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Evidence That Certain Retinal Bipolar Cells Use Both Glutamate and GABA

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ABSTRACT

Retinal bipolar neurons release the excitatory transmitter, glutamate. However, certain bipolar cells contain GABA, raising the question whether a neuron might release both transmitters and, if so, what function might the inhibitory transmitter play in a particular circuit? Here we identify a subset of cone bipolar cells in cat retina that contain glutamate, plus its vesicular transporter (VGLUT1), and GABA, plus its synthetic enzyme (GAD_{65}) and its vesicular transporter (VGAT). These cells are negative for a marker of ON bipolar cells and restrict their axons to the OFF strata of the inner synaptic layer. They do not colocalize with the neurokinin 3 receptor that stains a type (or two) of OFF bipolar cells. By "targeted injection," we identified two types of OFF bipolar cell with the machinery to make and package both transmitters. One of these types costratifies with a dopamine plexus. J. Comp. Neurol. 478:207–218, 2004.

Indexing terms: VGLUT; VGAT; GAD; dopamine; dye injection

Dale's law, which stated that a neuron should release only one type of transmitter (Dale, 1935), has been pretty well disproved. We now know that a synapse can release several different peptidergic neuromodulators (Hökfelt et al., 1984; reviewed by Nusbaum, 2001), or a catecholaminergic modulator + a fast transmitter, such as dopamine + glutamate (Sulzer and Rayport, 2000), or two fast transmitters, such as acetylcholine + GABA (Brecha et al., 1988; Vaney and Young, 1988; O'Malley et al., 1992), or GABA + glycine (Jonas et al., 1998; O'Brien and Berger, 1999). Even the brain's most abundant fast and antagonistic transmitters, glutamate and GABA, can be released by the same cell; for example, by hippocampal mossy fibers (Walker et al., 2001) and possibly by salamander bipolar cells (Yang and Yazulla, 1994; Yang, 1998; Yang and Wang, 1999; Yang et al., 2003). However, it is still not clear *why* a neuron releases two transmitters with apparently opposite effects. To address this, one needs to place such a neuron within the context of its circuitry. For this, mammalian retina offers a clear advantage because many of its cell types and connections are known (Masland, 2001; Sterling and Demb, 2004).

Bipolar cells, collecting input from photoreceptors and signaling to ganglion cells, use glutamate as the transmitter (Slaughter and Miller, 1983; Massey and Miller, 1988; reviewed by Massey, 1990). However, in salamander retina, certain bipolar cells contain GABA and express GABA's synthetic enzyme, GAD, and its transporter GAT (Yang and Yazulla, 1994; Yang, 1998). In mammalian retina, certain bipolar cells contain GABA (Wässle and Chun, 1989; Pourcho and Owczarzak, 1989; Grünert and Wässle, 1990; Vardi and Auerbach, 1995), and certain bipolar cells express GAD (reviewed by Freed, 1992; Vardi and Auerbach, 1995). However, it is not known whether these are the same cells, and whether they might satisfy other criteria for GABAergic transmission. Here we identify two types of OFF bipolar cell in the cat retina that satisfy several of the criteria for use of glutamate and GABA.

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MATERIALS AND METHODS

Tissue preparation

Eyes were obtained from adult cats under deep sodium pentobarbital anesthesia (40 mg/kg) at the end of acute physiological experiments. Following enucleation, the animal was sacrificed by an overdose of pentobarbital. These procedures were performed in accordance with guidelines of University of Pennsylvania and National Institutes of Health. The posterior half of the eye was fixed at room temperature by immersion in buffered 4% paraformaldehyde (10 minutes, light microscopy) or 4% paraformaldehyde containing 0.01% glutaraldehyde (for GAD_{65}, electron microscopy; for GABA and glutamate, light microscopy). Following rinse and overnight cryoprotection in 0.1 M phosphate buffer (pH 7.4) containing 30% sucrose, the central area (up to 2 mm eccentricity) was cut. For cryosections (10 μm), the tissue was incubated in a mixture of tissue freezing medium with buffered 30% sucrose, removed to fresh medium, and frozen in liquid nitrogen. For Vibratome sections (50–100 μm), tissue was frozen in liquid nitrogen, thawed, and embedded in 4% agarose.

Immunostaining

Immunocytochemistry was performed on radial sections following a standard protocol: preincubate in diluent containing 10% normal goat serum, 5% sucrose, and 0.15–0.3% saponin, or 0.1–0.7% Triton X-100 in 0.1 M phosphate buffer for 1 hour at room temperature; incubate in primary antibodies for 2–4 days at 4°C; wash and incubate in secondary antibodies conjugated to horseradish peroxidase (HRP) or to a fluorescent marker; incubate (HRP conjugated antibodies) in 0.05% 3, 3′-diaminobenzidine (DAB) + 0.01% hydrogen peroxide in PB for 15 minutes; and wash and mount in glycerin or Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA). For electron microscopy, fixed tissue was frozen and thawed three times prior to incubation in primary antibody; no detergent was added. DAB reaction product was intensified with gold-substituted silver. For double-labeling for GAD_{65} with GABA, VGAT, VGLUT1, glutamate, $G\alpha_o$, and neurokinin 3 receptor (NK3-R), the primary antibody against GAD_{65} (raised in mouse) and the other antibodies (raised in rabbit) were applied simultaneously. Fluorescence-conjugated secondary antibodies were also applied simultaneously. In control experiments, secondary antisera never gave neuronal staining, antirabbit never recognized the mouse antibody, and the antimouse antibodies never recognized the rabbit antibodies (Fig. 1). For double-labeling for NK3-R and tyrosine hydroxylase (TH), the antibodies (both raised in rabbit) were applied sequentially: anti-NK3-R followed by an excess of antirabbit-FITC F(ab′), then anti-TH followed by antirabbit-Cy3 (IgG).

Antibodies

Monoclonal antibody against GAD_{65} raised in mouse was used at high concentration (GAD-6; 1:20) to maximize soma staining. GAD-6, originally prepared by Chang and Gottlieb (1988), was obtained from the Developmental Studies Hybridoma Bank (Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and Department of Biology, University of Iowa, Iowa City, IA, contract N01-HD-6-2915). The specificity of the antibody in cat and monkey retinas has been thoroughly established (Vardi et al., 1992; Vardi and Auerbach, 1995). Its use at the present concentrations and even higher (tested up to 1:5) does not increase background staining. Polyclonal antibody against GABA was raised in guinea pig (1:500–1,000; Chemicon, Temecula, CA). Polyclonal antibody against glutamate was raised in rabbit (1:100; Chemicon). Polyclonal antibody against $G\alpha_o$ was raised in rabbit against the peptide

