Published in final edited form as:

Peptides. 2016 October ; 84: 22–35. doi:10.1016/j.peptides.2016.08.007.

Wide-field Diffuse Amacrine Cells in the Monkey Retina Contain Immunoreactive Cocaine- and Amphetamine-Regulated Transcript (CART)

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Abstract

The goals of this study were to localize the neuropeptide Cocaine- and Amphetamine-Regulated Transcript (CART) in primate retinas and to describe the morphology, neurotransmitter content and synaptic connections of the neurons that contain it. Using in situ hybridization, light and electron microscopic immunolabeling, CART was localized to GABAergic amacrine cells in baboon retinas. The CART-positive cells had thin, varicose dendrites that gradually descended through the inner plexiform layer and ramified extensively in the innermost stratum. They resembled two types of wide-field diffuse amacrine cells that had been described previously in macaque retinas using the Golgi method and also A17, serotonin-accumulating and waterfall cells of other mammals. The CART-positive cells received synapses from rod bipolar cell axons and made synapses onto the axons in a reciprocal configuration. The CART-positive cells also received

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Contributors

Ye Long carried out in situ hybridization and immunolabeling experiments, acquired and analyzed light microscopic images and wrote the manuscript.

Andrea S. Bordt carried out immunolabeling experiments, acquired and analyzed light microscopic images and wrote the manuscript.

Weiley S. Liu carried out electron microscopic immunolabeling experiments and cut ultrathin sections.

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Luke Tseng analyzed the morphology and spatial distribution of the labeled cells.

Alice Z. Chuang carried out the statistical analyses.

Christopher M. Whitaker made intracellular injections and analyzed the morphology of the labeled cells.

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Michael B. Sherman helped with the acquisition of electron microscopic images.

David W. Marshak designed the experiments, acquired and analyzed the electron microscopic images and wrote the manuscript.

synapses from other amacrine cells. Some of these were located on their primary dendrites, and the presynaptic cells there included dopaminergic amacrine cells. Although some CART-positive somas were localized in the ganglion cell layer, they did not contain the ganglion cell marker RNA binding protein with multiple splicing (RBPMS). Based on these results and electrophysiological studies in other mammals, the CART-positive amacrine cells would be expected to play a major role in the primary rod pathway of primates, providing feedback inhibition to rod bipolar cells.

Keywords

Neuropeptide; Primate; Rod Bipolar Cell; Reciprocal Synapse; GABA

1. Introduction

Cocaine- and Amphetamine-Regulated Transcript (CART) is a neuropeptide expressed at high levels in many different areas of the brain. In humans there are two major forms, consisting of amino acids 42-89 and 49-89 of the propeptide. Although CART was named for its role in the response to psychostimulants and plays an important role in reinforcement and reward, it also contributes to many other neural circuits, including those in the retina [1]. CART was first localized to rat amacrine cells, inhibitory local circuits of the inner retina, and retinal ganglion cells, the projection neurons, using immunolabeling techniques and in situ hybridization [2, 3]. CART mRNA has been identified in dopaminergic amacrine cells isolated from mouse retina [4], and in frog retina, CART was localized to amacrine cells [5]. More recently, CART was localized to ganglion cells and to amacrine cells in mouse retina [6-8]. The goal of this study was to identify the neurons that contain CART in retinas of Old World Monkeys and, ultimately, to understand how they contribute to vision in primates.

2. Materials and Methods

2.1 Tissue Preparation

Tissue from 11 baboons (*Papio anubis*) and 5 macaques (1 *Macaca fascicularis* and 4 *M. mulatta*), of both sexes, were used in a total of 50 immunolabeling experiments examining CART-positive cells in the retina. The anterior half of the eye was cut away, the vitreous humor was removed with fine forceps, and the eyecups were incubated in Ames medium (Sigma-Aldrich, St. Louis, MO) equilibrated with 95% oxygen and 5% carbon dioxide for 0.5-4 hours at 20°C before fixation or injection. For immunolabeling, the tissue was immersion fixed in 0.1M phosphate buffer (PB) pH 7.4 containing 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for times ranging from 30 minutes to overnight at either room temperature (0.5-1 hour) or 4°C (overnight). In some instances, 0.05-0.1% glutaraldehyde (EM grade, Ted Pella, Inc. Redding, CA) was included, and those retinas were pre-treated for 60 minutes with 1% sodium borohydride (Sigma-Aldrich) in 0.01M phosphate buffered saline (PBS, Sigma-Aldrich).

For frozen sections, fixed primate retinas were incubated in 25-30% sucrose (Sigma-Aldrich) for more than 24 hours until the tissue sank. The tissue was embedded in Tissue-

Tek[®] OCT compound (Electron Microscopy Sciences, Hatfield, PA). Sections were cut at a thickness of 12 μ m using a Microm HM505E cryostat (Thermo Fisher Scientific, Inc., Waltham, MA) and then were mounted on histological slides. For vibratome (Leica VT1000 S, Bannockburn, IL) sectioning, retinal pieces were embedded in 4-4.5% low-melting temperature agarose (Sigma-Aldrich) in 0.01M phosphate-buffered saline with or without 0.3% sodium azide (PBSa), and then were cut into 30-100 μ m vertical sections.

