Published in final edited form as:

J Comp Neurol. 2003 June 16; 461(1): 76–90. doi:10.1002/cne.10672.

Synaptic Connections of Starburst Amacrine Cells and Localization of Acetylcholine Receptors in Primate Retinas

ELIZABETH S. YAMADA^{1,5}, NINA DMITRIEVA², KENT T. KEYSER², JON M. LINDSTROM³, LOUIS B. HERSH⁴, and DAVID W. MARSHAK^{1,*}

¹Department of Neurobiology & Anatomy, University of Texas Medical School, Houston, Texas 77225

²Vision Sciences Research Center, University of Alabama, Birmingham, Alabama 35294

³University of Pennsylvania Institute for Neurological Sciences, Philadelphia, Pennsylvania 19104

⁴Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40536

⁵Departamento de Fisiologia, Universidade Federal do Pará, Belém, Pará 66075-900, Brazil

Abstract

Starburst amacrine cells in the macaque retina were studied by electron microscopic immunohistochemistry. We found that these amacrine cells make a type of synapse not described previously; they are presynaptic to axon terminals of bipolar cells. We also confirmed that starburst amacrine cells are presynaptic to ganglion cell dendrites and amacrine cell processes. In order to determine the functions of these synapses, we localized acetylcholine receptors using a monoclonal antibody (mAb210) that recognizes human $\alpha 3$ - and $\alpha 5$ -containing nicotinic receptors and also antisera against the five known subtypes of muscarinic receptors. The majority of the mAb210-immunoreactive perikarya were amacrine cells and ganglion cells, but a subpopulation of bipolar cells was also labeled. A subset of bipolar cells and a subset of horizontal cells were labeled with antibodies to M3 muscarinic receptors. A subset of amacrine cells, including those that contain cholecystikinin, were labeled with antibodies to M2 receptors. Taken together, these results suggest that acetylcholine can modulate the activity of retinal ganglion cells by multiple pathways.

Indexing terms

acetylcholine; nicotinic; muscarinic; cholinergic; presynaptic receptors

The cholinergic neurons in the retina are a type of amacrine cells known as starburst cells because of their distinctive morphology (Famiglietti, 1983). These amacrine cells also contain and release gamma-aminobutyric acid (GABA; reviewed by Duarte et al., 1999). As in other mammals, two subtypes have been described in primates: one with cell bodies in the ganglion cell layer (GCL) and processes stratifying within the proximal sublamina (sublamina *b*) of the inner plexiform layer (IPL), and the other with cell bodies in the inner nuclear layer (INL) and processes stratifying within the distal sublamina (sublamina *a*) of the IPL (Rodieck and Marshak, 1992). Previous electron microscopic (EM) studies using Golgi-impregnated or choline acetyltransferase (ChAT) immunolabeled retinas have

revealed some aspects of their synaptic connectivity in primates and other mammals. Starburst cells receive input mainly from bipolar cell axons, and their major postsynaptic targets are ganglion cell dendrites (Brandon, 1987; Mariani and Hersh, 1988; Famiglietti, 1991), a finding consistent with known direct effects of acetylcholine on several ganglion cell types in mammalian retinas (Masland and Ames, 1976; Schmidt et al., 1987; Lipton et al., 1987; Downing and Kaneko, 1992; Kaneda et al., 1995). Synaptic contacts between starburst cells have also been described (Millar and Morgan, 1987; Mariani and Hersh, 1988), and a subset of the starburst cells express nicotinic receptors (Keyser et al., 2000).

In the past three decades, there have been a number of physiological studies of the effects of acetylcholine on the activity of retinal ganglion cells. ON and ON-OFF direction selective ganglion cells in the rabbit retina are particularly sensitive to acetylcholine (Ames and Pollen, 1969; Masland and Ames, 1976; Ariel and Daw, 1982; Kittila and Massey, 1997), and their dendrites costratify with the processes of starburst amacrine cells in the IPL (Famiglietti, 1992; He and Masland, 1998). However, other types of ganglion cells that do not costratify with starburst cells are also excited by acetylcholine. In the rabbit retina, these include both brisk and sluggish types (Ariel and Daw, 1982). In the cat retina, acetylcholine increases the maintained discharge rate in all ganglion cells studied, including Y cells, X cells and various types of sluggish cells with both concentrically organized and complex receptive-field properties (Schmidt et al., 1987).

Because ganglion cells receive input from bipolar cells and amacrine cells, cholinergic synapses onto these two cell types would also contribute to the effects of acetylcholine on ganglion cells. To describe these synapses, starburst cells were labeled in the macaque retina using antibodies to ChAT and their synaptic connections were studied by electron microscopy (EM). We confirmed the results from previous EM immunohistochemical studies in mammalian retinas, and we also found that ChAT-immunoreactive (IR) processes were presynaptic to axon terminals of bipolar cells and to processes of unlabeled amacrine cells. Acetylcholine receptors were also localized by light microscopy in macaque and baboon retinas. We labeled vertical sections using a monoclonal antibody (mAb210) that recognizes human $\alpha 3/\alpha 5$ -containing nicotinic receptors (nAChRs) and antisera to M1, M2, M3, M4, and M5 muscarinic receptors (mAChRs). A sparse population of bipolar cells was mAb210-IR, and a subset of cone bipolar cells was M3-IR. Amacrine cells were labeled with mAb210 and antibody to M2 receptors, and horizontal cells were labeled with antibody to M3 receptors. These results indicate that acetylcholine can also influence the activity of ganglion cells indirectly through horizontal cells, bipolar cells, or amacrine cells and that these indirect effects may be mediated by either nicotinic or by muscarinic receptors.

MATERIALS AND METHODS

Immunohistochemistry

The polyclonal goat antiserum to human placental choline acetyltransferase (ChAT) was made as described previously (German et al., 1985) except that a goat was immunized. Affinity-purified antibodies to ChAT were also obtained from Chemicon (Temecula, CA). Monoclonal antibody against $\alpha 3/\alpha 5$ -containing nAChR receptors (mAb210) was made against mouse and bovine muscle nicotinic receptors (Tzartos et al., 1987). It binds to the main immunogenic region of alpha subunits and recognizes $\alpha 3$ and/or $\alpha 5$ subunits of human neuronal nicotinic receptors (Lindstrom, 2000). Monoclonal antibodies to M2 (MAB367) and M4 (MAB1578) mACh receptors were obtained from Chemicon and raised against the third intracellular loop of the M2 or M4 receptor protein fused with glutathione S-transferase (Levey et al., 1995). Polyclonal antisera against M1 (AS-3701S), M3 (AS-3741S), and M5 (AS-3781S) mAChRs were obtained from Research and Diagnostic Antibodies (Berkeley, CA) and were raised against a unique synthetic peptide from the carboxyl terminal of each

receptor that had been covalently attached to the carrier protein, bovine thyroglobulin (Ndoye et al., 1998).

