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Research Article

Immunocytochemical localization of cholinergic amacrine cells in the bat retina

Eun-Bee Park, Ya-Nan Gu, Chang-Jin Jeon*

Department of Biology, School of Life Sciences, BK21 Plus KNU Creative BioResearch Group, College of Natural Sciences, and Brain Science and Engineering Institute, Kyungpook National University, Daegu, 41566, South Korea

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ABSTRACT

The purpose of this study was to localize the cholinergic amacrine cells, one of the key elements of a functional retina, in the retina of a microbat, *Rhinolophus ferrumequinum*. The presence and localization of choline acetyltransferase-immunoreactive (ChAT-IR) cells in the microbat retina were investigated using immunocytochemistry, confocal microscopy, and quantitative analysis. These ChAT-IR cells were present in the ganglion cell layer (GCL) and inner part of the inner nuclear layer (INL), as previously reported in various animals. However, the bat retina also contained some ChAT-IR cells in the outer part of the INL. The dendrites of these cells extended into the outer plexiform layer, and those of the cells in the inner INL extended within the outer part of the inner plexiform layer (IPL). The dendrites of the ChAT-IR cells in the GCL extended into the middle of the IPL and some fibers ramified up to the outer IPL. The average densities of ChAT-IR cells in the GCL, inner INL, and outer INL were 259 ± 31 cells/mm^2, 469 ± 48 cells/mm^2, and 59 ± 8 cells/mm^2, respectively. The average total density of the ChAT-IR cells was 788 ± 58 cells/mm^2 (mean ± S.D.; n = 3; 2799 ± 182 cells/retina). We also found that the cholinergic amacrine cells in the bat retina contained calbindin, one of the calcium-binding proteins, but not calretinin or parvalbumin. As the cholinergic amacrine cells play key roles in the direction selectivity and optokinetic eye reflex in the other mammalian retinas, the present study might provide better information of the cytoarchitecture of bat retina and the basic sources for further physiological studies.

1. Introduction

Bats are the only mammalian species with true and sustained flying ability; they represent approximately one fifth to one quarter of all mammals (Fenton and Simmons, 2015). Bats are divided into two suborders based on their size: *Megachiroptera* (megabats) and *Microchiroptera* (microbats) (Tudge, 2000). Megabats generally have large eyes with excellent vision and navigate using their sight, while the microbats are known to rely on echolocation rather than visual abilities to monitor their surroundings, navigate, and locate their prey (Altringham, 2011; Jones and Rayner, 1989). However, microbats still have functional eyes that are capable of ultraviolet and scotopic vision (Müller et al., 2009; Eklöf, 2003; Winter et al., 2003). Many investigations have been focused on the microbat retina, but the functional structure of their retina and visual abilities are still not well understood.

The microbat, greater horseshoe bat (*Rhinolophus ferrumequinum*), is mainly cave-dwelling, insectivorous, and typically a nocturnal species with tiny eyes like other microbats (Fenton and Simmons, 2015; Ransome and Hutson, 2000). We showed in previous studies that *Rhinolophus ferrumequinum* retina has AII amacrine cells (Jeon et al., 2007) and M/L cone photoreceptors (Kim et al., 2008). Other studies have reported the presence of ON and OFF cone bipolar cells (Butz et al., 2015), rod bipolar cells (Müller et al., 2013), rod and S cone photoreceptors (Feller et al., 2009; Müller et al., 2009), and retinal neurotransmitters such as gamma-aminobutyric acid (GABA), substance P, and serotonin (Studholme et al., 1987) in the microbat retina. These studies show that microbats have functional eyes with a well-organized neural circuit.