2.2 Immunofluorescent labeling with CART antisera

Immunolabeling experiments were carried out on frozen and vibratome sections, as well as on flatmounts. Several vibratome and flatmount experiments began with a one hour blocking step in 5-10% ChemiBLOCKER[™] (Millipore, Billerica, MA). Incubation conditions for primary and secondary antibodies varied depended on the tissue format. Primary antibodies (see Table 1) were dissolved in PBS containing 5-20% ChemiBLOCKER[™] and 0.3% Triton-X 100 (Sigma-Aldrich), with or without sodium azide. The SV2 monoclonal antibody developed by Dr. Kathleen M. Buckley was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Frozen sections were incubated overnight at room temperature or 4°C; vibratome sections and flatmounts were incubated for 4-10 days at 4°C. Following three washes with PBS of at least 30 min each, tissue was incubated with Cy3-, Cy5-, Alexa Fluor[®] 488-, Alexa Fluor[®] 594-, or Alexa Fluor[®] 647-conjugated, affinity-purified secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a dilution of 1:200 for at least 1 hour at 20°C (frozen sections) or 2-4 days at 4°C (vibratome sections and flatmounts). For experiments using biotin/streptavidin, the tissue was first incubated in 1:100 biotinylated antibody (Jackson ImmunoResearch Laboratories, Inc.) specific for the primary antibody host, and then with 1:200 Streptavidin-conjugated fluorophore. Following a wash step, tissue was mounted in VECTASHIELD Antifade Mounting Medium with or without 4, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA).

Two different polyclonal antibodies directed against CART were used. The majority of experiments were done using the rabbit anti-CART antibody (Phoenix Pharmaceuticals, Inc., Burlingame, CA, Catalog No.H-003-62). In order to demonstrate the specificity of this antibody, a peptide blocking experiment was carried out. Briefly, antibody was concurrently subjected to two conditions for 45min at room temperature: (1) Preincubation of 1:5,000 rabbit anti-CART antibody (Phoenix Pharmaceuticals, Catalog No. H-003-62) with a 50-fold higher concentration of the antigen (CART (55-102)(Rat, Mouse, Bovine), Catalog No. 003-62, Phoenix Pharmaceuticals, Inc., Burlingame, CA) in 0.01M phosphate buffered saline (PBS, Sigma-Aldrich, Saint Louis, MO, Catalog No. P-3813) with 0.3% Triton[™] X-100 (SIGMA-ALDRICH, Saint Louis, MO, Catalog No. X100), and (2) 1:5,000 rabbit anti-CART antibody (Phoenix Pharmaceuticals, Catalog No.H-003-62) alone in PBS with 0.3% Triton[™] X-100. Solutions were added to frozen sections of baboon retina and incubated overnight at 4°C in a humidity chamber. After washing, sections were incubated with 1:200 Alexa Fluor[®] 594 AffiniPureF(ab')₂ Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour at room temperature.

2.3 Confocal microscopy

Fluorescent images were collected with Zeiss LSM 510 and LSM 780 confocal laser scanning microscopes (Carl Zeiss, Thornwood, NY). All sections were imaged with dye-appropriate spectral acquisition parameters. 40x or 63x oil immersion objective with numerical apertures of 1.40 were used to collect images. Each channel (DAPI, 488, 647, Cy5, or Cy3) was scanned separately in sequential frame mode, usually using an average of 4 and low scan speed. Single optical sections were 0.5-0.6 μm . A 20x dry objective with a numerical aperture of 0.8 was also used to measure the labeled somas in flat mount retina and to visualize injected cells. The images were analyzed as single optical sections and as stacks of optical sections projected along the y- or z-axes. Stacks were created using the Maximum Intensity Projection function within the Zen 2012 software (Carl Zeiss Microscopy GmbH 1997-2013), and some were subsequently refined using the Median Filter function. The Ortho feature in Zen 2012 was also used for orthogonal imaging. Images were processed with Photoshop (Adobe Systems, Mountain View, CA) for brightness and contrast adjustment and to change pseudocolors for some images. Photoshop was also used to create montages made by aligning tiles of high-power z-image projections. For montages, tiles were arranged either by lining up adjoining sections from sequentially acquired images or else using a low-power image as a guide.

2.4 Immunoperoxidase labeling with rabbit anti-CART

Retinas from 2 baboons were fixed in 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M PB pH 7.4 for 60 minutes. The tissue was treated 1 hour with 1% sodium borohydride with PBS and an ascending and descending series of graded ethanol solutions in PBS (10%, 20% and 40%). Vibratome sections 100 μm thick were prepared as described above and labeled as described previously [9]. After rinsing for several hours in PBS the tissue was incubated for 10-18 days in 1:1,000 rabbit anti-CART in PBS with 0.3% sodium azide. Next the tissue was rinsed in PBS and incubated for 2 days in 1:100 biotinylated donkey anti-rabbit IgG (Jackson Research Laboratories) in PBS. To visualize the biotin, the tissue was incubated in 1:50 avidin-biotin-peroxidase (Standard Kit, Vector Laboratories, Burlingame, CA) overnight, followed by histochemical reaction with 0.5 mg/ml 3, 3'-diaminobenzidine tetrahydrochloride (Polysciences, Warrington, PA) and hydrogen peroxide (0.0025%, Sigma-Aldrich) for 60 minutes. The tissue was treated with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M PB pH7.4 for 60 minutes and embedded in Epon (Ted Pella, Inc., Redding, CA). Serial sections of 80–100 nm thickness were cut on a Reichert Ultracut E ultramicrotome (Leica Microsystems, Buffalo Grove, IL) collected on Formvar-coated FF2010-AU, single-hole grids (Electron Microscopy Sciences) and stained with 1% aqueous uranyl acetate (Ted Pella, Inc.) for 20 minutes followed by 0.2% aqueous lead citrate [10] for 10-15 minutes. Labeled processes were imaged at 5,000 X using a JEOL 1400 electron microscope (JEOL USA, Peabody, MA). A macaque retina (*M. mulatta*) was processed similarly, except that the primary fixative did not contain glutaraldehyde, the retina was treated with 0.01% osmium tetroxide for 5 min, and the retina was embedded flat with ganglion cells upward.