Electron microscopy

Retinas from one adult macaque monkey (*Macaca mulatta*) were used. The eyes were obtained at the conclusion of an unrelated anatomical experiment at the University of Texas Medical School. The animal was euthanized with an overdose of pentobarbital sodium (Nembutal, 50 mg/kg i.v.) and then enucleated. The eyes were then cleaned of surrounding tissue, hemisected, and the posterior eyecups were cut into halves. The vitreous was removed with fine forceps and the pieces were fixed in 4% paraformaldehyde (PFA) / 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.2, at 37°C for 60 minutes. The tissue was then fixed overnight in 4% PFA in PB, pH 10, at 4°C. After rinsing the tissue in phosphate-buffered saline (PBS), the retina was carefully dissected from the choroid and sclera and was then treated with 1% sodium borohydride / PBS for 1 hour followed by an ascending and descending series of graded ethanol solutions in PBS (Marshak et al., 1990). After rinsing for several hours in PBS the retina was incubated for 11 days in 1:1,000 goat anti-ChAT in PBS with 0.3% sodium azide. Next the retina was rinsed in PBS and then incubated for 2 days in 1:200 biotinylated donkey antigoat IgG (Jackson Research Laboratories, West Grove, PA). To visualize the biotin the retina was incubated in 1:100 avidin-biotin-peroxidase (Standard, Vector Laboratories, Burlingame, CA) overnight, followed by histochemical reaction with diaminobenzidine (DAB, 0.5 mg/ml) and hydrogen peroxide (0.005%) for 60 minutes. Next the tissue was treated with 1% osmium tetroxide in PB for 60 minutes and embedded in Epon. Serial sections of 80–100 nm thickness were collected on Formvar-coated, single-hole grids and stained with 2% uranyl acetate in 50% methanol for 60 minutes followed by 0.2% lead citrate for 1–2 minutes. Labeled processes in each section were photographed at 10,000× (for analysis of serial sections) or 20,000× (for illustrations) in a JEOL 100CX electron microscope using a rotating, goniometer stage to orient the sections and align the synaptic membranes.

Light microscopy

ChAT and muscarinic receptors—Five eyes from *Macaca mulatta*, one eye from *M. fascicularis*, and one eye from *Papio papio* were used in these experiments. No interspecies differences in labeling were observed. After the initial dissection as described above, the pieces of tissue were fixed in 2–4% PFA overnight. The choroid, retinal pigment epithelium and vitreous were removed, and the retina was rinsed in PBS. Antiserum to ChAT (affinity-purified, 1:200) was used in both vertical sections and whole mounts; only vertical sections were labeled with muscarinic receptor antibodies (1:1,000). Using a tissue chopper, 100 µm-thick vertical sections were obtained and then incubated free-floating in the primary antibodies for 5–10 days with 1:100 normal donkey serum and 0.3% Triton at 4°C. Next, after PBS rinses sections were incubated in biotinylated secondary antisera raised in donkeys at 1:100 for 2 days, rinsed again in PBS, followed by incubation in Cy3-conjugated Streptavidin for 1–2 days (Jackson ImmunoResearch Laboratories). For whole-mount preparations reacted for ChAT, the same incubation times as above were applied for primary and secondary antibodies. All antibody solutions were diluted in PBS with 0.3% sodium azide and 0.3% Triton X-100. Both sections and whole mounts were coverslipped in a 3:1 mixture of glycerol and PBS with 0.3% sodium azide and 0.01% paraphenylenediamine (Sigma, St. Louis, MO).

Some double-labeling experiments were also performed. In this procedure sections were incubated in a mixture of the two primary antibodies. One of the primary antibodies was visualized with Cy3 as above, and the other was labeled with Cy5-conjugated or FITC-conjugated affinity-purified secondary antibodies (Jackson ImmunoResearch Laboratories).

In control experiments to determine the specificity of the secondary antibodies, one of the primary antibodies was omitted. Labeled tissue was examined in a Zeiss confocal laser-scanning microscope with a kryptonargon laser. Excitation was at 568 nm for Cy3, 488 for FITC and 647 nm for Cy5. Emission filters were 515–540 nm for FITC, 590–610 nm for Cy3 and 670–810 nm for Cy5. Using a 40× or a 63× oil immersion objective, the retina was scanned and 30–50 0.5 μm - or 1 μm -thick optical sections were obtained.

Markers for amacrine and bipolar cells—Several other primary antibodies were used in experiments to identify the amacrine and bipolar cells that were labeled with antisera to muscarinic receptors. We used a monoclonal mouse antibody to tyrosine hydroxylase (Sigma) to label dopaminergic amacrine cells, a polyclonal rabbit anti-calretinin (Chemicon) to label AII amacrine cells, two polyclonal antisera against 65 and 67 KD isoforms of glutamic acid decarboxylase (GAD; Chemicon) to label GABAergic amacrine cells and a polyclonal rabbit antibody raised against the glycine-extended cholecystokinin precursor (Marshak et al., 1990) to label CCK-containing amacrine cells. Double-labeling experiments were performed using antiserum against ChAT and two different markers for bipolar cells: anti-protein kinase C α (Chemicon), which labels rod bipolar cells and DB4 cone bipolar cells, and anti-calbindin (Sigma), which labels DB3 and DB5 diffuse bipolar cells. These experiments used vertical sections and followed the same double-labeling protocol described for ChAT and muscarinic receptors.

$\alpha 3/\alpha 5$ nAChR receptor—Retinas were obtained from *Macaca mulatta* perfused intracardially with 2–4% PFA. After enucleation, pieces of tissue were postfixed in cold 2–4% PFA for 4–6 hours. Next the choroid and vitreous were removed, and the retina was rinsed in cold PBS. Immunoreactivity was demonstrated in vertical frozen sections. Before sectioning, retinas were immersed in 30% sucrose in PBS for 24 hours at 4°C and were then repeatedly frozen and thawed. Vertical sections (30 μm -thick) were obtained on a cryostat and were collected on gelatinized slides, rinsed in PBS and then incubated in 1:1,000 mAb210 for 2 days at 4°C. Next, after PBS rinses sections were incubated in biotinylated donkey antimouse IgG at 1:100 for 2 hours and rinsed again in PBS. The biotin was visualized using the avidin-biotin-peroxidase method as described above. Double-labeling experiments were performed using mAb210 and anti-protein kinase C. These experiments used vertical sections and followed the same double-labeling protocol described above.

RESULTS

Amacrine cells were labeled using antiserum to ChAT in macaque retinal whole-mounts (Fig. 1). Starburst cells ramifying in sublamina *b* were more numerous (Fig. 1A), forming a network of highly overlapping dendrites that was more dense than that of the starburst cells ramifying in sublamina *a*. As a result, the typical starburst morphology of individual amacrine cells can be appreciated better in sublamina *a* (Fig. 1B). In vertical sections ChAT-IR dendrites form relatively narrow bands (Fig. 1C) situated at depths of 30% ($\text{SD} \pm 7\%$) and 70% ($\text{SD} \pm 9\%$) of the inner plexiform layer, corresponding to strata 2 (S2) and 4 (S4), respectively. The proximal dendrites usually take a direct course towards the IPL. Therefore, the ChAT-IR bands in S2 and S4 consist mainly of the distal cholinergic dendrites and parts of the intermediate zone dendrites (Famiglietti, 1991). As reported previously, we found very few labeled starburst cells ramifying in sublamina *a* in the central retina (Mariani and Hersh, 1988; Rodieck and Marshak, 1992).

