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DENSITY AND TYPES OF CALRETININ-CONTAINING RETINAL GANGLION CELLS IN RABBIT

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Abstract—Single-cell injection with lipophilic dyes following immunocytochemistry is extremely valuable for revealing the morphology of a cell expressing a protein of interest, and is a more reliable technique for cell type classification than standard morphological techniques. This study focuses on calretinin (CR), which is used as a selective marker for distinct subpopulations of neurons in the rabbit retina. The present study used single-cell injection after immunocytochemistry to describe the density and types of CR-containing retinal ganglion cells (RGCs) in rabbit. The density of CR-immunoreactive cells in the rabbit RGC cell layer was 426 cells/mm^2. CR-containing RGCs were identified by immunocytochemistry and were then iontophoretically injected with a lipophilic dye, DiI. Subsequently, confocal microscope was used to characterize the morphology of CR-immunoreactive RGCs based on their dendritic field size, branching pattern, and stratification of the inner plexiform layer. Our results show that 10 morphologically different types of rabbit RGCs expressed CR. CR-containing RGCs were heterogeneous in their morphology. This approach to integrate the selective expression of a particular protein with spatial patterns of dendritic arborization will lead to a better understanding of RGCs.

Key words: density, types, retinal ganglion cells, single-cell injection, calretinin.

INTRODUCTION

In the retina, visual signals originating in the photoreceptors project to different recipient visual areas of the brain through different classes of retinal ganglion cells (RGCs). These different RGC types are distinguished from one another by several morphometric parameters (e.g., dendritic morphology, dendritic thickness, and stratum of dendritic ramification).

Dendrites receive signals and process synaptic inputs. Dendrites also exhibit enormously diverse forms, but the organization of their arbors is not random (Wong and Ghosh, 2002). Since the shape of the dendritic arbor affects the connectivity of the neuron, many studies have focused on dendritic morphology and ramification depth in an attempt to understand the neuron's functional roles. Indeed, the functions of some RGCs with specific dendritic branching patterns have been identified. Therefore, cells with distinct shapes likely have distinct physiological functions (Masland, 2001a,b). For example, the dendritic arbors of direction-selective RGCs curve back toward the cell body, giving a uniform honeycomb-like appearance (Yang and Masland, 1994). Intrinsically photosensitive RGCs exhibit a unique morphology featuring a sparse dendritic tree (Berson et al., 2002). Thus, morphological analyses of RGC dendrites have been extensively performed using a variety of methods in a number of species (Kolb et al., 1981, 1992; Rockhill et al., 2002; Sun et al., 2002; Badea and Nathans, 2004; Kong et al., 2005; Coombs et al., 2006; Völgyi et al., 2009).

The Rockhill group identified 11 anatomical types of rabbit RGCs using several different methods (Rockhill et al., 2002). The Roska group physiologically classified RGCs in the rabbit by measuring spatio-temporal responses, and then associated these types with specific anatomical types having specific dendritic morphology and stratification in the inner plexiform layer (IPL) (Roska et al., 2006). Since then, identified RGCs have been characterized in more detail, and novel RGC types that were not previously found in rabbit retina have been elucidated including S/M ganglion cells (Hoshi and Mills, 2009; Sivyer et al., 2010; Hoshi et al., 2011; Sivyer et al., 2011; Hoshi et al., 2013; Mills et al., 2014).

Particle-mediated transfer of lipophilic dyes, microinjection of Lucifer yellow, and other techniques can reveal RGC dendritic structures. However, existing methods have only focused on identifying neuronal morphology. Use of transgenic strains expressing green fluorescent protein is also a good method for revealing the morphology of neurons by expression of a specific protein, although the method is limited if the density of cells expressing the protein of interest in the retinal ganglion cell layer (GCL) is very high. In contrast to these

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Abbreviations: CBPs, calcium-binding proteins; CR, calretinin; FITC, fluorescein isothiocyanate; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IR, immunoreactive; RGCs, retinal ganglion cells.

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methods, single-cell injection of lipophilic dye following immunocytochemistry is a systematic and innovative approach to correlate the morphology of each RGC type with the expression of a specific protein. Therefore, we used single-cell injection after immunocytochemistry to identify each RGC type with its expression of a particular protein, resulting in a detailed functional anatomy.

Calcium-binding proteins (CBPs) are well-known Ca^{2+} buffers controlling Ca^{2+} concentration in the cytosol. CBPs are also involved in numerous cellular functions as Ca^{2+} transporters or as Ca^{2+}-modulated sensors (Schwaller, 2009, 2010). In addition, CBPs are present in distinct subgroups of neurons in CNS, thus making them very useful anatomical markers for these subgroups. Calretinin (CR), a CBP, has a species-, tissue-, and cell-type-specific expression. Many papers have focused on characterizing these patterns and revealing their function in the nervous system. Although the physiological roles of CR have not been established, considerable attention has been focused on its localization within the retina. CR antibodies label amacrine cells, including AII amacrine cells, cone bipolar cells, and numerous cell bodies in the GCL of the rabbit retina (Völgyi et al., 1997; Jeon and Jeon, 1998; Kwon et al., 2005).