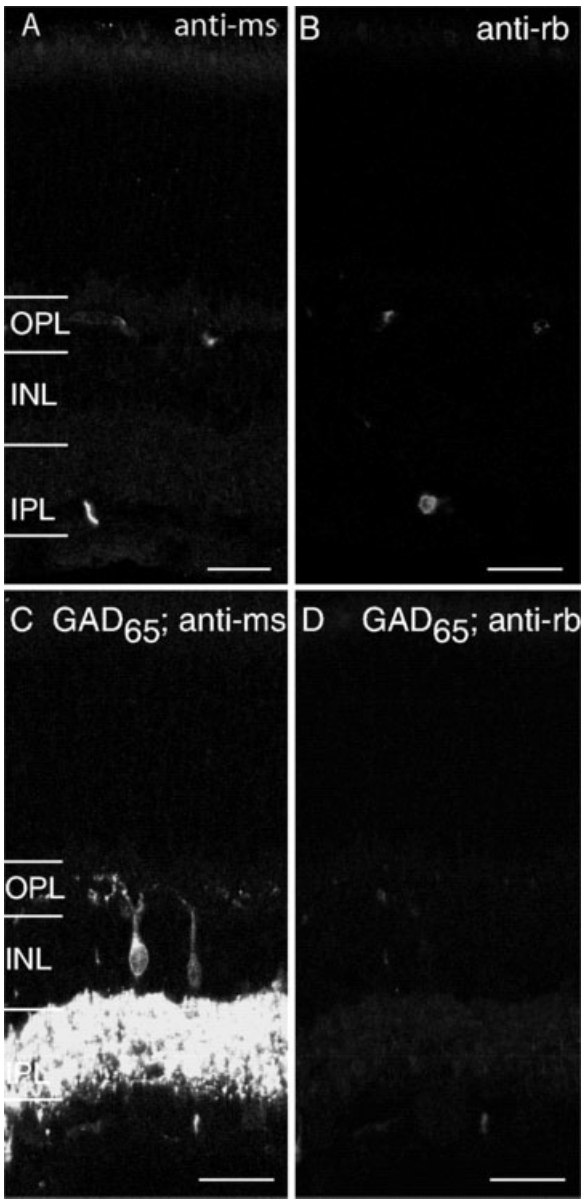

Fig. 1. Secondary antisera give very low background activity. **A,B:** Cat retina stained with antimouse conjugated to FITC (A) or antirabbit conjugated to Cy3 (B); only blood vessels are stained. **C,D:** Cat retina stained for GAD_{65} and labeled with antimouse conjugated to FITC (C) followed by antirabbit Cy3 (D). For this and rest of figures: OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bars = 25 μm in A–D.

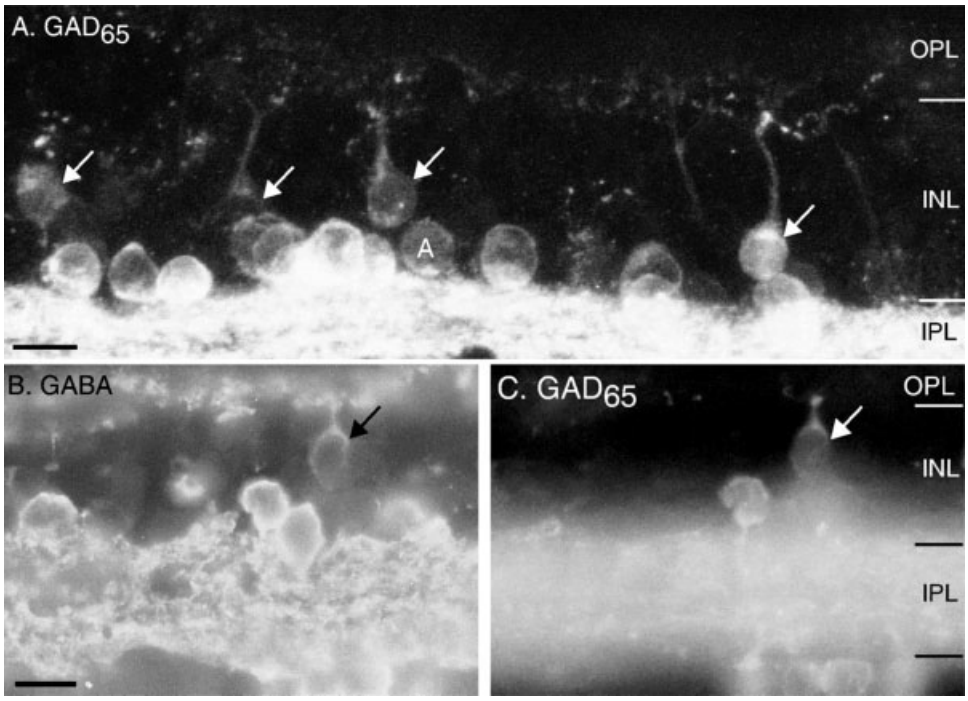

Fig. 2. GABA containing bipolar somas express the GABA synthetic enzyme, GAD_{65}. **A:** Many bipolar cells (arrows) in cat retina immunostain for the GABA-synthesizing enzyme isoform, GAD_{65}. A, amacrine cell soma (projection of 21 optical sections spanning a depth of 21 μm). **B,C:** GAD_{65}-positive bipolar cell is also positive for GABA (arrow; epifluorescence, 100× 1.32 NA oil). Scale bars = 10 μm in A,B.

ANNLRGCGLY located at the C terminus (1:1,000, gift of David Manning, University of Pennsylvania). Polyclonal antibody against the vesicular GABA transporter (VGAT) was generated at the lab of Dr. Edwards and raised in rabbit against 99 amino acids located at the N-terminus (1:100, Chaudhry et al., 1998). Polyclonal antibody against the vesicular glutamate transporter (VGLUT1) was also generated at the laboratory of Dr. Edwards, it was raised in rabbit against the last 68 amino acids (C-terminus) (1:100, Bellocchio et al., 1998). A second polyclonal antibody raised in guinea pig against rat VGLUT1 (C-terminus) was commercially available (Chemicon). Polyclonal antibody against NK3-R was raised in rabbit against amino acids 438–452 (AB "94192") of the intracellular portion of rat NK3-R (Grady et al., 1996) (1:2,000, gift of Dr. Nigel Bunnett, UCSF). Polyclonal antibody against TH was raised in rabbit (1:500, Chemicon). *Secondary antibodies:* Goat antimouse peroxidase-conjugated F(ab′) fragment (Protos Immunoresearch, Burlingame, CA). Goat antimouse Alexa 488 (1:100) was from Molecular Probes (Eugene, OR), and goat antirabbit Cy3 (1:300) and donkey antirabbit Cy5 (1:100) were from Jackson ImmunoResearch Lab (West Grove, PA). Incubation with all secondary antibodies (omitting the primaries) gave a very low background (Fig. 1).

Bipolar cell injection

Retina was lightly fixed in 4% paraformaldehyde for 10–30 minutes and a small piece (4 × 4 mm) was immunostained overnight for GAD_{65}. The tissue was washed (3× 10 minutes in 0.1 M phosphate buffer) and then incubated for 30 minutes with antimouse Alexa 488 and 0.3% Tween 20. The tissue was embedded in 4% agarose and sliced at 200 μm on a Vibratome. A sharp microelectrode (~30 M° measured with 1 M KCl) was filled at the tip with 1% DiI (Molecular Probes) in 100% ethanol, then the shank and barrel were filled with 100% ethanol. Bipolar cells identified by immunostaining for GAD were injected by expelling dye with 1–50 nA positive current for ~10 seconds. Tissue was imaged by confocal microscopy; most images represent single optical sections taken under 40× oil immersion, NA = 1.25; 63× water immersion, NA = 1.2; or 100× oil immersion NA = 1.4.

Cell counts

Cell counts were made on sections stained for GAD. One retina was sectioned at 60 μm thick, reacted with DAB, and photographed with DIC optics. Another retina was sectioned at 100 μm thick, stained with Cy3, and analyzed from confocal sections (Leica, 100× objective). Soma positions were plotted and the mean nearest-neighbor distance was calculated (Wässle and Riemann, 1978). Images were cropped and adjusted for contrast with Adobe PhotoShop (San Jose, CA).

