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Identification of parvalbumin-containing retinal ganglion cells in rabbit

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ABSTRACT

A calcium-binding protein, parvalbumin (PV), is widely distributed in the central nervous system and is expressed in the retinal neurons of various vertebrate species. The present study was aimed at describing the types and density of PV-containing retinal ganglion cells (RGCs) in rabbits by using single-cell injection after immunocytochemistry. PV-containing RGCs were first identified by immunocytochemistry and were then iontophoretically injected with a lipophilic dye, DiI. Subsequently, confocal microscopy was used to characterize the morphological classification of the PV-immunoreactive (IR) ganglion cells on the basis of their dendritic field size, branching pattern, and stratification within the inner plexiform layer. The results indicated that at least 8 morphologically different types of rabbit RGCs express PV. They were heterogeneous in terms of their morphology. The present study showed that the proportion of RGCs that contained PV was between 17% and 19% of the total number of ganglion cells. The density of PV-IR RGCs in the rabbit retina was 144 cells/mm^2. Also, it was found that PV was present in all cholinergic amacrine cells in the ganglion cell layer (GCL) and the inner nuclear layer (INL). This integrated approach of characterizing the cell morphology and the selective expression of a particular protein will lead to a better understanding of the properties of RGCs.

1. Introduction

The morphological and physiological analyses of retinal ganglion cells (RGCs) are essential steps in the comprehension of the visual system as the RGCs collectively transmit visual information from the neural retina to the visual processing zone in the brain (Rodieck, 1998). As the rabbit retina is a more accessible retina in terms of ease of isolation, staining and recordings than some other mammalian retinas, it has been intensively studied. For this reason, quantitative studies of properties including cell density and distribution of RGCs have already been accomplished in rabbits (Oyster et al., 1981; Provis, 1979; Vaney and Hughes, 1976). The physiological types, as determined through analysis of the receptive field of RGCs and its anatomical types have also been the subject of many advanced studies in rabbits (Amthor et al., 1984, 1989a, 1989b; Barlow et al., 1964; Caldwell and Daw, 1978; Devries and Baylor, 1997; Famiglietti, 2004; Levick, 1967; Roska and Werblin, 2001; Sivyer et al., 2011; Vaney et al., 1981). Rockhill et al. (2002) scrupulously identified 11 different types in the entire rabbit RGC population with four different mechanistic bases: photofilling, microinjection, gene encoding of a green fluorescent protein, and particle-mediated introduction of DiI.

The approaches that aim to identify the selective expression of a protein in combination with the neuronal structure will be extremely valuable compared to the strict morphological approaches, as different proteins confer different physiological properties. We previously developed a systematic approach of single-cell injection of lipophilic dye following immunocytochemistry to correlate the morphology of each type of RGC with its expression of a specific protein. This method provided the means to identify each RGC type along with a detailed functional anatomy of each RGC with its expression of a particular protein (Kim and Jeon, 2006; Lee et al., 2010).

Intracellular free calcium ions (Ca^{2+}) are a significant element in RGC responses. Parvalbumin (PV), one of the intracellular Ca^{2+} binding buffers, is the critical determinants of the kinetic of fluctuations in intracellular Ca^{2+} concentration (Baimbridge et al., 1992). Although the physiological roles of PV in the retina have not been well established, considerable attention has been focused on its localization within neural tissues. PV is a good marker of AII amacrine cells in rabbit, rat, and bat retinas (Casini et al., 1995; Jeon et al., 2007; Wässle et al., 1993). PV is also expressed in subpopulations of ganglion, amacrine, bipolar, and horizontal cells in

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vertebrate retinas, though there is some degree of species-specific variation (Casini et al., 1998; Haverkamp and Wässle, 2000; Sanna et al., 1993).

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

2. Materials and methods

2.1. Animal procedures

Adult New Zealand white rabbits (mixed sex; 2.5–3 kg, $n = 25$) were used for these experiments. The rabbits were obtained from a local vendor. The rabbits were intramuscularly anesthetized with a mixture of ketamine hydrochloride (30–40 mg/kg) and xylazine (3–6 mg/kg). Proparacaine HCl (200–300 ul) was applied to the cornea to suppress blink reflexes. Each eye was enucleated and they were immersed in 0.1 M phosphate buffer (pH 7.4). The animals were euthanatized by an overdose of the same anesthetics. The experiments were carried out in accordance with the Association for Research in Vision and Ophthalmology (ARVO) guidelines for the use of animals with the approval of the committee of Kyungpook National University.