Amacrine cells of the mammalian retina are classified into around 30 different types; they form multiple synapses with bipolar cells and ganglion cells, and synapse with each other (Masland, 2012). Although the specific functions of the different types of amacrine cells have not yet been fully identified, many researches have focused on the functional and anatomical aspects of the AII and starburst amacrine cells. Starburst amacrine cells, the second most numerous amacrine cell type in the retina, are cholinergic neurons releasing two neurotransmitters, the inhibitory neurotransmitter GABA and the excitatory neurotransmitter acetylcholine (ACh); thus, they transmit both inhibi-

* Corresponding author at: Neuroscience Lab., Department of Biology, College of Natural Sciences, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu, 41566, South Korea.
E-mail address: cjjeon@knu.ac.kr (C.-J. Jeon).

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tory and excitatory signals to the postsynaptic neurons (Taylor and Smith, 2012; Wei and Feller, 2011; Briggman et al., 2011; Zhou and Lee, 2008; Demb, 2007; Masland, 2005; Famiglietti, 1983b). Cholinergic starburst amacrine cells are known to play key roles in direction selectivity and optokinetic eye reflex of a moving stimulus in the retina (Ding et al., 2016; Taylor and Smith, 2012; Vaney et al., 2012; Wei and Feller, 2011; Briggman et al., 2011; Zhou and Lee, 2008; Masland, 2005; Yoshida et al., 2001; Rodieck, 1998). Starburst amacrine cells have been found in the retina of various species including rabbits (Lee and Jeon, 2013; Famiglietti and Tumosa, 1987; Famiglietti, 1983a), cats (Schmidt et al., 1985), rats (Gábriel and Witkovsky, 1998; Voigt, 1986), mice (Kang et al., 2004; Haverkamp and Wässle, 2000; Jeon et al., 1998), primates (Moritoh et al., 2013; Rodieck and Marshak, 1992; Mariani and Hersh, 1988), tree shrews (Sandmann et al., 1997), ground squirrels (Cuenca et al., 2003, 2002), chicken (Spira et al., 1987; Millar et al., 1987, 1985), turtles (Nguyen et al., 2000), and dogfish (Brandon, 1991).

Calcium-binding proteins (CBPs) are one of important proteins in calcium-mediated neurotransmissions of the nervous system. Cholinergic starburst amacrine cells have also been confirmed to release neurotransmitters GABA and ACh via a calcium-dependent mechanism (Zheng et al., 2004). Distribution of CBPs has been well-studied in the retina of many different species (Haverkamp and Wässle, 2000; Pochet et al., 1991; Pasteels et al., 1990), and CBPs have been used as specific markers of specific subpopulations of neurons. The functions of various CBPs in the retinal neurons are yet to be defined; however, the types of CBPs present in the cholinergic amacrine cells have been revealed in previous studies and showed variation among different species (Lee and Jeon, 2013; Morona et al., 2011; Cuenca et al., 2003, 2002; Gábriel and Witkovsky, 1998).

To date, although two cholinergic populations in the microbat *Carollia perspicillata* retina have been shown in a previous study (Müller et al., 2013), cholinergic amacrine cells, one of the most important elements of the visual function of the retina, have not been described in detail in the bat retina. Therefore, the primary aim of this study was to investigate the localization and distribution of cholinergic amacrine cells in the retina of *Rhinolophus ferrumequinum*, a species of nocturnal microbats. Our study will further the knowledge of neuronal cytoarchitecture of the bat retina as a foundation for future studies to understand the mechanisms of bat vision.

2. Materials and methods

2.1. Animal and tissue preparation

Adult greater horseshoe bats (*Rhinolophus ferrumequinum*, mixed sex, weighing 15–20 g) were deeply anesthetized with a mixture of ketamine hydrochloride (30–40 mg/kg) and xylazine (3–6 mg/kg). Proparacaine HCl (100–200 μl) was applied to the cornea to suppress the blink reflex. The eyes (axial length, 1.8–1.9 mm) were immediately enucleated and the retinas were isolated in the 0.1 M phosphate buffer (PB, pH 7.4). The isolated retinas were fixed for 2 h at 4 °C with a solution containing 4% paraformaldehyde in 0.1 M PB (pH 7.4). After three rinses of 10 min each in 0.1 M PB, the retinal tissues were processed as whole mounts and 50 μm-thick vertical sections using a vibratome. The animals were euthanized by administering an overdose of the same anesthetics. All investigations involving animals conformed to the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. This research was approved (permission no. 2016-151) by the Institutional Animal Care and Use Committee of Kyungpook National University, Daegu, South Korea.