RESULTS

Certain bipolar cells contain GABA, GAD_{65}, and the vesicular GABA transporter

Although certain bipolar cells in cat were known to contain GABA (Chun and Wässle, 1989; Pourcho and Owczarzak, 1989), it was unknown whether they also contain GAD. We tested this with an antibody for GAD_{67} and found bipolar cells to be negative (see also Vardi and Auerbach, 1995). However, an antibody to GAD_{65} stained numerous bipolar somas in the middle of the inner nuclear layer (Fig. 2A). Their dendritic stalks and finer dendritic processes were also stained. When we costained for GABA and GAD_{65}, all GABA-positive cells were also GAD_{65}-positive (Fig. 2B,C; see also fig. 1 in Vardi and Auerbach, 1995). The reverse was not true because the antibody

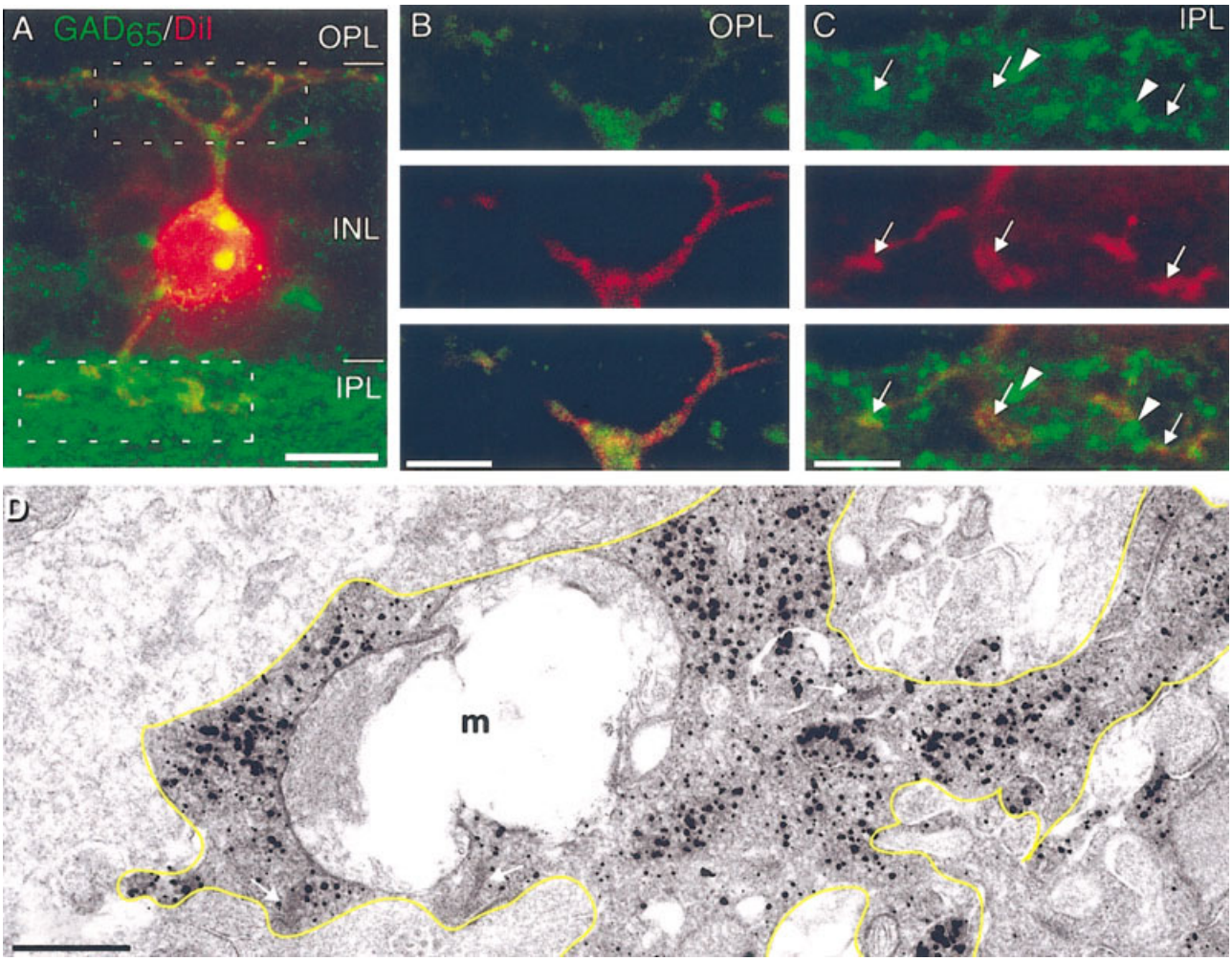

Fig. 3. Dendrites and axons of GABA containing bipolar cells express GAD_{65}. **A:** DiI injected GAD_{65}-bipolar cell. Dendrites are GAD_{65}-positive (arrowheads) (confocal, 100× 1.4 NA oil, the cell is a projection of 16 optical sections). **B:** Higher magnification of dendrites (single optical section of region enclosed in dashed rectangle). GAD_{65} is present in these dendrites. **C:** Higher magnification of IPL (single optical sections of the region enclosed in dashed rectangle). Axon terminals (arrows) contain GAD_{65}: the left and right arrows point to bipolar terminals (red) whose corresponding green stains are almost identical. The middle arrow points to a bipolar axon terminal, which in this case is outlined by the diI (because diI stains the membrane). The green puncta are present either on top of the red or inside the profile. Bright green structures near the DiI-stained terminals (arrowheads) might be processes of GABAergic amacrine cells feeding back onto the bipolar. **D:** Electron micrograph of bipolar terminals located in stratum 1 stained for GAD_{65}. White arrows point to synaptic ribbons which identify the structure as a bipolar axon terminal. m, mitochondria. For clarity, the membrane was outlined in yellow. Note that the gold particles are clustered. This probably corresponds to the puncta seen by light microscopy. Scale bars = 10 μm in A; 5 μm in B, C; 0.5 μm in D.

against GABA penetrated less deeply than the antibody against GAD_{65}. GAD_{65}-staining in the bipolar cell somas was weaker than in some amacrine somas, but was of similar intensity to others. Colocalization of GABA and GAD_{65} in these bipolar cells suggests that the GAD_{65} staining is specific and that the bipolar cells indeed synthesize GABA.

To localize GAD_{65} at the subcellular level we used a new method, "targeted injection" (Kao and Sterling, 2003), and confirmed it by electron microscopy. First, we identified a GABA bipolar cell by immunostaining for GAD_{65}; then we injected it with DiI to reveal the dendritic and axonal arbors. Anti-GAD_{65} was used because it penetrates well and does not stain horizontal cells. The bipolar dendrites examined at high magnification (100× objective, NA = 1.4) were confirmed to express GAD_{65} (Fig. 3A,B; also Fig. 10). The axon terminals also stained for GAD_{65}, although less intensely than amacrine processes (Fig. 3C), and it sometimes appeared punctate within the bipolar axon. GAD_{65}-staining in bipolar axon terminals was confirmed at the EM level, using tissue processed for standard preembedding immunocytochemistry (no dye injection), where bipolar axon terminals were identified by the presence of a synaptic ribbon. Most terminals were negative for GAD_{65}, but in stratum 1 of the inner plexiform layer we found a few GAD_{65}-positive terminals (Fig. 3D). The lack of staining in most terminals can be easily explained by the presence of the non-GAD-containing bipolar cells,