2.2. Antibody characterization

The primary antibodies used in this study are described in Table 1.

(1) A monoclonal antibody against PV was generated using purified frog muscle PV as the immunogen. This antibody has been found to react with PV from human, bovine, goat, pig, rabbit, dog, cat, rat, frog, and fish. It does not react with other members of the EF-hand family of calcium-binding proteins. Immunoblotting shows a single band corresponding to 12 kD (information provided by manufacturer). The anti-PV-antibody labeled numerous cell bodies in the ganglion cell layer (GCL) and some horizontal and amacrine cells (Casini et al., 1995). Patterns of immunoreactivity in the present study matched those presented in previous reports on the retina (Kim and Jeon, 2006; Klump et al., 2009; Kwon et al., 2005).

(2) The rabbit polyclonal choline acetyltransferase (ChAT) antibody was raised against human placental ChAT. The specificity of this antibody has been demonstrated through Western blot analysis of human brain tissue, which showed single binding of 68 kD (Bruce et al., 1985). ChAT is a marker of cholinergic amacrine cells, which can be categorized into two subpopulations. One is found in the inner nuclear layer (INL) and the dendritic plexus, where it is confined to sublamina a. The other forms a displaced population in the GCL and the dendritic plexus, in which it is confined to sublamina b (Brandon, 1987; Famiglietti, 1983).

(3) We stained the intrinsically photosensitive retinal ganglion cells (ipRGCs) of the rabbit retina with a commercial antibody to Connexin 45 (MAB3101; Millipore, Bedford, MA), which fortuitously stains not Connexin 45 puncta but ipRGCs (Hoshi et al., 2009; Kim et al., 2012) in the rabbit retina. ipRGC types have a unique morphology with sparse dendritic trees in the rabbit retina. Anti-Connexin 45 antibody was raised against amino acids 354–367 of human Connexin 45.

2.3. Immunocytochemistry

Immediately after enucleation, the retinas were carefully isolated from the eye cup and cut from the mid-periphery of the ventral retina, 6–10 mm inferior to the optic disk. Retinal pieces were mounted onto a non-fluorescent filter membrane (HABP; Millipore) with the GCL upper. The filter membrane and the attached retinas were fixed in 4% paraformaldehyde for 30 min. For the immunocytochemical study, the retinas were incubated in 1% sodium borohydride ($NaBH_4$) for 30 min. Standard immunocytochemical techniques and immunocytochemical methods that have been described in detail in our previous reports were used (Jeon and Jeon, 1998). The primary antibodies and their concentrations were as follows: mouse/rabbit anti-PV (1:2000, Sigma–Aldrich, Temecula, CA), rabbit anti-ChAT (1:250, Millipore) and rabbit anti-Connexin 45 (1:1000, Millipore). For detection by immunofluorescence, the secondary antibodies used were FITC/Cy3-conjugated horse anti-mouse IgG (1:100, Vector Laboratories, Burlingame, CA), and FITC/Cy5-conjugated goat anti-rabbit IgG (1:100, Jackson ImmunoResearch, West Grove, PA).

2.4. Cell injection

Previous reports give a detailed description of the procedure of single-cell injection following immunocytochemistry (Kim and Jeon, 2006; Lee et al., 2010). For single-cell injection following immunocytochemistry, we did not use any detergents such as Triton X-100, which will prevent the dye from spreading in the neuronal membrane (Kao and Sterling, 2003). The fixed tissues were incubated in a 1:200 dilution of the primary antiserum, a monoclonal antibody against PV (Sigma–Aldrich), in 0.1 M phosphate buffer for 2 h at 25 °C. After three rinses, the tissues were incubated in a 1:50 dilution of fluorescein (FITC)-conjugated horse anti-mouse immunoglobulin G (IgG) (Vector Laboratories) in 0.1 M phosphate buffer for 2 h. The immunolabeled retina was placed on a microscope stage, and the 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 GCL were

Table 1
Information of primary antibodies.

Antigen	Immunogen	Manufacturer	Dilution
Parvalbumin	Purified frog muscle parvalbumin	Sigma–Aldrich Mouse monoclonal (moue IgG1 isotype) is derived from the PARV-19 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunized mouse. #P3088	1:1000–1:2000
Choline acetyltransferase (ChAT)	Human placental ChAT	Millipore Rabbit polyclonal #AB143	1:250
Connexin 45	Amino acids 354–367 of human Connexin 45	Millipore Mouse monoclonal #MAB3101	1:1000