localized puncta along the OPL. Confocal images were scanned at 0.5 μm intervals and a total of eight optical images were obtained and compressed for viewing. Scale bar: 10 μm.
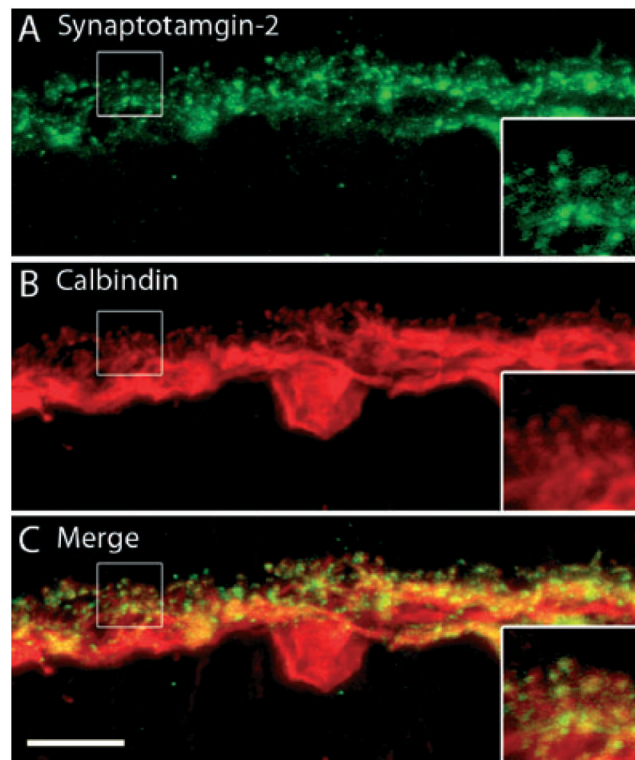
Fig. 3.
Synaptotagmin-2, a sensor for Ca^{2+}-triggered vesicular release, localizes to horizontal cell processes and their terminals. A vertical section of guinea pig retina was double labeled with antibodies to synaptotagmin-2 and CaBP. (A) Synaptotagmin-2 antibodies labeled the processes in the OPL, with more intensely labeled dots throughout OPL. (B) CaBP immunostaining is in horizontal cell somata, processes and horizontal cell endings. (C) Merged images show co-localization of synaptotagmin-2 and CaBP immunostaining in horizontal cell processes and endings. The inset reveals a digital magnification of the boxed region indicating horizontal cell processes extending from the OPL. Confocal images were scanned at 0.7 μm intervals and a total of 10 optical images were obtained and compressed for viewing. Scale bar: 10 μm.

Fig. 4.
(A–C) Synapsin I, a marker of conventional synapses, is present in horizontal cells. A vertical section of guinea pig retina was double labeled with antibodies to synapsin I and CaBP. (A) Synapsin I immunolabeling was present throughout the OPL, with labeled puncta just above the OPL. Synapsin I also labels bipolar cell bodies just below the OPL. (B) CaBP immunoreactivity is strongest in the cell bodies, but also along the horizontal cell processes and their endings. (C) Merged images reveal the co-localization of synapsin I and CaBP immunoreactivity in the horizontal cell fine processes and endings emerging from the main processes. Confocal images were scanned at 0.5 μm, and a total of 11 optical images were obtained and compressed for viewing. Scale bar: 20 μm. (D–F) Synapsin I is also localized to rod bipolar cell dendrites. A vertical section of guinea pig retina was double labeled with antibodies to synapsin I and PKCa. (D) Synapsin I immunolabeling is present throughout the OPL with labeling of the bipolar cell somata as well. There are also punctate-like areas of more intense immunostaining throughout the OPL. (E) Protein kinase Ca (PKCa), a marker of rod-bipolar cells labels bipolar cell soma and the dendritic tree. (F) Merged images reveal co-localization between synapsin I labeling and PKCa at the rod bipolar dendrites. Confocal images were scanned at 0.8 μm intervals, and a total of 10 optical images were obtained and compressed for viewing. Scale bar: 10 μm.