2.5 In Situ Hybridization

Tissue from baboon retina was used for in situ hybridization. RNA in situ hybridization to detect Papio anubis CART prepropeptide (CARTPT) mRNA (http://www.ncbi.nlm.nih.gov/nuccore/XM_003899785.1) was performed manually using the RNAscope® 2.0 HD Detection Kit (RED) (Advanced Cell Diagnostics, Inc., Hayward, CA) [11], according to the manufacturer's instructions. Target probe pairs were designed to anneal to the region between bases 30-883 of the 917nt-long baboon CARTPT transcript. A piece of central retina (5 × 5 mm) was fixed in fresh 10% neutral buffered formalin (Fisher Scientific, Kalamazoo, MI) for 16 hours at room temperature and then embedded in paraffin. The embedded tissue was cut into 5µm sections and mounted on Superfrost Plus slides (Fisher Scientific). The slides were air-dried over night at room temperature and baked at 60°C for 1 hour. The slides were then deparaffinized by immersion in fresh xylene twice for 5 minutes each, followed by immersion in 100% ethanol twice for 1 min each, and air drying for 5 min at room temperature. Peroxidase blocker was added to cover the tissues and the slides were incubated for 10 min at room temperature and then were washed twice with distilled water. After boiling in citrate buffer (10 nmol/L, pH 6) for 15 min, the slides were washed with distilled water twice and 100% ethanol once and were allowed to air dry. Tissue was circled using a hydrophobic barrier pen (Advanced Cell Diagnostics) and allowed to dry completely. The tissues were then treated with protease (10 µg/mL) for 30 min at 40°C in a humidity chamber followed by two washes with distilled water. For the probe hybridization step, the tissue was incubated with probes (CARTPT target, baboon hypothalamus positive control, and DapB bacterial gene negative control (all Advanced Cell Diagnostics) in hybridization buffer A [6X SSC (1X SSC (saline sodium citrate) in 0.15 mol/L NaCl, 0.015 mol/L Nacitrate), 25% formamide, 0.2% lithium dodecyl sulfate, blocking reagents] for 2 hours at 40°C in a humidity chamber. Slides were then washed in Wash Buffer (0.1X SSC, 0.03% lithium dodecyl sulfate) twice for 2 min each at room temperature. Next, slides were incubated with preamplifier (2 nmol/L) in hybridization buffer B (20% formamide, 5X SSC, 0.3% lithium dodecyl sulfate, 10% dextran sulfate, and blocking reagents), a series of amplifier molecules (2 nmol/L) in hybridization buffer B was completed, and label probe (2 nmol/L) in hybridization buffer C (5X SSC, 0.3% lithium dodecyl sulfate, blocking reagents). Slides were incubated in each solution for 15-30 minutes each at 40°C in a humidity chamber with two washes in Wash Buffer for 2 min after each step. Signal was detected by treating slides with alkaline phosphatase solution (1:60 ratio of Red B to Red A) for 10 min at room temperature, followed by two washes in distilled water. Slides were counterstained with 50% Gill's Hematoxylin I (Sigma-Aldrich) in distilled water] for 2 min at room temperature, and then rinsed several times in distilled water until the slides were clear. After being immersed in 0.02% ammonia water (Sigma-Aldrich), slides were washed in distilled water 3-5 times and dried in a 60°C dry oven for 15 min. Slides were then dipped briefly into fresh pure xylene and immediately mounted in EcoMount mounting medium (Biocare Medical, Concord, CA), coverslipped and then air dried for 5 min. Tissue sections were evaluated using a Zeiss Axioskop with a 40x oil-immersion objective. Positive controls were performed for all runs using baboon hypothalamus tissue with the CART mRNA probe.

2.6 Intracellular injection of Neurobiotin

Eyecups were incubated in Ames medium equilibrated with 95% oxygen and 5% carbon dioxide for 30 minutes with 30 μ M DAPI (Molecular Probes, Eugene OR). The retina was isolated and placed on a black membrane filter paper with ganglion cell side up. The retina was superfused in carboxygenated Ames medium on an upright, fixed stage microscope Olympus BX50WI (Tokyo, Japan). Microelectrodes were pulled from borosilicate glass (O.D.:1.2mm, I.D.: 0.69mm, with filament, Sutter Instruments, Novato, CA) with a Brown-Flaming P-97 horizontal micropipette puller (Shutter Instruments, Novato, CA). The microelectrode resistance was between 100-120 M Ω . Microelectrodes were tip filled with 0.5% Lucifer Yellow (Invitrogen, Carlsbad, CA, Catalog No. L-453) and 4% Neurobiotin (Vector Laboratories, Inc. Burlingame, CA) in 0.1 M phosphate buffer and back filled with 3 M LiCl (Sigma-Aldrich). Amacrine cells were targeted based on their intensity of DAPI staining, size, and shape using a 40x water-immersion objective. Lucifer Yellow and Neurobiotin were iontophoresed with biphasic current (± 1 nA) for 5-10 min using a Warner Instruments (Hamden, CT) 1E-210 amplifier. After injection, the tissue was superfused for at least 20 min to allow for tracer diffusion and then fixed with 4% paraformaldehyde in 0.1 M PB, 30 min at room temperature. The tissue was incubated in 1:250 streptavidin-Cy3 (Jackson ImmunoResearch, West Grove, PA) overnight at room temperature. Fluorescent images of filled amacrine cells were acquired with a Zeiss LSM 780 confocal microscope.