2.2. Immunocytochemistry

A polyclonal antibody against choline acetyltransferase (ChAT) (goat anti-ChAT, 1:500; Catalog #AB144P, Millipore, Temecula, CA, USA) was used as the primary antibody. The primary antiserum was diluted to 1:1000–1:2000. For detection by immunofluorescence, the secondary antibody used was a Cy3-conjugated donkey anti-goat IgG antibody (1:250; Catalog #705-165-147, Jackson ImmunoResearch, West Grove, PA, USA). As a negative control, some sections were incubated in the same solution without the addition of the primary antibody and these control tissue sections showed no ChAT immunoreactivity. Negative control test and preabsorption test for the specificity of this antibody conducted in the nervous system of the microbat have been described in a previous report (Kruger et al., 2010). For comparison, retinal tissues from adult mice were used in the same process. The methods for the standard immunohistological techniques performed in the bat and mouse retinas have been described in detail in one of our previous reports (Jeon et al., 1998). For double-labeling experiments with CBPs, monoclonal mouse anti-calbindin-D-28K (1:500; Catalog #C9848, Sigma Chemical, St. Louis, MO, USA), monoclonal mouse anti-calretinin (1:500; Catalog #MAB1568, Millipore), and monoclonal mouse anti-parvalbumin (1:500; Catalog #P3088, Sigma Chemical) were used as the primary antibodies. Fluorescein isothiocyanate (FITC) anti-mouse (1:200; Catalog #FI-2000, Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody. Immunofluorescence images were obtained using a laser-scanning confocal microscope (LSM 700; Carl Zeiss, Jena, Germany). To identify the retinal layers, the nuclei of the retina were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000; Catalog #0100-20, SouthernBiotech, Birmingham, AL, USA) for 15 min. The tissues were also examined and photographed on a Zeiss Axioplan microscope (Carl Zeiss) using differential interference contrast (DIC) optics to recognize the layers.

2.3. Quantitative analysis

Three Cy3-immunostained whole mount retinas were used to determine the density of ChAT-immunoreactive (ChAT-IR) cells in the GCL, inner INL, and outer INL of the bat retina. These ChAT-IR cells were viewed using a Zeiss Plan-Apochromat 63× objective (Carl Zeiss) along the central dorsoventral and nasotemporal axes passing through the optic nerve head. To count the ChAT-IR cells, 16–17 sample areas of 200 × 200 μm^2 were selected at evenly distributed positions across the retina. A total of 50 sample areas from the three retinas were analyzed. The cell density was expressed as the number of ChAT-IR cells/mm^2 of each retinal layer. The cell density was multiplied by the retinal area to determine the total number of cells. The topographical distribution of ChAT-IR cells was mapped by additional cell counting through one Cy3-immunostained whole mount retina. The density distribution map was coded by different-sized dots to clarify the densities of the ChAT-IR cell populations in the bat retina. The density values were as the number of cells/mm^2.

3. Results

3.1. ChAT-IR cells in the bat retina

DAPI-stained 50 μm-thick vertical sections of the adult microbat retina showed that ChAT-IR cells were expressed both in the GCL and the INL (Fig. 1A–C). For comparison, the same immunocytochemical process was performed on 50 μm-thick vertical sections of the adult mouse retina (Fig. 1D–F). While ChAT-IR cells were expressed both in the GCL and INL in bat and mouse, some ChAT-IR cells were also expressed in the outer INL in the bat (Fig. 1A–C, arrowheads) but not in the mouse retina. An additional distinctive difference between mouse and bat retina was observed: while the ChAT-IR dendritic stratum of the mouse retina formed two distinct bands within the inner plexiform layer (IPL; Fig. 1E), the ChAT-IR dendritic stratum showed weak immunoreactivity in the bat retina (Fig. 1B).