Electron microscopy

We analyzed serial, ultrathin sections of peripheral macaque retina labeled with antiserum to ChAT. All the synapses involving immunolabeled dendrites in the selected area were

reconstructed using serial electron micrographs taken at 10,000 $\times$ in the vertical plane. We analyzed 791 micrographs, including 39 labeled dendrites with 20 completely reconstructed varicosities. The reconstructed dendrites were all situated in stratum 4 of the IPL. Because the distal dendrites of starburst cells overlap extensively, it was not possible to determine how many cells contributed these dendrites. ChAT-IR varicosities had irregular contours and were frequently found in close apposition to other ChAT-IR dendrites. At the synapses made by starburst cells onto other types of cells, the postsynaptic processes were frequently invaginated into the ChAT-IR dendrites, particularly when the postsynaptic element was smaller than the ChAT-IR dendrite. The unlabeled processes making synapses with ChAT-IR processes were identified as axon terminals of bipolar cells, dendrites of ganglion cells, or amacrine cell processes by their characteristic ultrastructure (Dowling and Boycott, 1966). Of the 152 labeled synapses identified, the vast majority (93%, $n = 141$) involved an unlabeled process; only 7% of the synapses ($n = 11$) were between two labeled processes. For calculations involving only inputs or only outputs, these 11 synapses between labeled dendrites were counted twice, once as inputs and again as outputs. Overall, output synapses and input synapses each constituted 50% of all identified synapses of labeled cells. A few (8.6%, $n = 7$) of the cholinergic output synapses were onto processes too small to positively identify. These were probably dendritic spines of ganglion cells. The results are summarized in Table 1.

Synapses from starburst cells onto bipolar cell axon terminals

Figure 2 shows two examples of ChAT-IR dendrites making synapses onto bipolar cell axon terminals. Synapses like these represented 15% ($n = 12$) of the output of cholinergic cells. The bipolar cell axon terminals can be readily recognized by the presence of synaptic ribbons. Typically, the synaptic contacts from the ON-cholinergic cell dendrite onto the bipolar cell were located more than 1 μm (mean = $1.2 \mu\text{m} \pm 0.45 \text{ SD}$) away from the ribbon where the bipolar cell was presynaptic to ganglion cells or unlabeled amacrine cells (Fig. 2A,B). In two cases, however, we found labeled dendrites making reciprocal synapses onto bipolar cell axon terminals (Fig. 2C,D). That is, the ChAT-IR dendrite received a ribbon synapse from a bipolar cell (Fig. 2C, top arrow) and made a conventional synapse onto the same bipolar cell (Fig. 2C,D, arrowheads). At the points where they received synapses the bipolar cell profiles were usually large (mean = $0.87 \pm 0.48 \mu\text{m SD}$), with an electron-dense cytoplasm containing numerous synaptic vesicles and mitochondria. The postsynaptic bipolar cell profiles we reconstructed contained 25 ribbons and the majority made typical dyads. The most common type of postsynaptic element at these bipolar cell dyads was a process of unlabeled amacrine cells (47%), followed by ganglion cells (31%) and other ChAT-IR dendrites (14%); the rest were unidentified. One ribbon had three postsynaptic processes and another was not apparently associated with a synapse. Their pattern of connectivity, level of termination in the IPL, and shape indicated that they were cone bipolar cells.

Synapses from starburst cells onto amacrine cells

Synapses onto unlabeled amacrine cell profiles represented 15% ($n = 12$) of ChAT-IR output synapses (Fig. 3). At least two types of unlabeled amacrine cells were postsynaptic to ChAT-IR dendrites. One type had many vesicles (Fig. 3A) and relatively small profiles (mean = $0.53 \pm 0.21 \mu\text{m SD}$) at the sites of the synapses and made synapses onto other processes with similar features or onto ganglion cell dendrites (Fig. 3B). The second type of amacrine cell process was more electron-lucent and had larger diameter profiles (mean = $0.72 \pm 0.18 \mu\text{m SD}$). They were typically ovoid in shape and contained very few vesicles, but many microtubules (Fig. 3C). The second type of amacrine cell process also made homologous gap junctions onto electron-lucent processes of the same type (Fig. 3D). To positively identify these profiles as amacrine cells, we followed them through consecutive

sections and demonstrated that they made conventional synapses. The synaptic contacts from ChAT-IR cells onto these electron-lucent amacrine cells were concave on the side of the starburst cell. However, the output synapses made by these unlabeled amacrine cells onto other processes were not invaginated (Fig. 3C).

As previously reported (Brandon, 1987; Millar and Morgan, 1987; Mariani and Hersh, 1988; Famiglietti, 1991), we also found conventional synapses between starburst amacrine cells (Fig. 4). We found 11 examples of these synapses, which represented 14% of the total output and 13% of the total input from ChAT-IR processes (Table 1). We only counted the synapses where a clear thickening of the synaptic membranes and an accumulation of synaptic vesicles were visible. Because the reaction product was often very dense, some of the synapses between cholinergic processes may have gone undetected. It was common to find other types of synapses nearby. For example, one of the ChAT-IR amacrine cells makes a synapse onto an unlabeled amacrine cell process (Fig. 4A). The same is true of the cell illustrated in Figure 3B, which also receives an input from a bipolar cell axon.

Synapses from starburst cells onto ganglion cells

The major output from ChAT-IR dendrites was onto ganglion cell dendrites (48%, $n = 39$). The synaptic contacts of the starburst cells onto ganglion cells are typically concave in the presynaptic side (Fig. 5B,C), with the ChAT-IR process often surrounding the smaller ganglion cell dendritic spine, as described previously in the rabbit retina (Famiglietti, 1991). About half (47%) of the ChAT-IR synapses were onto very small, electron-lucent ovoid profiles with diameters in their short axes ranging from 0.2–0.45 μm (mean = $0.31 \pm 0.1 \mu\text{m}$ SD). By following these small profiles through the series it was possible to confirm in many cases that they were small protrusions emerging from larger dendrites. The remaining synapses (53%) were onto larger electron-lucent dendritic profiles (Fig. 5A–C) with diameters in their short axes ranging from 0.5–1 μm (mean = $0.83 \pm 0.21 \mu\text{m}$ SD). These larger dendrites are likely to belong to parasol ganglion cells for several reasons. First, parasol cell dendrites are very narrowly stratified at the same depth in the IPL as the cholinergic cell dendrites and make more appositions with them than would be expected by chance (Jacoby et al., 1996). Second, Neurobiotin-injected parasol cells receive synapses from amacrine cells with the same ultrastructural features as the ChAT-IR processes (Marshak et al., 2002). EM double-labeling experiments would be necessary to test this hypothesis rigorously, however.

Synaptic inputs to starburst cells

The input to ChAT-IR dendrites originated mainly from bipolar cells (68%, $n = 56$); these are illustrated in Figs. 2C and 4B. Ribbon synapses onto labeled dendrites were found in profiles of various sizes, ranging from very small ones (0.2–0.4 μm), which were probably regions between varicosities, to larger profiles, which were varicosities (0.5–1.85 μm). As described above, the presynaptic bipolar cells were classified as cone bipolar cells. The bipolar cell profiles presynaptic to ChAT-IR dendrites were usually irregular in shape, and most of them (71%) made one or more synapses onto ganglion cell dendrites. There were 94 synaptic ribbons in the sample, and each had two postsynaptic processes, forming typical dyads. About half of these dyads (51%) had at least one ChAT-IR dendrite, and the most common second element was a ganglion cell dendrite (44%), followed by unlabeled amacrine cells (27%) and other ChAT-IR dendrites (19%). The remaining postsynaptic elements were unidentified.