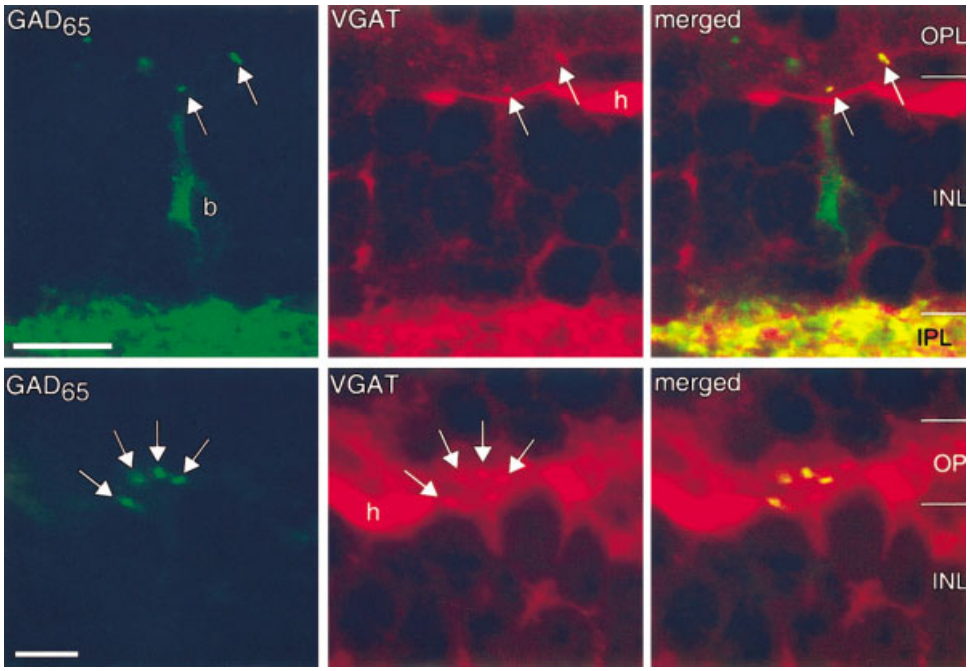

Fig. 4. GAD_{65}-positive bipolar cells contain vesicular GABA transporter (VGAT). Costaining for GAD_{65} (green) and VGAT (red). VGAT is found in bipolar soma and dendrites (arrows) and also in horizontal cell somas and processes. Note that the bright red horizontal cells are not seen in green and certain green dendrites are not red. This shows that the secondary antibodies did not cross react with the wrong primaries. b, bipolar cell; h, horizontal cell. Scale bars = 10 μm in top panel; 5 μm in bottom panel.

but it is also possible that GAD_{65}-containing bipolar cells remained unstained because of the stronger fixation and lack of detergent required for EM.

Next, we immunostained for VGAT, the vesicular GABA transporter (Chaudhry et al., 1998). In the outer plexiform layer VGAT was strongly expressed, both in horizontal processes, as previously reported for rat (Cueva et al., 2002), and also in GABA bipolar dendrites (Fig. 4). As neither horizontal cell processes nor bipolar dendrites contain synaptic vesicles, this finding suggests that VGAT might act to transport from cytoplasm to the extracellular space (Schwartz, 1987). In the inner plexiform layer VGAT was strongly expressed throughout all strata, frequently costaining with GAD_{65}. However, because GABA bipolar terminals intermingle with the far more numerous GAD_{65} amacrine processes, it was impossible to determine which of the costained processes were bipolar. Attempts to stain first for GAD_{65} + VGAT and then inject DiI failed because VGAT's penetration was insufficient. Nonetheless, the presence of VGAT in bipolar cells that also contain GABA and GAD suggests that these cells are likely GABAergic.

GABA bipolar cells are also glutamatergic

We next tested whether GABA bipolar cells also use glutamate. Double-staining showed that the somas of all GAD_{65}-positive bipolar cells also contained glutamate (Fig. 5A). However, glutamate is a precursor for GABA synthesis, so we also tested for vesicular glutamate transporter (VGLUT). As expected, anti-VGLUT1 (with both the rabbit and guinea pig antibodies) strongly stained both synaptic layers. Positive structures in the outer plexiform layer were the photoreceptor terminals. Positive structures in the inner plexiform layer were distinct lobules that colocalized with anti-kinesin, which stains synaptic ribbons, and therefore identifies bipolar axon terminals. Rod bipolar axon terminals in stratum 5 were particularly strong (Fig. 5C), and cone bipolar terminals in both ON and OFF strata were also clearly evident. GAD_{65} colocalized with VGLUT1 (rabbit antibody) in some bipolar axon terminals located in the OFF stratum, but not in the ON stratum (Fig. 5C). Also, although somas typically did not stain for VGLUT1, we observed a few somas which were positive for both VGLUT1 and GAD_{65} (Fig. 5B). We conclude that GAD_{65} bipolar cells also contain and package glutamate for release.

GABA bipolar cells comprise two types of OFF cell

To determine whether GAD_{65} bipolar cells belong to the ON or OFF class, we immunostained for GAD_{65} and the ON bipolar cell marker, $G\alpha_o$ (Vardi, 1998). GAD_{65}-positive bipolar cells were negative for $G\alpha_o$, suggesting that they are OFF cells (Fig. 6A). To further classify them we stained for NK3-R, which stains two types of OFF bipolar cells in rat and mouse (Casini et al., 2000, 2002; Oyamada et al., 1999; Haverkamp et al., 2003; Ghosh et al., 2004). This identified what seemed like one type of OFF bipolar cell, but this bipolar was negative for GAD_{65} (Fig. 6B,C). Lacking other markers (standard OFF cell markers such as recoverin do not work in cat), we made further targeted injections.

DiI staining of GAD_{65} bipolar somas revealed the full dendritic and axonal arbors (Fig. 7). Twenty-four GAD_{65}-positive bipolar cells were injected and all sent axons to the OFF layer. Most cells (20) arborized mainly in stratum 1 with the primary axon emitting branches nearly perpendicular to the stalk (type I; Fig. 8A). Four cells arborized in stratum 2 (type II; Fig. 7B). Reconstructions of the cells from optical sections (of injected cells and NK3-R cells) suggest that the branching patterns of NK3-R and type I cells are similar but NK3-R stratifies slightly lower than type I, bordering stratum 2 (Fig. 7C). The GABA bipolar cell morphology clearly differs from that of the interplexiform cell shown previously in cat and shown here by DiI