2.7 Data analysis

The distribution of labeled varicosities as a function of depth in the inner plexiform layer was described quantitatively. Confocal images of vertical sections from baboon retina were analyzed by hand. The position of each dendritic varicosity was measured and expressed as a percentage of the total depth of the inner plexiform layer at that point, with 0 being the lower boundary of the inner nuclear layer and 100 being the upper boundary of the ganglion cell layer. The distribution of varicosities as a function of depth was fitted by mixture of 2 normal distributions, corresponding to descending and stratifying dendrites. The parameters, including the proportion of varicosities in each normal distribution as well as mean and standard deviation (s.d.) for each normal distribution, were estimated using an expected-maximization algorithm (<http://www.jstatsoft.org/v32/i06/paper>). Initially, 50% of the varicosities were assumed to be descending and 50% stratifying. The mean and s.d. for each distribution were estimated. Next, each varicosity was re-assigned to the descending or the stratifying group by calculating the probability that it belonged to the group using the estimated means and standard deviations from the preceding step. This was repeated until the algorithms converged. The function `normalmixEM`, in the `mixtools` package of R software version 3.0.1 (R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2015, <https://www.R-project.org>) was used to perform the analysis.

To analyze the spatial organization of CART-IR somas, images were acquired from a fluorescent-labeled flat mount of far peripheral baboon retina using a Zeiss LSM780 with a 20x objective. The images were converted to a Maximum Intensity Projection using Zen 2012 software. Soma areas were measured using the Particle Analysis function of ImageJ 1.47 (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,

<http://imagej.nih.gov/ij/>, 1997-2014). When all the CART-positive somas were analyzed together, their spatial distribution was indistinguishable from a random distribution (not illustrated). Because two types varying in size had been described in primate retinas previously [12], the areas of the labeled somas were fit by 2 normal distributions; the intersection of the two curves was used to divide the populations. The proportion of cells in each normal distribution as well as the mean and standard deviation for each normal distribution were estimated using an expected-maximization algorithm, as described above. The Dip Test in R was also used to determine whether or not the distribution of soma areas was multimodal. Large and small types were analyzed separately, as described previously [13]. The spatial distributions of the large and small somas were plotted separately and converted into BMP files using Paint version 6.1 (Microsoft, Redmond, WA). These were loaded into WinDRP (<http://wvad.mpimf-heidelberg.mpg.de/departments/biomedicalOptics/softwareDevelopment/WinDRP/WinDRPManual/index.html>). This program calculates the density-recovery profiles and also performs nearest-neighbor analyses.

3. Results

3.1 CART-positive amacrine cells

CART-positive somas were observed in the inner nuclear layer (INL) and, occasionally, in the ganglion cell layer (GCL). Because of the punctate nature of the immunolabeling and the extensive overlap of the labeled dendrites, it was not possible to trace the dendrites of a single immunolabeled cell through the inner plexiform layer (IPL). However, it was possible to observe that individual dendrites varied in their trajectories through the IPL. Some ran in the outermost stratum (S1) and gradually descended to the innermost stratum (S5), and others descended more directly to S5. A dense plexus of CART-positive dendrites was observed in S5 (Fig. 1). In order to more completely describe the distribution of CART-positive varicosities within the IPL, a statistical analysis was performed using a 2-normal-mixture model. Depth was defined as a percentage of the distance from the INL to the GCL. From 0-60% through the IPL, there was a relatively low density of CART-positive varicosities. There was a major peak at 82.5% with a standard deviation of 10.1, indicating that the density of CART-positive varicosities was highest in S5 (Fig. 2).

CART mRNA expression in the retina was studied to provide an additional control for the specificity of the immunolabeling. Sections from central baboon retina were hybridized with an RNAscope target probe recognizing CART mRNA (Fig. 3). CART mRNA was found in somas in the INL and GCL but not in the outer nuclear layer. Most labeled somas were in the outermost row of the GCL or innermost row of the INL. A few (< 1%) labeled somas were found in the inner half of the INL, and one cell was found in the outermost row of the INL. The somas varied in size; diameters ranged from 5-12 μm . In a few cells, the primary dendrites were also labeled.

Because some CART-positive somas were observed in the GCL and some were relatively large, it was essential to determine whether or not they were retinal ganglion cells. The ganglion cell marker RNA-binding protein with multiple splicing [14](RBPMS) was applied in frozen sections (12 sections, 12 μm /section), vibratome sections (23 sections, 50 μm /section), and flatmounts (three samples from two baboons). No somas in which CART was

co-localized with RBPMS were observed. CART was not colocalized with choline acetyltransferase, a marker for starburst cells, another type of amacrine cell with somas in the GCL (Fig. 4) [15].

3.2 Spatial distribution of CART-positive amacrine cells

All of the CART-positive cells in the INL had thin, varicose dendrites and similar stratification patterns in the IPL. However, the variation in the soma size of CART-positive cells and previous work using the Golgi method suggested that there were two types. To test this hypothesis, CART-positive somas in both the INL and GCL were analyzed in the far periphery (>10 mm from the fovea) of a flatmount baboon retina. The soma diameters of CART-positive cells varied, but cells in peripheral baboon retina could be classified as either small (mean diameter 6.15 μm) or large (mean diameter 8.98 μm). Although the distribution of soma areas was not multimodal ($p = 0.89$), it could be fit by 2 normal distributions (Fig. 5). The sampled area contained 171 large somas in the INL and GCL, giving a spatial density of 154/mm². There were no instances of superposition of large somas in the two layers, a finding suggesting that they were a single type. However, the density recovery profile, a plot of the spatial density of other large somas versus the distance from each cell, indicated that the effective radius in this region of the retina was only 8.9 μm , a value very similar to the mean soma diameter. Likewise, there were no instances of superposition of small somas in the two layers. However, the distribution of the small cells ($n = 179$, spatial density 171/mm²) appeared to be random based on the density recovery profile (Fig. 6). For both types, the spatial distribution was essentially the same as random (Weibull) distributions having the same means (data not shown). These findings suggested that there were at least two types of CART-positive cells, which varied in the sizes of their somas. Both populations had a few cells with somas in the GCL, but these did not appear to be distinct types. The spatial distributions of CART-positive large and small somas appeared to be random.