Unlabeled amacrine cells provided 18% ($n = 15$) of the input to ON-starburst amacrine cells. The majority of these amacrine cell processes (71%) were electron-lucent profiles that made small, flat synapses (Fig. 6A). Amacrine cells having a more electron-dense cytoplasm and a

larger number of synaptic vesicles made up the remaining 29% of the amacrine cells providing input to ChAT-IR dendrites (Fig. 6B).

Glutamic acid decarboxylase in starburst cells

It is possible that GABA is the neurotransmitter released at some of the synapses made by starburst cells, rather than acetylcholine. In order to test this hypothesis, we performed double-label experiments using antisera to ChAT and the two isoforms of GAD in vertical sections. In agreement with previous reports, we found that GAD65 and GAD67 were abundant in processes throughout the IPL, many perikarya in the INL and some perikarya in the GCL, and the patterns of labeling were the same with both antisera (Vardi et al., 1994; Vardi and Auerbach, 1995). The majority of the labeled cells were amacrine cells, but there were also some labeled bipolar cells and, perhaps, a few ganglion cells. We found very low levels of expression of both isoforms of GAD in starburst cells, with only a few perikarya very weakly labeled. We were unable to detect expression of these enzymes in the ChAT-IR dendrites (Fig. 7). Indeed, the labeling with GAD antisera in the IPL left two gaps which seemed to be filled by the ChAT-IR dendrites (Fig. 7C).

Localization of nicotinic and muscarinic cholinergic receptors

To determine whether bipolar cells and amacrine cells express acetylcholine receptors, vertical sections of macaque or baboon retinas were labeled with antibodies against $\alpha 3$ and $\alpha 5$ nicotinic receptor subunits (mAb210) and antibodies against M1, M2, M3, M4 and M5 muscarinic receptors. In frozen sections of macaque retina, mAb210 labeling was most intense in the INL, IPL and GCL (Fig. 8A). In the IPL, mAb210 labeled two diffuse bands centered on the ChAT bands but broader, approximately 4 μ m thick. In the GCL, the sizes of the labeled perikarya indicated that ganglion cells were labeled. In the INL, many perikarya were labeled, the majority situated close to the IPL, where amacrine cell perikarya are located. There were also some labeled perikarya situated in the middle of the INL, each giving rise to a single descending axon running towards the IPL; this morphology is characteristic of bipolar cells (Fig. 8A). We performed a series of double-labeling experiments using mAb210 and one marker for bipolar cells, anti-protein kinase C, which labels rod bipolar cells and DB4 cone bipolar cells. Rod bipolar cells were not labeled with mAb210 (not shown) and, therefore, we concluded that the mAb210-IR bipolar cell was a cone bipolar cell.

Immunoreactive M3 muscarinic receptor was present in both plexiform layers and in the perikarya of bipolar cells and horizontal cells in the INL (Fig. 8B–E). Sometimes it was possible to follow bipolar cell axons into sublamina *a* of the IPL, but it was difficult to follow the axons to their terminals because of the extensive labeling of other processes in the IPL. To test the hypothesis that the M3-IR bipolar cells included the DB3 type, we carried out a series of double-label experiments with antibody to calbindin. In the peripheral retina, DB3 bipolar cells were never labeled with M3 antisera (Fig. 8B,C). However, in central retina, some M3-IR bipolar cells contained immunoreactive calbindin, and some stratified at the same level of the IPL as the DB3 bipolar cells (Fig. 8D,E). We could never follow bipolar cell axons all the way into sublamina *b*, but it is likely that ON-subtypes of bipolar cells were also labeled because there were M3-IR processes throughout the IPL.

Double-label experiments using anti-ChAT and anti-PKCa (Fig. 8F) or anti-calbindin (Fig. 8G) showed that DB4 axon terminals (Fig. 8F) ramified slightly above the ChAT-IR band in sublamina *b*. A few varicosities of rod bipolar terminals also stratified at the same level as ChAT-IR band in S4 (Fig. 8F), but we found no evidence of contacts between ChAT-IR dendrites and rod bipolar terminals at the EM level. DB5 bipolar cells (Fig. 8G) had axon

terminals stratifying at the same level as the dendrites of starburst cells. Therefore, DB5 bipolar cells are the best candidates to make contacts with ChAT-IR dendrites.

Amacrine cells and processes in the IPL were labeled with antiserum to the M2 muscarinic receptor. To identify the specific types of amacrine cells, we performed double-label experiments using antisera to tyrosine hydroxylase, calretinin, the glycine-extended cholecystokinin precursor G6-gly and GAD65 (Fig. 9). In the central 3–4 mm the outermost M2-IR band occurs at the same depth as the dopaminergic processes, but the two markers were not colocalized (Fig. 9D–F). The outermost M2-IR band overlapped with lobular AII dendrites and the two innermost M2 bands surrounded the bands formed by the labeled dendrites of AII amacrine cells (Fig. 9A–C), but M2 immunoreactivity was not detected in any AII amacrine cells. In the peripheral retina, most of the M2 immunoreactivity was localized in G6-gly-IR varicosities in both sublaminae *a* and *b* (Fig. 9G–I). There were also some M2-IR puncta on unlabeled processes, a finding suggesting that there are other, unidentified types of M2-IR amacrine cells. Perikarya of amacrine cells labeled with anti-GAD65 were not double-labeled, but some GAD65-IR processes were M2-IR (Fig. 9J–L). Starburst amacrine cells were not among the M2-IR amacrine cells in the peripheral retina, but in more central retina a subset of weakly labeled ChAT-IR perikarya in the INL were also labeled with M2 antiserum (not shown). Although these cells had their perikarya in the INL, a few of them sent dendrites to the ChAT-IR band in sublamina *b*. Starburst cells with misplaced perikarya have been described previously in the primate retina (Rodieck and Marshak, 1992). Antibodies to M1, M4, and M5 muscarinic receptors gave no specific labeling in baboon or macaque retinas.

DISCUSSION

Synaptic organization of starburst cells

We have shown that starburst amacrine cells in the macaque retina make synapses onto axon terminals of bipolar cells and processes of amacrine cells; taken together, these represent 44% of the output of starburst cells. Synapses onto starburst and other types of amacrine cells have been observed in mammalian retinas (Famiglietti, 1991; Mariani and Hersh, 1988; Millar and Morgan, 1987), but none of the previous EM studies of starburst cells reported synapses onto bipolar cell axon terminals. However, the postsynaptic profile identified as an amacrine cell by Mariani and Hersh (1988) in their figure 9 closely resembles the bipolar cell terminals we have identified postsynaptic to the starburst cells, although it does not contain a ribbon in the section illustrated. Without analyzing serial sections, bipolar cell terminals could have been mistakenly identified as amacrine cells because the synaptic ribbons of bipolar cell terminals are typically found several sections away from the synapses by cholinergic cells. Because bipolar cells and amacrine cells, in turn, make synapses onto other amacrine and ganglion cells, starburst cells can influence the neural activity of those cells indirectly.