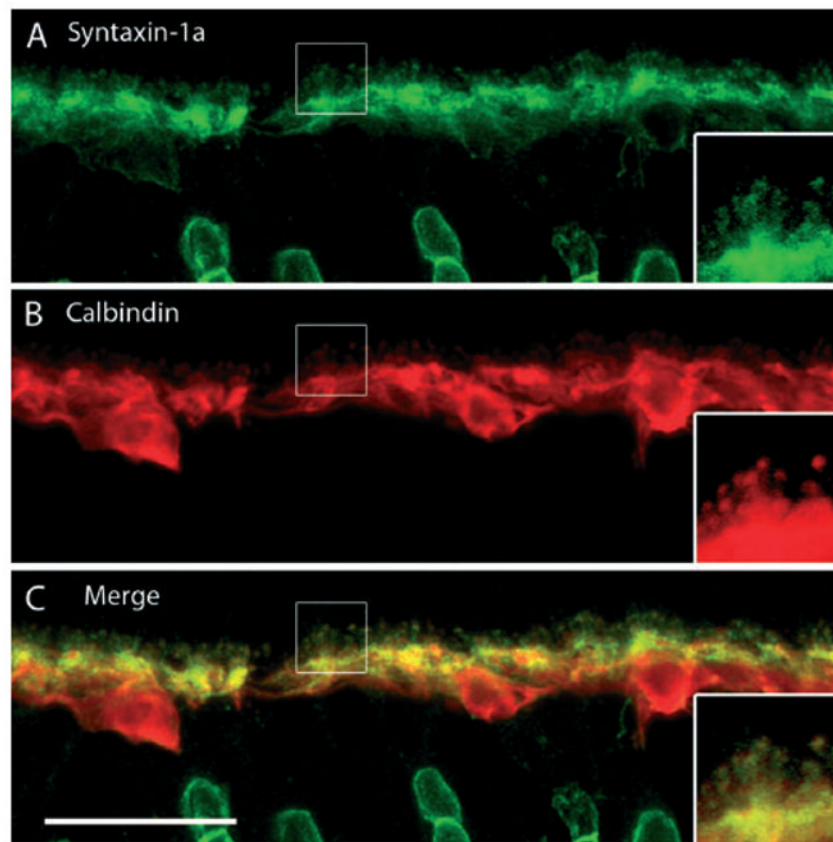
Fig. 5.
Syntaxin-1a, a SNARE core protein, localizes to amacrine cells and horizontal cell processes and endings. (A) Syntaxin-1a immunolabeling labels horizontal cell processes and endings in the OPL, as well as amacrine cells in the inner nuclear layer (INL). The horizontal cell somata are more faintly labeled. Labeled amacrine cells can be seen in the INL at the bottom of the image. (B) CaBP immunoreactivity is present in horizontal cell somata, their processes and terminal endings. (C) Merged images reveal the co-localization of syntaxin-1a and CaBP immunoreactivity in the OPL to the laterally running horizontal cell processes, as well as the finer endings that emerge from them. Confocal images were scanned at 0.6 μm intervals and a total of nine optical images were obtained and compressed for viewing. Insets reveal digital magnification of the boxed region, highlighting the horizontal cell process and endings. Scale bar: 20 μm.

Fig. 6.
Syntaxin-4, a SNARE complex protein, localizes to horizontal cell processes and endings. (A–C) A vertical section of guinea pig retina was double labeled with antibodies to syntaxin-4 and VGAT. (A) Syntaxin-4 immunolabeling was robust along the OPL with a punctate pattern. There were also regions of more intense staining in the OPL that were located along horizontal cell processes and near the soma. (B) VGAT immunolabeling was localized to the laterally running processes of horizontal cells, as well as the finer processes and endings emerging from them. (C) Merged images reveal the co-localization of syntaxin-4-labeled puncta and VGAT-labeled endings of horizontal cells. The insets are a digital magnification of the boxed region, which demonstrates staining of the delicate processes and endings of horizontal cells by both syntaxin-4 and VGAT antibodies. Confocal images were scanned at 0.5 μm intervals and a total of 10 optical images were obtained and compressed for viewing. Scale bar: 20 μm. (D–F) Syntaxin-4 does not label cone photoreceptor terminals. (D) Dense spots of syntaxin-4 immunoreactivity occur at regular intervals along the OPL. (E) Cone pedicles, labeled with peanut-agglutinin (PNA), produce a similar punctate pattern of densities along the OPL. (F) Merged images reveal that the syntaxin-4-immunoreactive clusters correspond to horizontal cell endings grouped together just underneath cone pedicles. The arrow points to a horizontal cell ending that continues up past the cone pedicle (indicated by the asterisk) to terminate at a rod spherule, which is more distal to horizontal cells than cone pedicles. Confocal images were scanned at 0.5 μm intervals and a total of eight optical images were obtained and compressed for viewing. Scale bar: 10 μm.