3.3 CART-positive cells contain GABA

Double labeling experiments (34 frozen sections, 12 μm /section; 14 vibratome sections, 50 μm /section) were carried out to investigate the relationship between CART- and GABA-positive amacrine cells (Fig. 7). All CART-positive cells both in the INL and GCL were GABA-positive, and in these double-labeled cells, CART and GABA immunoreactivity were present within both the somas and dendrites. However, only a subset of GABA-positive cells contained immunoreactive CART. GABA-positive processes were present throughout the IPL and also formed the three dense bands in the IPL. To determine whether CART-positive cells use glycine as a neurotransmitter, another set of experiments was carried out with antibodies to CART and the glycine transporter Glyt-1. There was no co-localization (data not shown).

3.4 Synaptic connections of CART-positive cells

The dendrites of CART-positive cells occupied the entire IPL, terminating in a dense plexus in the innermost stratum (S5), where rod bipolar cell axon terminals are located. To determine whether the two types of cells interact, double label experiments were done using antibody to protein kinase C (PKC; Figs. 8 and 9). The axon terminals of rod bipolar cells

were surrounded by CART-positive dendrites in S5. CART-positive dendrites were associated with both the descending axons and their terminals. Some of these experiments utilized a third label, SV2A, a marker for the presynaptic cells at conventional, but not at ribbon, synapses [16]. Punctate SV2A labeling was observed at contacts between CART-positive processes and rod bipolar cell axon terminals in S5 (Fig. 8B), a finding suggesting that CART-positive amacrine cells are presynaptic at these synapses.

CART-positive primary and secondary dendrites ramified in the most distal stratum of the IPL, where dopaminergic amacrine cells ramify. To test the hypothesis that the dopaminergic cells were presynaptic to the CART-positive cells, triple label experiments were done using antibodies to CART, tyrosine hydroxylase and SV2. Contacts between the two cell types were observed, and there was punctate SV2 labeling at these sites (Fig. 10).

The synaptic connections of CART-positive amacrine cells were also studied by electron microscopy. The profiles labeled with rabbit anti-CART had ultrastructure typical of amacrine cell dendrites in primate retinas [17]. They contained clear synaptic vesicles and also, in some instances, large, dense-core vesicles, which were very well-labeled. The sample included 82 identified synapses, some of which were followed through a short series of sections (Fig. 11). There were 45 input synapses to CART-positive profiles in all, 22 where axons of bipolar cells were presynaptic and 23 where amacrine cell processes were presynaptic. The primary dendrites of CART-positive cells received large synapses from amacrine cells in S1, but most synaptic inputs from amacrine cells were found in S5. The same was true of synaptic inputs from bipolar cells. The axon terminals of bipolar cells typically made ribbon synapses onto the CART-positive dendrites in S5, but there were also instances where the axon, itself, made atypical ribbon synapses onto the labeled dendrites. Virtually all of the output synapses of the CART-positive dendrites ($n = 37$) were onto bipolar cell axon terminals, often the same bipolar cells that had provided their input.

3.5 Morphology of CART-positive amacrine cells

In order to see the dendritic morphology of individual cells more clearly, five wide-field diffuse amacrine cells from mid-peripheral retina were injected intracellularly with Neurobiotin (Fig. 12). Of these, four were subsequently labeled with rabbit anti-CART, and the fifth was labeled with goat anti-CART and rabbit anti-PKC. The injected cells all had three primary dendrites, measuring 1-2.5 μm in diameter. The primary dendrites branched into two, or occasionally three, secondary dendrites, typically at acute angles. The injected cells were classified as large or small based on the diameters of their dendritic fields. The large cells ($n = 2$) had a dendritic fields $>3\text{mm}$ in diameter. Their dendrites were straighter and less branched with varicosities 0.5-1 μm in diameter. Dendrites of one of the large cell made contacts with axon terminals of rod bipolar cells (Fig. 12). The small cells ($n = 3$) had more curving dendrites that were also more highly branched, particularly near the somas. Varicosities of the small cells were 1-2 μm in diameter, and their dendritic field diameters were 0.8 and 1.2 mm. Lightly labeled, tracer-coupled somas were observed near the injected small somas (data not shown).

4. Discussion

4.1 Localization of immunoreactive CART in Old World Monkey retinas

Immunoreactive CART was localized exclusively to amacrine cells in baboon and macaque retinas. The labeled amacrine cells contain GABA, and there appear to be two morphological types. Both types have thin, varicose dendrites ramifying primarily in stratum 5 of the inner plexiform layer (IPL), like the wide-field diffuse amacrine cells of macaque retina [12]. The labeled amacrine cells receive input from rod bipolar cell axon terminals at dyad synapses and also make reciprocal synapses onto the axon terminals. Immunoreactive CART has not been isolated and characterized in primate retinas, but three lines of evidence suggest that the neuropeptide CART is, in fact, present in Old World Monkey retinas. First, the labeling patterns in baboon retina were identical using two antisera raised against different portions of the molecule. Second, the labeling was completely blocked when the rabbit antiserum to CART was preincubated with synthetic peptide. Third, amacrine cells were labeled using *in situ* hybridization with a probe directed against the baboon form of CART mRNA.