Localization of nicotinic receptors

We have also shown that ganglion cells as well as subsets of bipolar cells and amacrine cells were labeled with an antibody that recognizes human $\alpha 3/\alpha 5$ nAChR subunits, a finding suggesting that some of the synapses from starburst cells onto bipolar cells and amacrine cells utilize nicotinic ACh receptors. All subtypes of nicotinic receptors are cation-selective channels that open upon binding of acetylcholine leading to membrane depolarization (Dani, 2001). Therefore, synapses involving $\alpha 3$ - and/or $\alpha 5$ -containing receptors are likely to be excitatory. Previously, nAChRs have been localized in the retinas of rats, ground squirrels, and rabbits (Swanson et al., 1987; Britto et al., 1994; Keyser et al., 2000; Dmitrieva et al., 2001). In rabbits, most of the labeled neurons were ganglion cells and amacrine cells, but

there were some immunoreactive perikarya localized in the middle of the INL. However, it was not possible to detect inwardly or outwardly directed processes originating from these perikarya in order to prove that they were bipolar cells. In the chicken retina, a subset of bipolar cells express $\alpha 8$ -nAChR subunits (Keyser et al., 1993; Hamassaki-Britto et al., 1994), one of the types that binds α -bungarotoxin, and it is possible that nicotinic receptors like these are also present in mammalian retinas. An EM study in the mouse retina reported α -bungarotoxin binding sites at synapses from amacrine cells onto bipolar cells (Pourcho, 1979).

Recently, Neal et al. (2001) showed that stimulation of nicotinic receptors increases GABA release from amacrine cells. This is supported by the finding that GABAergic amacrine cells in the rabbit retina, including a subset of the cholinergic amacrine cells, were nAChR-IR (Dmitrieva et al., 2001). Nicotinic receptors would also be expected to stimulate glutamate release from bipolar cell axon terminals, and there is evidence that this occurs in the chicken retina. The nicotinic agonists nicotine and cytisine evoke spreading depression indirectly through the activation of NMDA receptors, and the effect is blocked by the nicotinic antagonist mecamylamine (Sheardown, 1993).

Localization of muscarinic receptors

We also localized immunoreactive M3 muscarinic receptors to a subset of bipolar cells and processes in the IPL, a finding suggesting that the synapses from starburst cells onto bipolar cells might utilize receptors of this subtype. These are G-protein-coupled receptors that activate phospholipase C β (Nahorski et al., 1997) and, consequently, release calcium from intracellular stores by inositol 1,4,5 triphosphate (IP3) and activation of protein kinase C by diacylglycerol. In some systems, muscarine-sensitive potassium channels are closed, which increases excitability of neurons (Brown et al., 1997). In other systems, calcium-gated potassium channels are activated, and the net effect of stimulating M3 receptors is inhibitory (Jones et al., 1988; Barbara et al., 1998; Bordey et al., 2000). It is uncertain which of these mechanisms operate in the retina. Muscarine increases intracellular calcium levels in rabbit retinal ganglion cells (Baldridge, 1996), but this is likely to be an indirect effect because there are no muscarinic receptors on isolated mammalian retinal ganglion cells (Lipton et al., 1987; Kaneda et al. 1995). M3 muscarinic receptors have also been localized to bipolar cells in the chicken retina (Fischer et al., 1998).

We localized another muscarinic receptor, the M2 subtype, to amacrine cells and processes in the IPL. There were two M2-IR bands that overlapped the ChAT-IR bands. Muscarinic receptors have been localized previously using radiolabeled ligands, but due to the limitations in resolution of this technique the receptors could only be localized to the IPL and not to sublayers or to specific types of neurons (Hutchins and Hollyfield, 1985; Zarbin et al., 1986). Muscarinic receptors have also been localized in the retina using immunohistochemical methods. In rat retina, M2-immunoreactivity was observed in amacrine cells and, perhaps, a few ganglion cells but not in bipolar cells; the labeling in the IPL was most dense in a band in the middle, between the ChAT-IR bands (Was-sélius et al., 1998). A similar pattern was observed in the rabbit retina (Zucker and Ehinger, 2001). M2 muscarinic receptors typically mediate inhibitory effects through opening of potassium channels or closing of calcium channels (Caulfield, 1993; Löffelholz, 1996).

We also identified one type of amacrine cell that expressed M2 receptors, the G6-gly-IR amacrine cell (Marshak et al., 1990). In the rabbit, DAPI-3 amacrine cells, which are similar in some respects to the G6-gly-IR amacrine cells, have M2 and nicotinic receptors (Zucker and Ehinger, 2001), and acetylcholine stimulates the release of glycine in the rabbit retina (Cunningham et al., 1983; Neal et al., 2001). Because the dendrites of DAPI-3 cells and of the starburst cells overlap only slightly and synapses from starburst cells onto other

amacrine cells have not been observed in rabbit retina, it has been proposed that acetylcholine acts on those receptors by volume transmission. This type of ephaptic interaction is common elsewhere in the CNS (Descarries et al., 1997) and may also occur in the monkey retina because we found M3-immunoreactivity far from the sites of acetylcholine release. However, our EM results indicate that cholinergic amacrine cells make synapses onto other types of amacrine cells in the monkey retina, and these may include the G6-gly-IR cells. Double-labeling experiments will be required to test this hypothesis.

Synapses between starburst cells

We also found synapses between dendrites of starburst cells, as described previously (Millar and Morgan, 1987; Mariani and Hersh, 1988), but the function of these synapses remains uncertain. Because starburst cells have GABA receptors (Wässle et al., 1998) and also contain and release GABA (Duarte et al., 1999), it is possible that the synapses between starburst cells are GABAergic. Indeed, the same is true of any of the output synapses of starburst cells. However, except for a weak signal in the perikarya of central starburst cells, we did not observe any colocalization of GAD and ChAT. One possible explanation for the low level of GAD-immunoreactivity in S2 and S4 is that an unidentified isoform of GAD is present in dendrites of starburst cells. Double-label experiments using antibodies to GABA itself would be required to answer this question (Kalloniatis and Marc, 1996). Our double-label experiments indicated that M2 and M3 muscarinic receptors were not localized to starburst cells, and we observed no labeling with antibodies to M1, M4 and M5 receptors. However, we cannot rule out the possibility that other types of muscarinic receptors are found at the synapses between starburst cells. In the rabbit, a subset of starburst cells was mAb210-IR (Keyser et al., 2000; Dmitrieva et al., 2001), but because we did not do any double-label experiments like these, we cannot say whether or not these synapses utilize nicotinic receptors. Physiological experiments so far have failed to find any direct effects of cholinergic agonists on starburst cells (Cunningham et al., 1983; Zhou and Fain, 1995; Baldrige, 1996).