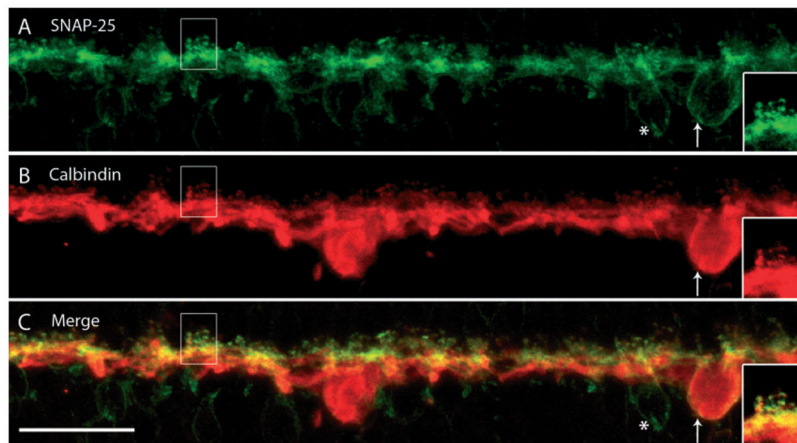
Fig. 7.
SNAP-25, a SNARE complex protein, is expressed in horizontal cell processes and endings. A vertical section of guinea pig retina was double labeled with anti-SNAP-25 and anti-CaBP antibodies. (A) SNAP-25 is expressed in the OPL at the fine processes and endings emerging from the laterally distributed processes throughout. There was also faint labeling of cell soma within the inner nuclear layer underneath the OPL. (B) CaBP is expressed in horizontal cell somata, processes and endings. (C) Merged images show the co-localization of SNAP-25 and CaBP to horizontal cell terminals. Although SNAP-25 faintly labeled a horizontal cell body, as indicated by co-localization with CaBP (arrow), it also weakly labeled nearby bipolar cell bodies (asterisk). The insets are a digital magnification of the boxed region. Confocal images were scanned at 0.7 μm intervals and a total of 10 optical images were obtained and compressed for viewing. Scale bar: 20 μm.
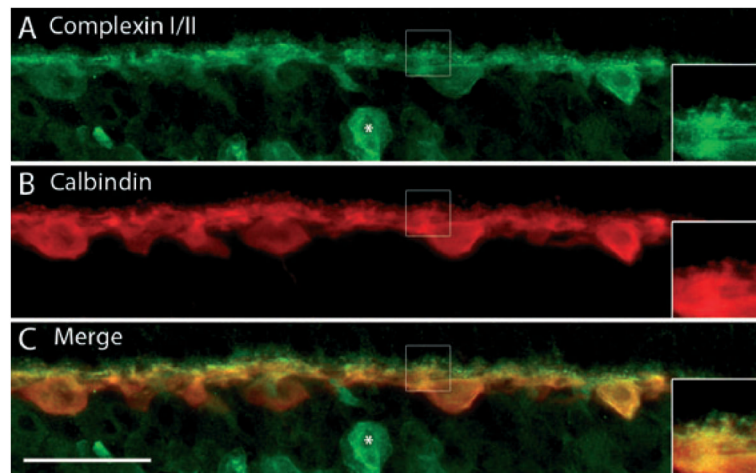
Fig. 8.
Complexin I/II, a cytosolic SNARE-associated protein, labels horizontal cell soma, processes and endings. A vertical section of guinea pig retina was double labeled with complexin I/II and CaBP antibodies. (A) Complexin I/II immunolabeling is very robust in horizontal cell somata and the larger laterally running processes, as well as the finer processes and endings emerging from them. A labeled amacrine cell is indicated by the asterisk. (B) CaBP expression is also robust in horizontal cell bodies, processes and terminals. (C) Merged images reveal the co-localization of complexin I/II to all parts of the horizontal cells, including the distal processes and endings. Insets are digital magnification images of the boxed region, highlighting several horizontal cell terminals and the localization of complexin I/II to the endings. Confocal images were scanned at 0.7 μm intervals and a total of 10 optical images were obtained and compressed for viewing. Scale bar: 20 μm.