4.2 Two types of labeled amacrine cells

The somas labeled with antibodies to CART varied considerably in size, as expected if there were multiple types. The intracellular injection experiments also provided clear evidence for at least two types. There were no obvious differences in the morphology and stratification patterns of the labeled dendrites or in their synaptic connections, however. These findings fit very well with the original observations of wide-field diffuse amacrine cells using the Golgi method in the macaque retina [12]. There were two types with fine, varicose dendrites that descended gradually through the IPL to form a dense plexus in the most proximal stratum, at the level of the rod bipolar cell axon terminals. The differences between the two types were particularly clear in the central retina. One type of wide-field diffuse cell there had a dendritic field approximately 300 μm in diameter. A second type, from the same region of the retina, had a much larger dendritic field, at least 600 μm in diameter. Another difference is that some dendrites of the second, larger type ran for a considerable distance in the most distal stratum, S1, of the IPL before descending. A later study of the macaque retina using the Golgi method also described two very similar types of amacrine cells, which were called spidery [18]. The majority of dendrites of both types ramified in S5, but spidery type 1 cells also had dendrites that terminated in S1. Another group using the Golgi method in human retina reached a different conclusion [19]. They argued that the spidery or wide-field diffuse cells were all a single type, which they called A17. They proposed that the dendrites that appeared to terminate in S1 had been incompletely stained and actually terminated in S5.

In some other mammalian retinas, there are two types of amacrine cells homologous to the wide-field stratified cells of primates. In rabbits, these amacrine cells can be labeled selectively because they take up serotonin and other indoleamines, and the two types are called S1 and S2 [13, 20]. S1 cells have much larger dendritic fields, express delta 1 ionotropic glutamate receptor subunits and are well-coupled to other S1 cells. S2 cells have smaller dendritic fields, are only weakly coupled to other S2 cells and have a higher density of varicosities on their dendrites [21-24]. In mice, two types of waterfall cells were identified

using a genetically directed labeling technique [25]. Waterfall type 1 cells are relatively small and were proposed as homologues of S2 cells in rabbits. The larger waterfall type 2 cells were proposed as homologues of S1 cells. In the retinas of rats and cats, however, only one amacrine cell type resembling the wide-field stratified cells has been described; it is called A17 in both species [26-31].

Another finding suggesting that CART-positive cells were homologous to A17 and S1/S2 cells was that they contained immunoreactive GABA. A17 cells of cat retina take up ^3H muscimol, a GABA analog [32], and they contain immunoreactive GABA [33]. The same is true of serotonin-accumulating amacrine cells in rabbit retinas [34]. This finding is also consistent with a previous electron microscopic study showing that amacrine cells making reciprocal synapses in primate retina are GABAergic [35].

4.3 Synaptic connections of amacrine cells containing immunoreactive CART

The CART-positive amacrine cells also resembled A17 and S1/S2 cells in their synaptic connections. The majority of the labeled dendritic varicosities co-stratified with axon terminals of rod bipolar cells, and there were contacts between the two types of processes associated with the presynaptic marker of conventional synapses, SV2A [16]. The electron microscopic immunolabeling experiments confirmed that the CART-positive cells received input from rod bipolar cells at ribbon synapses and made reciprocal synapses onto the rod bipolar cells nearby. These findings were consistent with three previous electron microscopic studies of the output synapses of rod bipolar cells in primates. AII amacrine cells of human retina were labeled by uptake of ^3H glycine and visualized by electron microscopic autoradiography [36]. The other, unlabeled dendrites at those synapses made reciprocal synapses, like those of the CART-positive cells in the present study. Rod bipolar cells in central macaque retina were labeled using antibody to protein kinase C and reconstructed from serial, ultrathin sections [37]. There were two types of amacrine cell dendrites receiving input from the bipolar cells, one from an AII amacrine cell and one making reciprocal synapses. These authors also described en passant synapses with small, atypical ribbons in the middle of the IPL from the axons of rod bipolar cells onto amacrine cells like the CART-positive dendrite illustrated in Fig. 11 of the present study. AII amacrine cells in macaque retinas were labeled using antibody to calretinin, and their arboreal dendrites received input from rod bipolar cells at dyad synapses [38]. Like the CART-positive dendrites in the present study, the unlabeled dendrite made reciprocal synapses. Synaptic inputs to CART-positive dendrites from amacrine cells were also observed by electron microscopy. Light microscopic experiments indicated that the amacrine cells presynaptic to the somas and primary dendrites included the dopaminergic type. In this respect, the CART-positive cells of Old World Monkeys resembled A17 cells of cat retina [29, 39] and amacrine cells of mouse and rabbit retinas [40].

4.4 Spatial Distribution of CART-positive somas

The finding that the spatial distributions of CART-positive somas were apparently random was unexpected. Most types of mature retinal neurons are distributed regularly, though there is considerable variation in the indices of regularity for different populations [41]. Taken with evidence from earlier light microscopic studies, the results of the intracellular injection

studies reported here suggest that there are at least two types of CART-positive, wide-field stratified cells. However, this was difficult to demonstrate using immunolabeling alone. Even in the far peripheral retina, the plexus of overlapping labeled dendrites was very dense, and it was not possible to study the morphology of individual CART-positive cells. Instead, the labeled somas were divided into two groups based on size, small ones with a mean diameter of 6µm and large ones with a mean diameter of 9µm. But even then, their spatial distributions were apparently random.