CONCLUSIONS

In summary, we have described pathways in the primate retina through which acetylcholine might modulate the release of neurotransmitters by acting directly on presynaptic terminals, as it does elsewhere in the central nervous system (van der Zee and Luiten, 1999; Dani, 2001). These are summarized in Figure 10. Through cholinergic synapses onto bipolar cells, which release glutamate, acetylcholine could have excitatory effects on retinal neurons beyond the very narrow strata of the inner plexiform layer in which the cholinergic cells ramify. Through inhibitory synapses onto other amacrine cells, which release GABA or glycine, acetylcholine could excite ganglion cells via disinhibition. We also confirmed that more than half of the output of starburst cells is directed to retinal ganglion cells. This pathway could account for the direct excitatory effects of acetylcholine on ganglion cells that costratify with cholinergic amacrine cells. Finally, our findings that acetylcholine receptors are distributed over a wider area than the dendrites of starburst cells suggests that acetylcholine could also act via volume transmission.

Acknowledgments

Grant sponsor: National Institutes of Health; Grant number: EY06472; Grant number: EY07845; Grant number: AG-05893, NS11323; Grant number: Core Grant EY10608; Grant sponsor: Pew Charitable Trusts Latin American Fellows Program P0199SC and CNPq (Brazil); Grant number: 200647/96-2 (E.S.Y.).

We thank Mrs. Lillemor Krosby for excellent technical assistance.

LITERATURE CITED

- Ames A 3rd, Pollen DA. Neurotransmission in central nervous tissue: a study of isolated rabbit retina. *J Neurophysiol.* 1969; 32:424–442. [PubMed: 4308417]
- Ariel M, Daw NW. Effects of cholinergic drugs on receptive field properties of rabbit retinal ganglion cells. *J Physiol.* 1982; 324:135–160. [PubMed: 7097593]
- Baldrige WH. Optical recordings of the effects of cholinergic ligands on neurons in the ganglion cell layer of mammalian retina. *J Neurosci.* 1996; 16:5060–5072. [PubMed: 8756436]
- Barbara JG, Lemos VS, Takeda K. Pre- and post-synaptic muscarinic receptors in thin slices of rat adrenal gland. *Eur J Neurosci.* 1998; 10:3535–3545. [PubMed: 9824466]
- Bordey A, Sontheimer H, Trouslard J. Muscarinic activation of BK channels induces membrane oscillations in glioma cells and leads to inhibition of cell migration. *J Membr Biol.* 2000; 176:31–40. [PubMed: 10882426]
- Brandon C. Cholinergic neurons in the rabbit retina: immunocytochemical localization, and relationship to GABAergic and cholinesterase-containing neurons. *Brain Res.* 1987; 401:385–391. [PubMed: 3028578]
- Britto LR, Rogers SW, Hamassaki-Britto DE, Duvoisin RM. Nicotinic acetylcholine receptors in the ground squirrel retina: localization of the beta 4 subunit by immunohistochemistry and in situ hybridization. *Vis Neurosci.* 1994; 11:569–577. [PubMed: 8038129]
- Brown DA, Abogadie FC, Allen TG, Buckley NJ, Caulfield MP, Delmas P, Haley JE, Lamas JA, Selyanko AA. Muscarinic mechanisms in nerve cells. *Life Sci.* 1997; 60:1137–1144. [PubMed: 9121358]
- Caulfield MP. Muscarinic receptors—characterization, coupling and function. *Pharmacol Ther.* 1993; 58:319–379. [PubMed: 7504306]
- Cunningham JR, Dawson C, Neal MJ. Evidence for a cholinergic inhibitory feed-back mechanism in the rabbit retina. *J Physiol.* 1983; 340:455–468. [PubMed: 6136606]
- Dani JA. Overview of nicotinic receptors and their roles in the central nervous system. *Biol Psychiatry.* 2001; 49:166–174. [PubMed: 11230867]
- Descarries L, Gisiger V, Steriade M. Diffuse transmission by acetylcholine in the CNS. *Prog Neurobiol.* 1997; 53:603–625. [PubMed: 9421837]
- Dmitrieva NA, Lindstrom JM, Keyser KT. The relationship between GABA-containing cells and the cholinergic circuitry in the rabbit retina. *Vis Neurosci.* 2001; 18:93–100. [PubMed: 11347820]
- Dowling JE, Boycott BB. Organization of the primate retina: electron microscopy. *Proc R Soc Lond B Biol Sci.* 1966; 166:80–111. [PubMed: 4382694]
- Downing JE, Kaneko A. Cat retinal ganglion cells show transient responses to acetylcholine and sustained responses to L-glutamate. *Neurosci Lett.* 1992; 137:114–118. [PubMed: 1352631]
- Duarte CB, Santos PF, Carvalho AP. Corelease of two functionally opposite neurotransmitters by retinal amacrine cells: experimental evidence and functional significance. *J Neurosci Res.* 1999; 58:475–479. [PubMed: 10533040]
- Famiglietti EV. ‘Starburst’ amacrine cells and cholinergic neurons: mirror-symmetric on and off amacrine cells of rabbit retina. *Brain Res.* 1983; 261:138–144. [PubMed: 6301622]
- Famiglietti EV. Synaptic organization of starburst amacrine cells in rabbit retina: analysis of serial thin sections by electron microscopy and graphic reconstruction. *J Comp Neurol.* 1991; 309:40–70. [PubMed: 1894768]
- Famiglietti EV. Dendritic co-stratification of ON and ON-OFF directionally selective ganglion cells with starburst amacrine cells in rabbit retina. *J Comp Neurol.* 1992; 324:322–335. [PubMed: 1383291]
- Fischer AJ, McKinnon LA, Nathanson NM, Stell WK. Identification and localization of muscarinic acetylcholine receptors in the ocular tissues of the chick. *J Comp Neurol.* 1998; 392:273–284. [PubMed: 9511918]
- German DC, Bruce G, Hersh LB. Immunohistochemical staining of cholinergic neurons in the human brain using a polyclonal antibody to human choline acetyltransferase. *Neurosci Lett.* 1985; 61:1–5. [PubMed: 3908999]