Table 1

Primary antibodies

Antibody	Host	Immunogen	Source	Dilution	Reference
CaBP	Rabbit	Recombinant rat calbindin D-28k (CB)	Swant, Bellinzona, Switzerland CB38	1 : 10 000	Haverkamp & Wässle (2000), Strettoi et al. (2002), Loeliger & Rees (2005), Damiani et al. (2008), Gaillard et al. (2008)
	Mouse	Bovine kidney calbindin-D	Sigma-Aldrich, St Louis, MO, USA, C9858, clone CB-955	1 : 2500	Renteria et al. (2005), Deng et al. (2006), Lee et al. (2006), Gargini et al. (2007), Hirano et al. (2007), Damiani et al. (2008), Eitaiche et al. (2009), Kyhn et al. (2009)
Complexin I/II	Rabbit	Synthetic peptide EEERKAKHARMEAEREKVRQQIRDKYGLKKKEEKEAE (aa 45–81 in complexin II) coupled to key-hole limpet hemocyanin via an added N-terminal cysteine residue	Synaptic Systems, Göttingen, Germany, 122 102	1 : 15 000	Reim et al. (2001), Hirano et al. (2005), Xue et al. (2008)
PKCα	Rabbit	Synthetic peptide corresponding to aa 659–672 from the C-terminal variable (V5) region of rat PKCα	Sigma-Aldrich, P4334	1 : 30 000	Wang et al. (2001), Elshatory et al. (2007)
SNAP-25	Rabbit	Synthetic peptide corresponding to the N-terminal of human SNAP-25 (synaptosome-associated protein-25) aa 9–29 with C-terminally added lysine conjugated to KLH	Sigma-Aldrich, S9684	1 : 60 000	Frassoni et al. (2005), Szklarczyk et al. (2007)
SV2A	Rabbit	Synthetic peptide EEGFRDRAAFIRGAKD (aa 2–17 in human) coupled to key-hole limpet hemocyanin via an added N-terminal cysteine residue	Synaptic Systems, 119 002	1 : 500	Janz et al. (1999), Janz & Südhof (1999), von Kriegstein & Schmitz (2003), Wang et al. (2003), Witkovsky et al. (2008)
Synapsin I	Mouse	Recombinant human synapsin 1	Millipore, Billerica, MA, USA, MAB10137 clone 3C5	1 : 100	De Camilli et al. (1983), Mandell et al. (1990, 1992), Smith et al. (1993), Hirano et al. (2005)
Syntaxin-1a	Mouse	Synaptosomal plasma-membrane fraction from adult rat hippocampus	Sigma-Aldrich, S0664	1 : 1000	Barnstable et al. (1985), Inoue et al. (1992), Morgans et al. (1996), Hirano et al. (2005)
Syntaxin-4	Rabbit	Highly purified corresponding to residues 2–23 of rat or mouse syntaxin-4 (accession Q08850)	Millipore, AB5330	1 : 1000	Gouraud et al. (2002), Spurlin et al. (2004), Sherry et al. (2006), Spurlin & Thurmond (2006), Hirano et al. (2007)
VGAT	Mouse	Synthetic peptide AEPPVEGDIHYQR (aa 75–87 in rat) coupled to key-hole limpet hemocyanin via an added N-terminal cysteine	Synaptic Systems, 131 011, clone 117G4	1 : 200	McIntire et al. (1997), Sagné et al. (1997), Jellali et al. (2002), Johnson et al. (2003), Guo et al. (2009)
Zmp-1 (synaptotagmin-2)	Mouse	1–5-day zebrafish embryo	Zebrafish International Resource Center, Eugene, OR, USA	1 : 200	Fox & Sanes (2007), Wässle et al. (2009)

aa, amino acids; KLH, keyhole limpet hemocyanin; PKCα, protein kinase C α.