The goal of the present study was to determine whether CR is specifically expressed in certain neuronal RGC populations in rabbits using single-cell injection following immunocytochemistry. So far, no attempt has been made to estimate the density of CR-expressing RGCs in rabbit, which was performed for the first time in this study.

EXPERIMENTAL PROCEDURES

Animal procedures

Adult New Zealand white rabbits (mixed sex; 2.5–3 kg, $n = 40$) obtained from Dae-Han Biolink (Eumseong, Korea) were used for experiments. Rabbits were 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 blink reflexes. Eyes were quickly enucleated after a reference point was noted to label the superior pole and immersed in 0.1 M phosphate buffer (pH 7.4). Animals were euthanized by an overdose of the same anesthetics. All investigations involving animals were performed in accordance with Association for Research in Vision and Ophthalmology (ARVO) guidelines and were approved by the committee of Kyungpook National University.

Immunocytochemistry

Immediately after enucleation, retinas were carefully isolated from the eyecup in 0.1 M phosphate buffer and mounted ganglion-side-up on a black, non-fluorescent filter membrane (HABP; Millipore, Bedford, MA, USA). The filter membrane with the attached retina was fixed in 4% paraformaldehyde for 30 min. Retinal tissues were processed as whole mounts and cut into 50-μm-thick vertical sections using a Vibratome 3000 Plus Sectioning System (Vibratome, St. Louis, MO, USA). Standard immunocytochemical methods were used (described in detail in our previous report; Jeon and Jeon, 1998). For immunocytochemistry, tissues were incubated with rabbit antibody against CR (1:250; cat# AB5054; Millipore) for 2 h. Following three rinses in ice-cold 0.1 M phosphate buffer, tissues were incubated with fluorescein isothiocyanate (FITC)-conjugated horse anti-rabbit IgG (1:50; Vector Lab., Burlingame, CA, USA) for 1–2 h. However, for single-cell injection following immunocytochemistry, permeabilizing detergents were not used. The original paper by Kao and Sterling (2003) used a non-ionizing detergent (Tween 20) to facilitate entry of antibodies. Although Tween 20 is a relatively mild membrane solubilizer, we performed our present study without permeabilizing detergents to minimize leakage of DiI and to preserve the morphology of the injected cells. Although no detergents were used, the antibody against CR adequately labeled cells in the GCL in whole mounts.

Cell injection

The procedures for single-cell injection following immunocytochemistry have been described in detail in our previous reports (Kim and Jeon, 2006; Lee et al., 2010, 2013). The dish containing the immunolabeled retina was placed on a microscope stage, and FITC-labeled neurons were viewed with a Zeiss 40× Plan Achroplan (NA 0.80) water immersion lens, using a 100 W mercury source and Zeiss filter set 09 (excitation, 450–490 nm; emission, 515 nm). Single cells in the retinal GCL were selected using an indexed grid reticle, which referred to randomly generated grid coordinates. The stage was repositioned so that the randomly selected cell was in the center of the field of view. Cells were filled iontophoretically by passing a positive current of 5–20 nA through a micropipette containing a 1% solution of the lipophilic dye, DiI (Molecular Probes, Eugene, OR, USA). DiI-filled cells were viewed using Zeiss filter set 20 (excitation, 540–552 nm; emission, 575–640 nm) and the optimal filling time was usually 15–30 min. After the selected cell was completely filled with DiI, we systematically moved the grid reticle 350 μm laterally and injected another cell at the center of the grid. We only injected cells in the mid-periphery of the ventral retina, a zone 1.0–1.7 mm inferior to the optic disk, as dendritic field size varies according to retinal eccentricity. After several cells in the retina were filled, the tissue was rinsed briefly in 0.1 M phosphate buffer, and fixed for 2 h in 4% paraformaldehyde. To stain all cells, the retina was immersed for 1 h in a nucleic acid dye, SYTO13 (10–20 nM; Molecular Probes), in 0.1 M phosphate buffer, washed 3 times in 0.1 M phosphate buffer, and then cover slipped with Vectashield (Vector Lab.), and sealed with nail polish.

Data analysis

Cells were imaged using a Bio-Rad MRC 1024 laser scanning confocal microscope with a Nikon Plan Fluor 40× objective (NA 0.75) for small cells, and a Nikon Plan Fluor 20× objective (NA 0.50) for large cells, to