- Hamassaki-Britto DE, Brzozowska-Prechtl A, Karten HJ, Lindstrom JM. Bipolar cells of the chick retina containing alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors. *Vis Neurosci*. 1994; 11:63–70. [PubMed: 8011584]
- He S, Masland RH. ON direction-selective ganglion cells in the rabbit retina: dendritic morphology and pattern of fasciculation. *Vis Neurosci*. 1998; 15:369–375. [PubMed: 9605536]
- Hutchins JB, Hollyfield JG. Acetylcholine receptors in the human retina. *Invest Ophthalmol Vis Sci*. 1985; 26:1550–1557. [PubMed: 2865230]
- Jacoby R, Stafford D, Kouyama N, Marshak D. Synaptic inputs to ON parasol ganglion cells in the primate retina. *J Neurosci*. 1996; 16:8041–8056. [PubMed: 8987830]
- Jacoby RA, Wiechmann AF, Amara SG, Leighton BH, Marshak DW. Diffuse bipolar cells provide input to OFF parasol ganglion cells in the macaque retina. *J Comp Neurol*. 2000; 416:6–18. [PubMed: 10578099]
- Jones SV, Barker JL, Bonner TI, Buckley NJ, Brann MR. Electro-physiological characterization of cloned m1 muscarinic receptors expressed in A9 L cells. *Proc Natl Acad Sci USA*. 1988; 85:4056–4060. [PubMed: 2453885]
- Kalloniatis M, Marc RE, Murry RF. Amino acid signatures in the primate retina. *J Neurosci*. 1996; 16:6807–6829. [PubMed: 8824321]
- Kaneda M, Hashimoto M, Kaneko A. Neuronal nicotinic acetylcholine receptors of ganglion cells in the cat retina. *Jpn J Physiol*. 1995; 45:491–508. [PubMed: 7474530]
- Keyser KT, Britto LR, Schoepfer R, Whiting P, Cooper J, Conroy W, Brozowska-Prechtl A, Karten HJ, Lindstrom J. Three subtypes of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors are expressed in chick retina. *J Neurosci*. 1993; 13:442–454. [PubMed: 8426223]
- Keyser KT, MacNeil MA, Dmitrieva N, Wang F, Masland RH, Lindstrom JM. Amacrine, ganglion, and displaced amacrine cells in the rabbit retina express nicotinic acetylcholine receptors. *Vis Neurosci*. 2000; 17:743–752. [PubMed: 11153654]
- Kittila CA, Massey SC. Pharmacology of directionally selective ganglion cells in the rabbit retina. *J Neurophysiol*. 1997; 77:675–689. [PubMed: 9065840]
- Koontz MA, Hendrickson AE, Ryan MK. GABA-immunoreactive synaptic plexus in the nerve fiber layer of primate retina. *Vis Neurosci*. 1989; 2:19–25. [PubMed: 2487633]
- Lindstrom, J. The structures of neuronal nicotinic receptors. In: Clementi, F.; Faornasari, D.; Gotti, C., editors. *Handbook of experimental pharmacology*. Vol. 144. Berlin: Springer; 2000.
- Lipton SA, Aizenman E, Loring RH. Neural nicotinic acetylcholine responses in solitary mammalian retinal ganglion cells. *Pflugers Arch*. 1987; 410:37–43. [PubMed: 3684508]
- Levey AI, Edmunds SM, Hersch SM, Wiley RG, Heilman CJ. Light and electron microscopic study of m2 muscarinic acetylcholine receptor in the basal forebrain of the rat. *J Comp Neurol*. 1995; 351:339–356. [PubMed: 7706546]
- Loffelholz K. Muscarinic receptors and cell signalling. *Prog Brain Res*. 1996; 109:191–194. [PubMed: 9009706]
- Mariani AP, Hersh LB. Synaptic organization of cholinergic amacrine cells in the rhesus monkey retina. *J Comp Neurol*. 1988; 267:269–280. [PubMed: 3343401]
- Marshak DW, Aldrich LB, Del Valle J, Yamada T. Localization of immunoreactive cholecystokinin precursor to amacrine cells and bipolar cells of the macaque monkey retina. *J Neurosci*. 1990; 10:3045–3055. [PubMed: 2398370]
- Marshak DW, Yamada ES, Bordt AS, Perryman WC. Synaptic input to an ON parasol ganglion cell in the macaque retina: a serial section analysis. *Vis Neurosci*. 2002; 19:299–305. [PubMed: 12392179]
- Masland RH, Ames A 3rd. Responses to acetylcholine of ganglion cells in an isolated mammalian retina. *J Neurophysiol*. 1976; 39:1220–1235. [PubMed: 993829]
- Millar TJ, Morgan IG. Cholinergic amacrine cells in the rabbit retina synapse onto other cholinergic amacrine cells. *Neurosci Lett*. 1987; 74:281–285. [PubMed: 3550530]
- Nahorski SR, Tobin AB, Willars GB. Muscarinic M3 receptor coupling and regulation. *Life Sci*. 1997; 60:1039–1045. [PubMed: 9121345]

- Ndoye A, Buchli R, Greenberg B, Nguyen VT, Zia S, Rodriguez JG, Webber RJ, Lawry MA, Grando SA. Identification and mapping of keratinocyte muscarinic acetylcholine receptor subtypes in human epidermis. *J Invest Dermatol.* 1998; 111:410–416. [PubMed: 9740233]
- Neal MJ, Cunningham JR, Matthews KL. Activation of nicotinic receptors on GABAergic amacrine cells in the rabbit retina indirectly stimulates dopamine release. *Vis Neurosci.* 2001; 18:55–64. [PubMed: 11347816]
- Pourcho RG. Localization of cholinergic synapses in mammalian retina with peroxidase-conjugated alpha-bungarotoxin. *Vision Res.* 1979; 19:287–292. [PubMed: 442552]
- Rodieck RW, Marshak DW. Spatial density and distribution of choline acetyltransferase immunoreactive cells in human, macaque, and baboon retinas. *J Comp Neurol.* 1992; 321:46–64. [PubMed: 1613139]
- Schmidt M, Humphrey MF, Wässle H. Action and localization of acetylcholine in the cat retina. *J Neurophysiol.* 1987; 58:997–1015. [PubMed: 3694255]
- Sheardown MJ. The triggering of spreading depression in the chicken retina: a pharmacological study. *Brain Res.* 1993; 607:189–194. [PubMed: 8097663]
- Sheardown MJ. The triggering of spreading depression in the chick retina by nicotinic receptor agonists. *Eur J Pharmacol.* 1997; 337:209–212. [PubMed: 9430415]
- Swanson LW, Simmons DM, Whiting PJ, Lindstrom J. Immunohistochemical localization of neuronal nicotinic receptors in the rodent central nervous system. *J Neurosci.* 1987; 7:3334–3342. [PubMed: 2822866]
- Tzartos S, Hochschwender S, Vasquez P, Lindstrom J. Passive transfer of experimental autoimmune myasthenia gravis by monoclonal antibodies to the main immunogenic region of the acetylcholine receptor. *J Neuroimmunol.* 1987; 15:185–194. [PubMed: 3495549]
- van der Zee EA, Luiten PG. Muscarinic acetylcholine receptors in the hippocampus, neocortex and amygdala: a review of immunocytochemical localization in relation to learning and memory. *Prog Neurobiol.* 1999; 58:409–471. [PubMed: 10380240]
- Vardi N, Auerbach P. Specific cell types in cat retina express different forms of glutamic acid decarboxylase. *J Comp Neurol.* 1995; 351:374–384. [PubMed: 7706548]
- Vardi N, Kaufman DL, Sterling P. Horizontal cells in cat and monkey retina express different isoforms of glutamic acid decarboxylase. *Vis Neurosci.* 1994; 11:135–142. [PubMed: 8011576]
- Wasselius J, Johansson K, Bruun A, Zucker C, Ehinger B. Correlations between cholinergic neurons and muscarinic m2 receptors in the rat retina. *Neuroreport.* 1998; 9:1799–1802. [PubMed: 9665603]
- Wässle H, Koulen P, Brandstatter JH, Fletcher EL, Becker CM. Glycine and GABA receptors in the mammalian retina. *Vision Res.* 1998; 38:1411–1430. [PubMed: 9667008]
- Yamada ES, Jacoby RA, Marshak DW. Cholinergic amacrine cells contain calbindin and alpha-Cam kinase II. *Invest Ophthalmol Vis Sci.* 1999; 40:S439.
- Zarbin MA, Wamsley JK, Palacios JM, Kuhar MJ. Autoradiographic localization of high affinity GABA, benzodiazepine, dopaminergic, adrenergic and muscarinic cholinergic receptors in the rat, monkey and human retina. *Brain Res.* 1986; 374:75–92. [PubMed: 3013364]
- Zhou ZJ, Fain GL. Neurotransmitter receptors of starburst amacrine cells in rabbit retinal slices. *J Neurosci.* 1995; 15:5334–5345. [PubMed: 7623156]
- Zucker CL, Ehinger B. Complexities of retinal circuitry revealed by neurotransmitter receptor localization. *Prog Brain Res.* 2001; 131:71–81. [PubMed: 11420982]

Fig. 1. Monkey retina labeled with ChAT antiserum. **A:** Perikarya and overlapping dendrites of ChAT-IR amacrine cells in S4. **B:** Starburst morphology of a ChAT-IR cell in S2. **C:** Vertical section showing the two ChAT-IR bands in the IPL. Scale bar = 25 μ m.