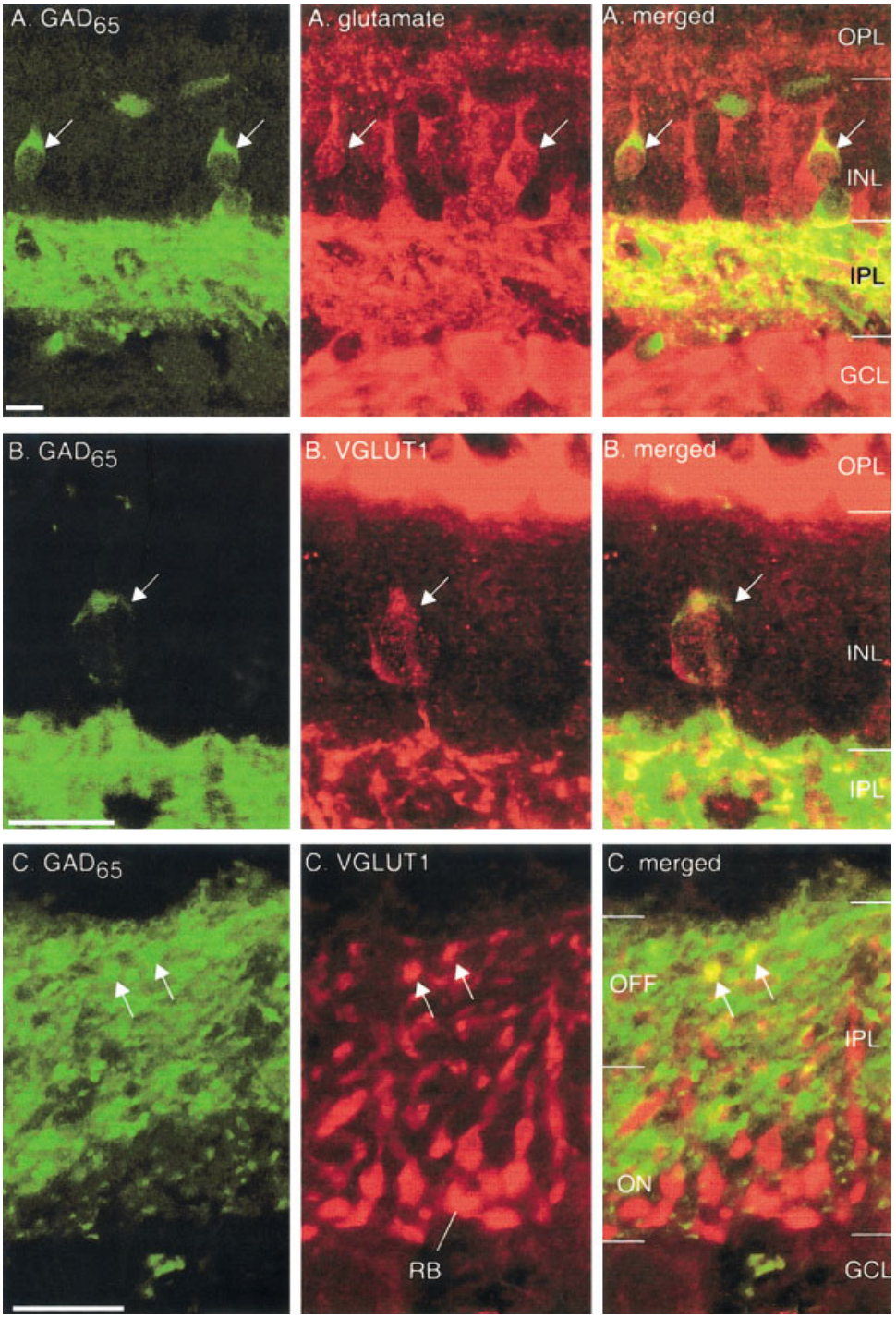

Fig. 5. GABA-bipolar cells contain glutamate and its vesicular transporter (VGLUT1). **A:** Double-staining for GAD_{65} and glutamate. All GAD_{65}-positive bipolar cells contain glutamate (arrows). **B,C:** Double-staining for GAD_{65} and VGLUT1. B: A GAD_{65}-positive bipolar soma stained for VGLUT1 (arrow). C: In general, VGLUT1 and GAD_{65} do not colocalize (indicating the antibodies do not cross-react). However, certain GAD_{65}-stained puncta in the OFF stratum of the IPL do contain VGLUT1 (arrows). These are probably axon terminals of the GAD_{65}-positive bipolar cell. Large puncta in the ON stratum are rod bipolar terminals (RB). Note that the bipolar axon terminals are clearly resolved (stained objects are about 2 μm, consistent with size of terminals as seen in Fig. 2). ON, ON stratum; OFF, OFF-stratum; GCL, ganglion cell layer. Scale bars = 10 μm in A–C.

injection (Fig. 8; Boycott et al., 1975; Nakamura et al., 1980; Pow and Hendrickson, 1999).

Distribution of GABA bipolar cells

The distribution density of GAD_{65}-positive bipolar cells was measured on 100-μm thick, vertical sections (rather than whole mount) to ensure complete antibody penetration. This plane also clearly showed the ascending dendrites, which allowed us to distinguish bipolar from amacrine somas. Bipolar somas tended to lie low in the inner nuclear layer, but their position varied. Cell density in the central area, determined from two retinas, was ~4,000 cells/mm^2, with a nearest-neighbor distance of 8.4 μm (Fig. 9, top). Cell density at 4–6 mm eccentricity, estimated in two sections from the same retina, was ~1,700 cells/mm^2, with a nearest-neighbor distance of 16.0 μm (Fig. 9, bottom). These densities are comparable to other cone bipolar cell types (Cohen and Sterling, 1990).

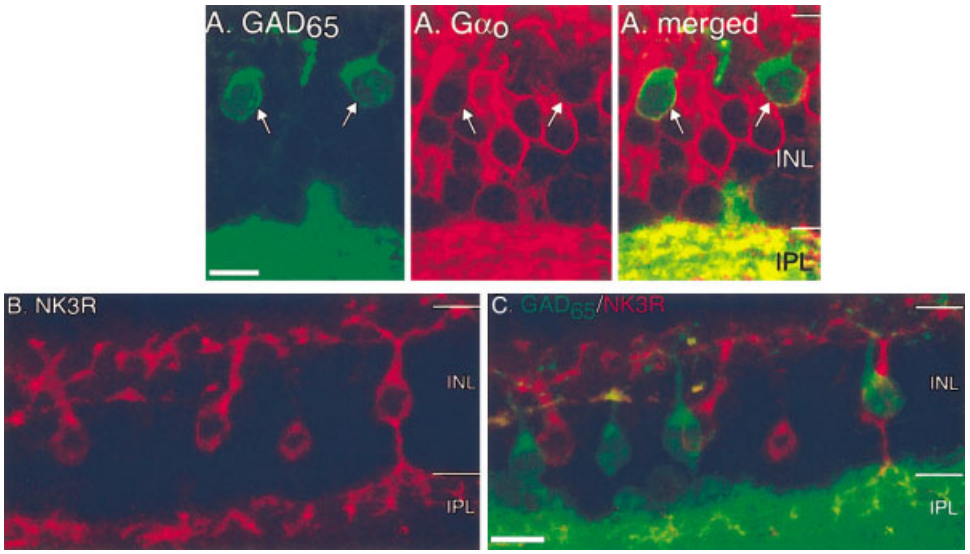

Fig. 6. GABA bipolar cells are OFF, but distinct from NK3-R-positive OFF bipolar cells. **A:** GAD_{65}-positive bipolar cells are negative for the ON bipolar marker $G\alpha_o$ and are therefore OFF cells (arrows). **B,C:** GAD_{65} and NK3-R do not colocalize in bipolar cell somas (projection of 23 optical sections, 23 μm). Scale bars = 10 μm.