There are several possible explanations for these results. 1) It is possible that some somas were not labeled, but this is unlikely. The experiments were done in the thinnest part of the retina, where antibody penetration is maximal, and the labeling was clearly distinguishable from the background based on the intensity. 2) It is possible that the antibody labeled somas containing a peptide structurally-related to CART. To rule this out, a protein blast search <https://blast.ncbi.nlm.nih.gov/Blast.cgi> was carried out using the sequence of 48 amino acids in the peptide antigen used to generate the rabbit antiserum. The only homologous proteins or peptides found were forms of CART from various species. It is possible that the two types contain different molecular forms of immunoreactive CART, however. 3) There may be more than two types of wide-field diffuse amacrine cells containing CART. A much larger sample of intracellularly injected amacrine cells and additional electron microscopic immunolabeling studies would be required to test this hypothesis. 4) The most likely explanation is that the spatial distributions of the two types of somas are, indeed, random. This is rare, but not unprecedented, in the retina; the spatial distribution of the somas of type 2 bipolar cells in mouse retina is random [42]. The random distribution of the CART-positive somas would be expected to have a negligible effect on their function. Each type of wide-field diffuse cell has a high spatial density and relatively large dendritic fields, and, therefore, the dendrites of each type overlap to a very large degree.

4.5 Conclusions

Based on their morphology and synaptic connections, CART-positive amacrine cells would be expected to depolarize in response to light stimuli in the scotopic range, as they do in cat retina [30]. The rod bipolar cells are likely to be the primary targets of the CART released by these amacrine cells. Based on work on rat hippocampal cells, one possible effect of CART is to block L-type voltage-gated Ca^{++} channels [43]. These channels are known to be present on the axon terminals of primate rod bipolar cells [44], and if they were blocked, direct signals from rods would not be transmitted to third-order neurons.

Acknowledgements

This project used biological materials funded by the Office of Research Infrastructure Programs/OD P51 OD011133. We wish to thank Dr. Michael J. Kuhar of Emory University for valuable discussions. This work was supported by Grants EY06472 and EY10608 from the National Eye Institute, the UTHHealth BRAIN Initiative and CTSA UL1 TR000371. Grant support: The funding agencies had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report or in the decision to submit the article for publication.

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Highlights

- Cocaine- and Amphetamine-Regulated Transcript (CART) was localized in monkey retina
- The neuropeptide was expressed in two very similar types of GABAergic local circuit neurons
- These amacrine cells received excitatory input from rod bipolar cells and also inhibited them
- They resembled A17, waterfall and serotonin-accumulating cells in other mammals
- Primate retinal ganglion cells do not contain CART, unlike those in rodents

Figure 1.

CART antibody labeled a subset of amacrine cells (red) with somas in the INL of baboon retina. Blue is DAPI. **A**, CART-positive dendrites (arrowheads) were found throughout the IPL (projection of 4 0.5 μm optical sections). **B**, Labeled dendrites were most common in stratum 5 close to the GCL. Note that there were relatively few labeled somas (arrows) in the GCL (projection of 10 0.5 μm optical sections). Scale bar = 50 μm .

Figure 2. Distribution CART-positive varicosities vs. depth in the baboon IPL; INL = 0, GCL = 100. The parameters of these distributions were estimated using an expected-maximization algorithm (see Methods 2.7). Varicosities were found throughout the IPL at a low density (red) but the peak (green) was in S5.

Figure 3. Localization of CART mRNA by in situ hybridization in the baboon retina. **A**, Hematoxylin & eosin-stained section of the central retina. **B**, In a corresponding region, somas expressing CART mRNA (red) were found in the innermost row of the INL, and one CART positive cell was found in the outermost row of the GCL. Scale bars = 5 μ m.

Figure 4.

CART does not colocalize with either choline acetyltransferase (ChAT) or RNA-binding protein with multiple splicing (RBPMS) in baboon retina. **A**, A single optical section in the inner nuclear layer (INL) labeled with antibodies to CART (red), RBPMS (green), and ChAT (cyan). **B**, The same image as in A, with ChAT signal removed (hollow arrowheads). **C**, A single optical section in the ganglion cell layer (GCL) labeled with the same antibodies. **D**, The same image as in C, with CART signal removed (filled arrowheads). Nuclei are labeled with DAPI (blue). Scale bar = 20 μm.

Figure 5. CART-immunoreactive soma areas in the far peripheral baboon retina. The distribution of CART-positive soma areas was fitted by two normal distributions based on previous studies with the Golgi method [3]; the parameters of these distributions were estimated using an expected-maximization algorithm (see Methods 2.7).

Figure 6.

Analysis of large and small CART-positive soma areas in far peripheral baboon retina using the density recovery profile. **A and B** Show the positions of the labeled somas whose areas are plotted in Fig. 5. The larger symbols indicate the large somas and the smaller symbols, the small somas. Scale bar = 200 μm . **C and D** show the results of the analysis. Based on the small effective radii, the spatial distributions of each type appear to be random.

Figure 7. CART positive (red) amacrine cells contain immunoreactive GABA (green) in baboon retina (z-axis projections of 3 optical sections). **A**, CART antibody labeled a subset of amacrine cell somas (arrows) in the INL and their dendrites in the IPL. **B**, GABA antibody labeled amacrine cells in the INL and in the GCL and their processes in the IPL. **C**, The merged image shows CART-positive cells that were also labeled by GABA antibody. Scale bar = 10 μm .

Figure 8.

CART-positive dendrites make synapses onto rod bipolar cell axon terminals in baboon retina. **A**, A single optical section in flat mount retina. CART positive dendrites (red) form a dense plexus in S5 and surround the axon terminals of rod bipolar cells labeled with antibody to PKC (green). CART-positive dendrites surround the axon terminals of labeled rod bipolar cells (arrows). Scale bar = 10 μ m. **B**, Triple labeling with CART, PKC, and SV2A antibodies in a single, vertical optical section of baboon retina. CART-positive cells (red) interact with axon terminals of rod bipolar cells labeled with antibody to PKC (green).

