Peptides 84 (2016) 22–35

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Wide-field diffuse amacrine cells in the monkey retina contain immunoreactive Cocaine- and Amphetamine-Regulated Transcript (CART)

Ye Long[a,1], Andrea S. Bordt[a,1], Weiley S. Liu[a], Elizabeth P. Davis[a], Stephen J. Lee[a], Luke Tseng[a], Alice Z. Chuang[b], Christopher M. Whitaker[b], Stephen C. Massey[b], Michael B. Sherman[c], David W. Marshak[a,b,*]

[a] Department of Neurobiology and Anatomy, McGovern Medical School, Houston, TX, United States
[b] Department of Ophthalmology and Visual Science, McGovern Medical School, Houston, TX, United States
[c] Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, United States

ARTICLE INFO

Article history:
Received 17 June 2016
Received in revised form 19 July 2016
Accepted 22 August 2016
Available online 25 August 2016

Keywords:
Neuropeptide
Primate
Rod bipolar cell
Reciprocal synapse
GABA

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 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.

1. Introduction

Cocaine- and Amphetamine-Regulated Transcript (CART) 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 vit-

* Corresponding author at: Department of Neurobiology and Anatomy, McGovern Medical School, P.O. Box 20708, Houston, TX, United States.
E-mail address: david.w.marshak@uth.tmc.edu (D.W. Marshak).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.peptides.2016.08.007
0196-9781/

reous 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 h at 20 °C before fixation or injection. For immunolabeling, the tissue was immersion fixed in 0.1 M phosphate buffer (PB) pH 7.4 containing 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for times ranging from 30 min to overnight at either room temperature (0.5–1 h) 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 min with 1% sodium borohydride (Sigma-Aldrich) in 0.01 M phosphate buffered saline (PBS, Sigma-Aldrich).

For frozen sections, fixed primate retinas were incubated in 25–30% sucrose (Sigma-Aldrich) for more than 24 h 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 μm 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 0.01 M phosphate-buffered saline with or without 0.3% sodium azide (PBSa), and then were cut into 30–100 μm 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 h 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 45 min at room temperature: (1) Preincubation of 1:5000 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 0.01 M 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. ×100), and (2) 1:5000 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 h 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. 40× or 63× 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 20× 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 min. The tissue was treated 1 h 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 1:1000 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 min. The tissue was treated with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M PB pH7.4 for 60 min 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 min followed by 0.2% aqueous lead citrate [10] for 10–15 min. Labeled processes were imaged at 5000× 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