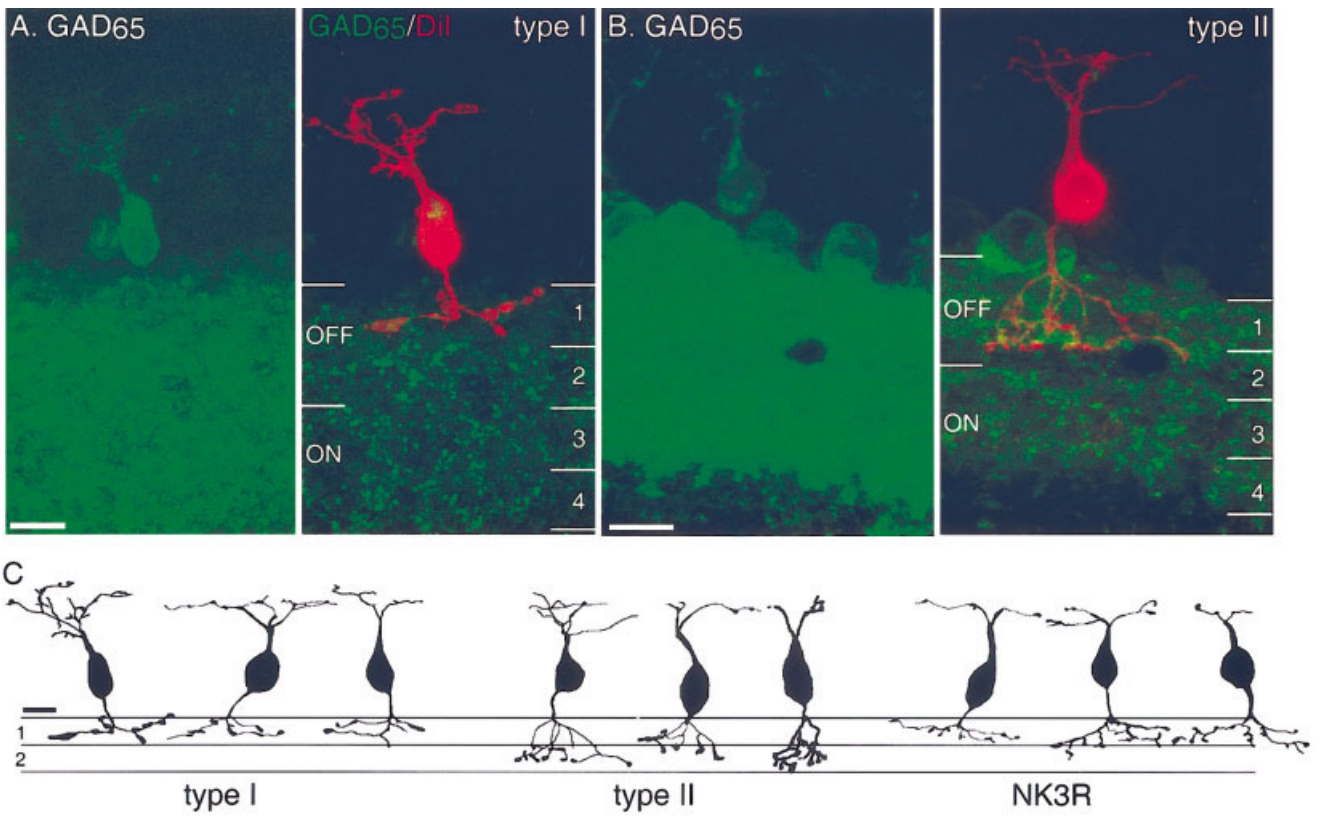

Fig. 7. GABA bipolar cells comprise two types. A,B: GAD_{65}-positive bipolar cells were first identified by immunostaining and then injected with DiI to reveal their complete morphology. **A:** Type I restricted its axon arbor to stratum 1 of the IPL (projection of 28 optical sections; 28 μm thick). **B:** Type II arborized in stratum 2 (projection of 21 optical sections; 21 μm). In the panels showing the injected cells, the intensity of the GAD_{65} staining (green) was reduced to permit clear visualization of the red cells. Numbers on the right designate strata. **C:** Tracings of injected (types I and II) and immunostained (NK3-R) cells; three types of OFF cone bipolar cells were identified here.

Ultrastructure of GAD_{65}-positive bipolar dendrites

We were surprised to find GAD_{65} and VGAT in the bipolar cell dendrites because dendrites are not expected to release transmitter. In tangential view the dendrites displayed a sparse network that tiled the plane continuously, preventing discrimination of individual dendritic arbors (Fig. 10A). The dendrites were fine (0.3–0.5 μm diameter) and regularly interrupted by prominent vari-

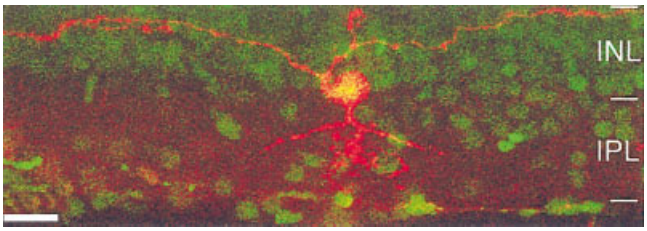

Fig. 8. Interplexiform cell injected with DiI cannot be mistaken for a GABA bipolar cell. Interplexiform soma is located in the amacrine stratum of the INL; its ascending processes spread more broadly than a bipolar dendritic arbor (projection of 20 optical sections, 20 μm). Retina was counterstained with Syto13 (green). Scale bar = 20 μm.

cosities (0.6–1 μm diameter) that contained both GAD_{65} and VGAT (Fig. 4). We surveyed numerous immunostained varicosities by EM, looking for clusters of synaptic vesicles, but found none.

Partially reconstructing three cone terminals from 21 serial EM sections, we observed eight dendritic tips apposed to the base of a cone. Each made a specialized contact ("basal contact"), which was smaller than the full apposition and was usually "triad-associated" (Fig. 10B–D). One dendrite invaginated the cone to form the *central* element of two different triads (under two ribbons) (Fig. 10B). We estimate that the 21 sections reconstructed about one-quarter of the area under a cone, and thus that each cone makes about 10 contacts with the GAD bipolar dendritic plexus. The GAD bipolar dendrites also formed specialized contacts with other processes; those were characterized by an electron-dense membrane of the GAD_{65}-positive dendrite, and often a dense striated material in the extracellular cleft (Fig. 10C,D). Five of the specialized contacts were with ON bipolar dendrites, one with a horizontal cell process, one with another GAD_{65}-stained dendrite, and nine unidentified. Membrane specializations between bipolar dendrites were also described between OFF midget bipolar dendrites and other bipolar and horizontal cells in monkey retina (Herr et al., 1997).

Possible connections of OFF bipolar axons

We investigated whether any of the OFF bipolar axons identified here might connect with the dense dopaminergic plexus that occupies the upper region of stratum 1 of the OFF synaptic layer. Visualizing this plexus with antibody against TH, we found the immunostained NK3-R bipolar axons to stratify just beneath it (Fig. 11A). Note that in the double-labeled material, the two staining patterns for TH and NK3-R are clearly discernable from each other, indicating that there was no cross-reaction between the Cy-3 antirabbit antibody and the rabbit anti-NK3-R. Careful examination of single optical sections did not reveal significant proximity between the NK3R dendrites and the dopaminergic arbor. Then targeted injection of type II GABA bipolars showed their axons to pass through the dopamine plexus, also arborizing just beneath it, mostly in stratum 2 (Fig. 11B), and they too were separated from the dopaminergic processes. Finally, targeted injection of type I GAD_{65} bipolars showed that although their axons arborize in

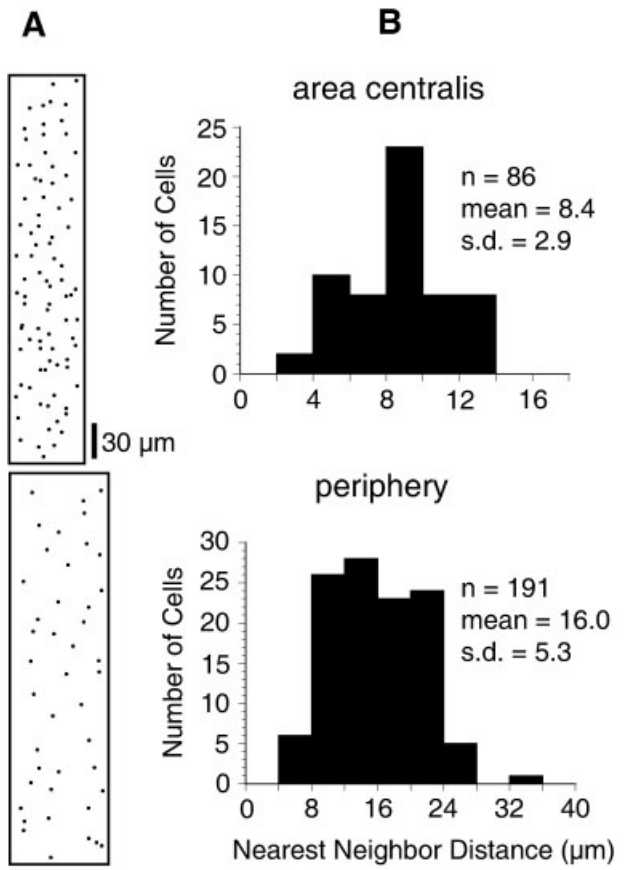

Fig. 9. Density distribution of GAD_{65}-labeled bipolar cells. **A:** Tangential views of soma location from the area centralis (top) and 4–6 mm eccentricity (bottom). For periphery, only half of the area used is shown. **B:** Nearest neighbor histograms of the two areas. n, total number of cells within reconstructed area. Number of cells whose nearest neighbor was calculated was 59 for central area and 113 for peripheral.