Puncta containing SV2A (blue) are found at several of these contacts (arrows). Scale bar = 5 μ m.

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Figure 9.

Rod bipolar cells make two types of contacts with the dendrites of CART-positive cells in baboon retina. Rod bipolar cells labeled with anti-PKC α (green) contact CART-positive dendrites (red) both at their axons (*en passant* synapses; B) and axon terminals (C). **A**, Z-axis maximum intensity projection of a stack of 6 optical sections. Retinal layers of interest are indicated: inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL). Filled arrowhead indicates an *en passant* contact; hollow arrowheads indicate contacts at the axon terminals. Scale bar = 10 μ m. **B**, Single optical section in which the *en passant* contact (filled arrowhead) has been enlarged. Scale bar = 5 μ m. **C**, Single optical section showing the axon terminal contacts (hollow arrowheads) at higher magnification. Scale bar = 5 μ m. Nuclei are visualized using DAPI (blue).

Figure 10.

Dopaminergic amacrine cells make synapses onto the soma and primary dendrites of CART-positive amacrine cells in baboon retina. **A**, Puncta containing SV2 (arrow, blue) are closely associated with the CART-positive soma and dendrites (red). **B**, The same area with dopaminergic processes labeled with antibody to tyrosine hydroxylase (green). **A** and **B** are z-axis projections from three optical sections. **C-E**, Single 0.5 μm optical sections showing that the three markers are colocalized (white). Scale bar = 5 μm .

Figure 11.

Bipolar cells and amacrine cells provided equal numbers of inputs to CART-positive amacrine cells in baboon retina. **A**, The axon terminals of rod bipolar cells (RB) typically made ribbon synapses (white arrowhead) like these onto the CART-positive dendrites in S5. Virtually all of the output synapses (black arrowheads) were onto bipolar cell axon terminals, often from the same cells that had provided their input. **B**, There were also instances where the axon, itself (B), made atypical ribbon synapses (white arrowheads) onto the labeled dendrites. **C**, The primary dendrites of CART-positive cells received large synapses (black arrowheads) from amacrine cells (A) in S1, but most amacrine cell synapses were found in S5.

Figure 12.

Intracellularly injected wide-field diffuse cells in flat mount preparations. The cells can be distinguished from one another by the diameters of their dendritic arbors. Scale bars = 200 μm. Figures A and B are montages made by arranging several high power z-axis projection images. Insets show varicosities along the lengths of dendrites (scale bars = 20 μm). **A**, A large cell from macaque retina with dendritic field diameter of > 3 mm. The proximal dendrites stratified in S1, and the distal dendrites stratified in S5. There are also four other, incompletely labeled cells. **B**, A small cell from baboon retina with a dendritic field diameter of approximately 800 μm. The distal dendrites stratified in S5. **C**, Another injected large cell from macaque retina (single optical section) is CART-positive (red). Scale bar = 10 μm.

Table 1

Primary antibodies used in this study.

Antibody	Antigen	Manufacturer, species, catalog number	Dilution used
CART (55-102) (Rat, Mouse, Bovine), polyclonal	Aa55-102 of the rat, mouse, bovine CART peptide	Phoenix Pharmaceuticals, Burlingame, CA, Catalog No. H-003-62, Rabbit	1:1,000 – 1:5,000
Human/Mouse/Rat CART (aa 28-116), Polyclonal	<i>E. coli</i> -derived recombinant human CART. Gln28-Leu116 Accession Number Q16568	R&D Systems, Inc., Minneapolis, MN, Catalog No. AF163, Goat	1:200 – 1:2,000
Choline acetyltransferase (ChAT)	Human placental ChAT	Chemicon, Billerica, MA, AB144P, lot 452882, Goat	1:200
GABA	gamma-amino butyric acid (GABA)	Gift from Prof. David V. Pow, RMIT University, Melbourne, Australia, Rat	1:500 – 1:5,000
Glycine Transporter I, polyclonal	Rat glial glyt-1, aa 614-633	Biogenesis, Brentwood, NH, Catalog No. 4710-8050, Goat	1:1000
Anti-Protein Kinase C (PKC) Antibody, monoclonal	Clone MC5, purified bovine brain protein kinase C	SIGMA-ALDRICH, St. Luis, MO, Catalog No. P5704, Mouse	1:100 – 1:1,000
Anti-Protein Kinase C α Antibody, polyclonal	Synthetic peptide, aa 659-672 of C-terminal V5 region of rat PKC α	SIGMA-ALDRICH, St. Luis, MO, Catalog No. P4334, Rabbit	1:500 – 1:1,000
Anti-Tyrosine Hydroxylase, polyclonal	Native tyrosine hydroxylase from rat pheochromocytoma (J. Biol. Chem., 1982, 257:9416-9423).	Chemicon International, Inc., Temecula, CA, Catalog No. AB1542, Sheep	1:1,000
SV2, polyclonal	Synthetic peptide aa 2 - 17 of human SV2 A	Synaptic Systems GmbH, Goettingen, Guinea pig, Cat. No. 119 004	1:500
SV2, monoclonal	Synaptic vesicle glycoprotein 2A; synaptic vesicles	Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, Mouse	1:500 – 1:1,000
Anti-RBPMS, polyclonal	Synthetic peptide corresponding to amino acid residues from the N-terminal region of the rat RBPMS sequence	PhosphoSolutions, Aurora, CO, 1832-RBPMS, Guinea Pig	1:500