Fig. 2. ChAT-IR dendrites making synapses (arrowheads) onto bipolar cell axon terminals in two sets of serial sections. **A,B:** The postsynaptic bipolar cell makes a ribbon synapse (arrow) onto unlabeled processes 50,000 $\times$. **C,D:** A reciprocal synapse; that is, the ChAT-IR dendrite receives an input from the bipolar cell axon and then makes a synapse nearby onto the same bipolar cell axon, which has two synaptic ribbons here; 30,000 $\times$. Scale bar = 0.5 μ m.

Fig. 3. ChAT-IR dendrites make synapses (arrowheads) onto amacrine cell processes. **A:** Some postsynaptic amacrine cell processes contained large numbers of synaptic vesicles. **B:** The postsynaptic amacrine cells make synapses (arrows) onto other unlabeled processes. **C:** Electron-lucent, microtubule-containing amacrine cell processes receive a synapse from a ChAT-IR dendrite. **D:** contacts through gap-junctions were also observed between these two types of postsynaptic processes (asterisk); 50,000 $\times$. Scale bar = 0.5 μ m.

Fig. 4.
A: ChAT-IR dendrites make synapses (black arrowheads) onto other ChAT-IR processes, which in turn make synapses (white arrowheads) onto unlabeled processes. The ChAT-IR amacrine cells are concave where they make synapses onto smaller processes. **B:** A synaptic ribbon is indicated with an arrow; 60,000 $\times$. Scale bar = 0.5 μ m.

Fig. 5.

A–C: ChAT-IR dendrites make synapses (black arrowheads) onto ganglion cell dendrites (G) 60,000 $\times$. **A:** The ganglion cell dendrite is sectioned longitudinally. **B,C:** The starburst cell dendrites are concave in the area where they make synapses. **C:** Another starburst cell is apposed to the presynaptic starburst cell.

Fig. 6. Inputs to starburst cells from amacrine cell processes 50,000 $\times$. **A:** ChAT-IR dendrite receives a synapse (arrowheads) from an electron-lucent amacrine cell. **B:** Input from a more electron-dense amacrine cell. Scale bar = 0.5 μ m.

Fig. 7.
In this double-labeled vertical section of monkey retina, ChAT-IR dendrites and perikarya (red, **B,C**) do not show GAD65 immunoreactivity (green, **A,C**). The bands labeled by the two markers in the IPL seem to alternate. Single optical sections, 1 μm thick. Scale bar = 25 μm .

Fig. 8.

A: mAB210-labeled amacrine cells (ac), ganglion cells (gc), and bipolar cells (bc) in vertical sections of monkey retina. **B–E:** M3 antiserum (red) labeled bipolar cells. They did not contain calbindin (green) in the periphery (B,C), but some did in the central retina (D,E). **F:** ON ChAT-IR dendrites (red) are slightly below DB4 axon terminals (arrows) labeled with antiserum to PKCa (green). **G:** A DB5 bipolar cell (bc) was labeled with antiserum to calbindin; its axon terminal ramifies in S4. Because starburst cells contain calbindin (Yamada et al., 1999), the ChAT-IR band in S4 (ACh) appears yellow. B and C are stacks of 20 and 25 1 μ m confocal images, respectively. D and E are single, 1 μ m optical sections. F is a stack of 17 0.5 μ m images. G is a stack of 11 0.8 μ m sections. Scale bar = 25 μ m in A–F; 10 μ m in G.

Fig. 9. Double-labeled vertical sections of monkey retina using antiserum to M2-muscarinic receptor (red) and three different markers for amacrine cells: anti-calretinin (green, **A,C**), anti-TH (green, **D,F**), anti-G6-gly (green, **G,I**), and anti-GAD65 (green, **J,L**). All amacrine cells (**A**), dopaminergic amacrine cells (**D**), and GAD65 cells are not labeled with M2 antiserum (**C,F,L**). GAD65 and M2 bands in the IPL seem to alternate and there is some colocalization where the bands intersect. M2-immunoreactivity was present in some G6-gly-IR amacrine cells (**I**, yellow). All images are single, optical sections. **A–H** are 1 μm thick, and **J–L** are 0.5 μm thick. Scale bar = 25 μm .

Fig. 10.

This circuit diagram illustrates some of the pathways through which starburst amacrine cells ramifying in S4 (ON-ChAT) might excite ganglion cells in the primate retina, which are represented here by the ON parasol cells. 1) In this study we demonstrate that starburst cells are presynaptic to bipolar cells; these synapses are expected to be excitatory (open triangles). The EM results indicate that these are cone bipolar cells, and two lines of evidence suggest that they include the DB5 type, which contains calbindin. First, the ChAT-IR cells ramify proximal to the plexus of DB4 axon terminals but in the same stratum as the calbindin-IR processes. Second, immunoreactive calbindin and M3 muscarinic receptors are colocalized in some bipolar cell perikarya and axons there. 2) The major output of starburst cells is onto ganglion cell dendrites, which express nicotinic acetylcholine receptors, and these synapses are expected to be excitatory. Previously, we reported evidence that these include ON parasol cells. ON parasol cells make more contacts with ChAT-IR dendrites than expected by chance (Jacoby et al., 1996), and they receive synapses from amacrine cells with ultrastructure characteristic of starburst cells (Marshak et al., 2002). 3) Starburst cells could also excite ganglion cells by disinhibition. In this study we confirm that starburst cells make synapses onto processes of amacrine cells, and these synapses are expected to be inhibitory (filled circles). We also present evidence that amacrine cells containing a precursor of the neuropeptide cholecystokinin (CCK) contain M2 muscarinic receptors.

TABLE 1

Synaptic Organization of Starburst Amacrine Cells in the Macaque Retina

Synaptic input	No. of input synapses	% of input	Synaptic output	No. of output synapses	% of output
Bipolar cells	56	68.3	Bipolar cells	12	14.8
Unlabeled amacrine cells	15	18.3	Unlabeled amacrine cells	12	14.8
ChAT-IR amacrine cells	11	13.4	ChAT-IR amacrine cells	11	13.5
			Ganglion cells	39	48.2
			Unidentified	7	8.6
Total	82			81	

In all, 152 synapses made by choline acetyltransferase-immunoreactive (ChAT-IR) amacrine cells were analyzed. Input synapses are synapses received by ChAT-IR amacrine cells and output synapses are synapses made by ChAT-IR cells. There were also synapses between ChAT-IR dendrites and in calculations involving only outputs or only inputs, these were counted twice.