stratum 1 slightly deeper than the dopaminergic processes, they clearly comingle and appose the dopaminergic processes as they descend to the inner plexiform layer (Fig. 11C). Thus, the NK3-R bipolar and the type II GABA bipolar have little opportunity to connect with the dopamine plexus. However, the type I GABA bipolar can potentially contact this cell. Because the dopaminergic amacrine cell ultrastructurally receives bipolar contacts (Hokoc and Mariani, 1988; Gustincich et al., 1997), and because comparison of the OFF bipolar cells described here to those in rat and mouse (Euler and Wässle, 1995; Ghosh et al., 2004) suggests that type I's axon terminals are closest to the INL/IPL interface, it is likely that the bipolar contacts are from the type I GABA bipolar cell.

DISCUSSION

Identification of the GABA bipolar cells

The GABA bipolar cells studied here were clearly distinguished from the GABAergic interplexiform cell whose processes also ascend to the outer plexiform layer

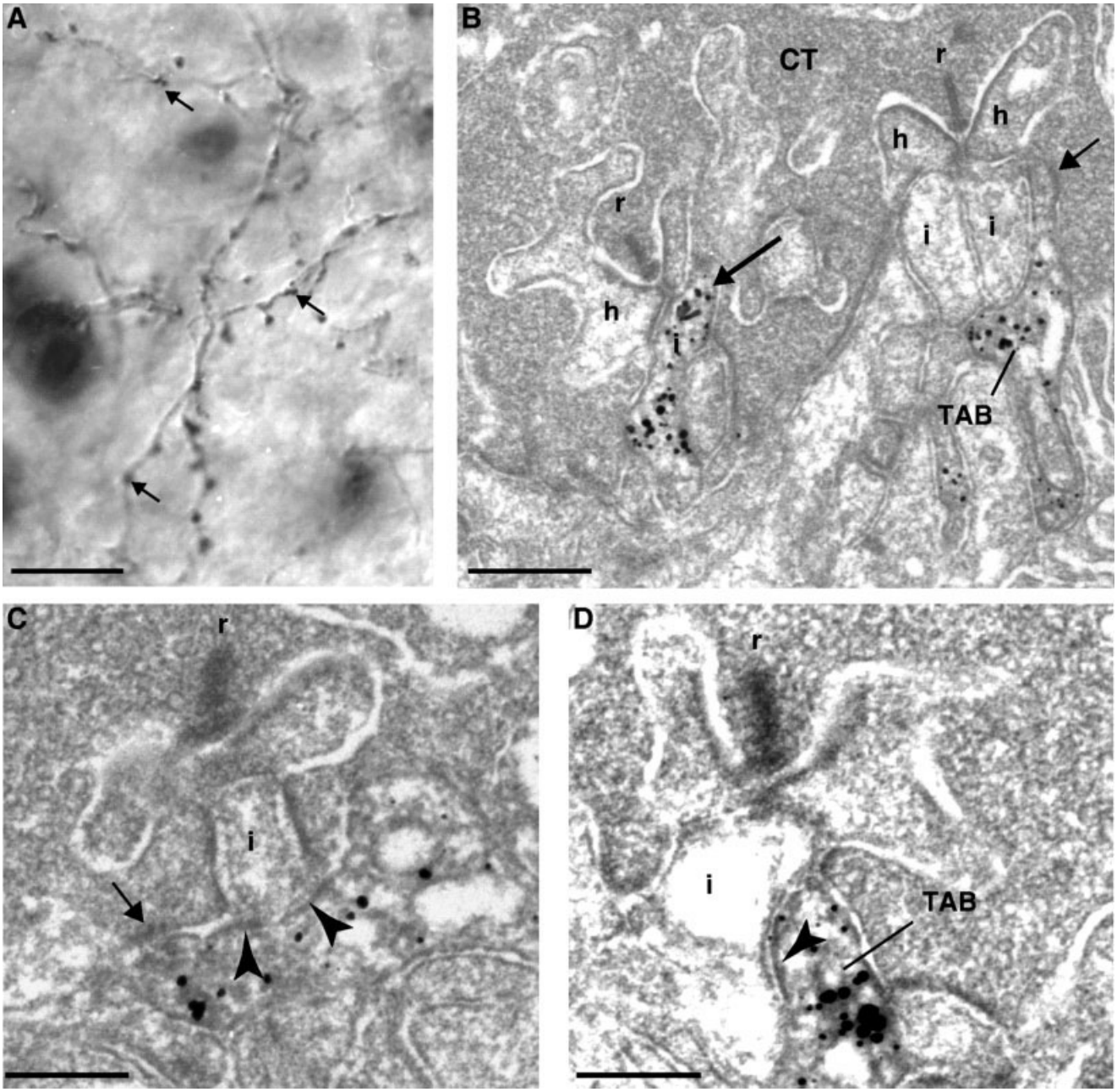

Fig. 10. Morphology of GAD_{65}-positive bipolar dendrites. **A:** Horizontal cryosection visualized with DAB reaction product. Dendrites express numerous prominent varicosities (arrows). Blurred dark patches are bipolar somas in a different plane of focus. **B:** Electron micrograph of cone terminal (CT) presynaptic to GAD_{65}-labeled dendritic processes. Most dendrites form basal contacts (short arrow) at the cone base (see also panel C), but some dendrites form a triad-associated basal (TAB) contact, and some are actually invaginating (long arrow). Most invaginating processes (i) are unstained. **C,D:** GAD_{65}-positive dendrites often form an electron-dense membrane specialization in apposition to other processes; here the electron-dense membranes (arrowheads) are in apposition to invaginating dendrites (i). Arrow in C points to a basal contact with the cone. r, synaptic ribbon; h, horizontal cell. Scale bars = 10 μm in A; 0.5 μm in B; 0.3 μm in C, D.

(Nakamura et al., 1980; Pow and Hendrickson, 1999). The interplexiform soma is located in the amacrine layer (rather than just above it), distributes at much lower density, and forms conventional chemical synapses with bipolar dendrites. The main type of GABA bipolar cell studied here, type I, matches a bipolar type previously identified by other methods. This type corresponds, by axonal stratification and dendritic ultrastructure, to type CBa1 (McGuire et al., 1984), also termed cb2 (Kolb et al., 1981). Type II may correspond to type CBa3 and the NK3-R bipolar may correspond to type CBa2, also termed cb1.

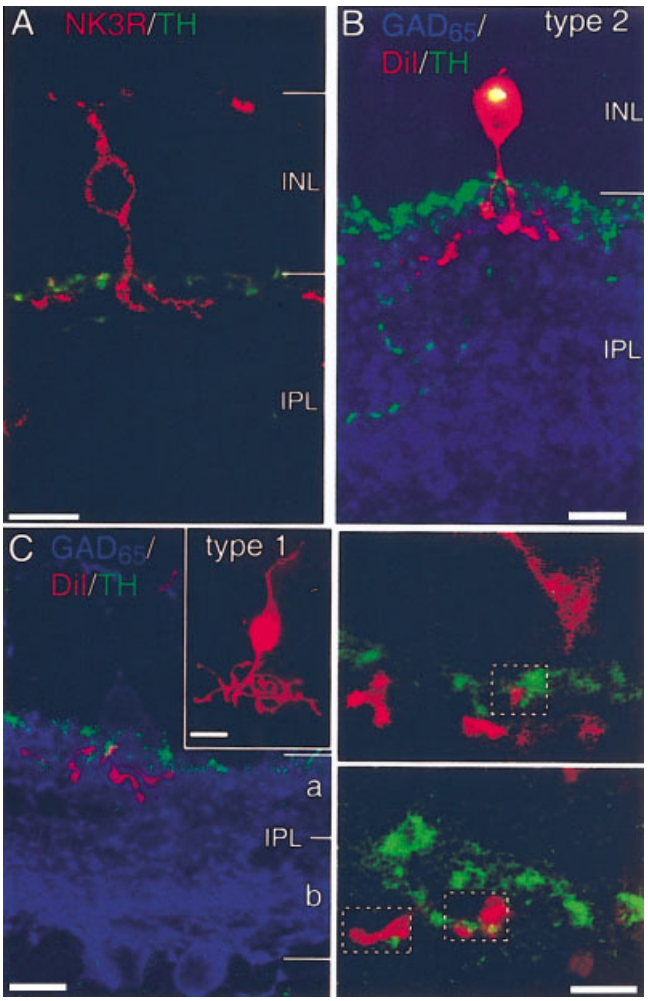

Fig. 11. GAD_{65}-positive cell (type I) associates with the dopaminergic amacrine cell. **A:** Double-staining for NK3-R (red) and tyrosine hydroxylase (TH; green), which identifies the dopaminergic amacrine cells. Bipolar axons stratify below the dopaminergic layer and make no apparent contacts. **B:** GAD_{65}-positive bipolar cell (type II) injected with DiI (red) and stained for TH (green). Only soma and axons are visible. Axons pass through the TH-stained dendrites and make no apparent contact. GAD_{65} staining (blue) marks the IPL. **C:** Left: GAD_{65}-positive bipolar cell type I injected with DiI (red) and stained for TH (green). Most axons terminate below the main dopaminergic level, but those that terminate high in stratum 1 may make contacts. This picture is a projection of nine optical sections spanning a depth of 9 μm, cell soma not included. The full cell spanning a depth of 28 μm is shown in the inset. Right: Higher magnification of selected single optical sections (GAD-stain omitted). Small dashed rectangles show putative contacts between the type I-injected bipolar axon terminals (red) and the dopaminergic processes (green). Scale bars = 10 μm in A–C (left), inset; 5 μm in C (right).

The distribution density of GABA bipolar somas (4,000 cells/mm^2) seems insufficient to account for two types, since each type of ON bipolar distributes at about this density, and the ON and OFF bipolar cells in cat retina are equally numerous (Cohen and Sterling, 1986, 1990). Thus, two OFF types with similar dendritic and axonal field size should distribute at ~8,000 cells/mm^2. We suspect that the missing somas belong to type II cells because our targeted injections yielded rather few of them. This would occur if type II somas express GAD_{65} relatively weakly, as is known to occur in other retinal cell types (Vardi and Auerbach, 1995; Johnson and Vardi, 1998). Alternatively, the density of 4,000 cells/mm^2 for each cell type might have been overestimated due to the small sample size in the quoted study.

Possible release sites

The most likely site for the bipolar cell to release GABA is at the axon terminal. However, for technical reasons we could not prove that the terminal expresses VGAT. Another possible site for GABA release is the bipolar dendrite, whose varicosities unexpectedly express both GAD_{65} and VGAT. The targeting of both proteins to these sites suggests that GABA might be released there. Although bipolar dendrites lack synaptic vesicles, VGAT might transport GABA directly from cytosol to extracellular space—as may also be true for horizontal cell processes that release GABA by transporter and express VGAT (Cueva et al., 2002). VGAT's conventional action in transporting GABA from cytosol to vesicular lumen is voltage-dependent, with cytosol negative to vesicle lumen (McIntire et al., 1997; Reimer et al., 1998). Because this voltage gradient also exists across the plasma membrane, it is plausible that VGAT could transport GABA from bipolar dendrite to extracellular space.

Possible functions of a glutamate/GABA bipolar cell

Unquestionably, glutamate is released from the synaptic terminal of the CBa1 OFF bipolar cell to excite ganglion cells (Brandstätter et al., 1997, 1998; Cohen and Miller, 1994; Cohen et al., 1994; Lukasiewicz et al., 1997; Shen and Slaughter, 1998; Massey and Miller, 1988; Pourcho et al., 2001). But if this type also releases GABA, as the evidence here suggests, what would that accomplish? First, we consider the possibility that GABA is released from the dendrites. These dendrites make numerous electron-dense appositions with other processes, raising the possibility that the electron-dense membrane represents the actual release site. If so, the OFF bipolar cell might provide a feedback inhibition nonspecifically to neighboring dendrites, reminiscent of feedback provided by horizontal cells.

If, on the other hand, release is from the axon terminals, then GABA from the CBa1 OFF bipolar might modulate dopamine release, which regulates day/night shifts in retinal sensitivity (Li and Dowling, 2000; reviewed by Witkovsky and Dearry 1990; Marshak, 2001). The dopaminergic amacrine cell fires action potentials and releases dopamine in the light (Iuvone et al., 1978; Witkovsky and Dearry, 1990; Djamgoz and Warner, 1992; Dong and McReynolds, 1991; Besharse, 1992; Gustincich et al., 1997), but what drives this cell has been a puzzle because its processes are generally restricted to the OFF stratum (Dacey, 1988, 1990). The dopamine cell receives bipolar contacts (Hokoc and Mariani, 1988; Gustincich et al., 1997), and expresses $GABA_A$ receptors (Feigenspan et al., 2000). If the bipolar contacts are from CBa1, it is possible that in darkness the dopamine cell is tonically inhibited by GABA from CBa1. When light suppresses GABA from CBa1, the dopamine cell is disinhibited and resumes its release of dopamine. Under this model, the contacts from CBa1 to ganglion cells and to the dopaminergic cell should be segregated to different sites, as glutamate would be

released onto ganglion cells, but not onto the dopaminergic cell. Although rare, release of different transmitters from different sites of the same cell has been reported in mesoaccumbens projections. These projections consist of three subsets of varicosities: those that contain only glutamate, only dopamine, and both (Joyce and Rayport, 2000). Consistent with this model of GABAergic input to the dopaminergic cells, the isolated dopamine cell fires spontaneous spikes that release dopamine and the spontaneous activity is inhibited by GABA (Kamp and Morgan, 1981; Marshburn and Iuvone, 1981; O'Connor et al., 1986; Feigenspan et al., 1998; Puopolo et al., 2001). A similar mechanism was proposed for lower vertebrates (Critz and Marc, 1992). Whereas this model is plausible, it is possible that in cat, as in certain other species, dopamine is released via input from the ON bipolar cells (Boelen et al., 1998; Dong and McReynolds, 1991, 92; Besharse, 1992). If so, the proposed model might represent merely an additional mechanism. On the other hand, it appears that the GABA-containing bipolar cells in different species are different; for example, in monkey these are the rod bipolar cells (Grünert and Wässle, 1990; Lassová, unpubl. results). Thus, for thorough understanding of the purpose of the GABA-containing bipolar cells, each species should be investigated independently of others.

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