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Research article

Types and density of calbindin D28k-immunoreactive ganglion cells in mouse retina

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ABSTRACT

Single-cell injection after immunocytochemistry is a reliable technique for classifying neurons by their morphological structure and their expression of a particular protein. The aim of the present study was to classify the morphological types of calbindin D28k-immunoreactive retinal ganglion cells in the mouse using single-cell injection after immunocytochemistry, to estimate the density of calbindin D28k-immunoreactive retinal ganglion cells in the mouse retina. Calbindin D28k is an important calcium-binding protein that is widely expressed in the central nervous system. Calbindin D28k-immunoreactive retinal ganglion cells were identified by immunocytochemistry and then iontophoretically injected with the lipophilic dye, DiI. Subsequently, the injected cells were imaged by confocal microscopy to classify calbindin D28k-immunoreactive retinal ganglion cells based on their dendritic ramification depth within the inner plexiform layer, field size, and morphology. The cells were heterogeneous in morphology: monostratified or bistratified, with small to large dendritic field size and sparse to dense dendritic arbors. At least 10 different morphological types (CB1–CB10) of calbindin D28k-immunoreactive retinal ganglion cells were found in the mouse retina. The density of each cell type was quite variable (1.98–23.76%). The density of calbindin D28k-immunoreactive cells in the ganglion cell layer of the mouse retina was 562 cells/mm^2, 8.18% of calbindin D28k-immunoreactive cells were axon-less displaced amacrine cells, 91.82% were retinal ganglion cells, and approximately 18.17% of mouse retinal ganglion cells expressed calbindin D28k. The selective expression of calbindin D28k in cells with different morphologies may provide important data for further physiological studies of the mouse retina.

1. Introduction

Retinal ganglion cells (RGCs) are the sole portal for conveying visual information to higher visual centers, and therefore have gained the attention of investigators. Different RGC types are suggested to be involved in different visual information processes and the transmission of information to related areas of the brain (Sanes and Masland, 2015). In a strict sense, neurons should be classified by their distinct functions. However, it is difficult to perform functional classifications, as this information is still unknown for most neurons.

The morphology of the dendritic arbors of RGCs is diverse, and their organization is not random (Jan and Jan, 2010; Sanes and Masland, 2015; Wong and Ghosh, 2002). The branching pattern of dendritic arbors is also thought to affect the connectivity of RGCs (Jan and Jan, 2010). Many studies have shown that the dendrites of RGCs ramify in the ON sublamina and OFF sublamina of the inner plexiform layer (IPL) of the mammalian retina in response to ON stimuli or OFF stimuli, respectively (Bolz et al., 1984; Crooks and Kolb, 1992; DeVries and Baylor, 1995; Pourcho and Owczarzak, 1991; Soucy et al., 1998; Tsukamoto et al., 2001). Thus, the dendritic morphology and ramification depth have been widely used as classification parameters in many studies, in an attempt to understanding the functional roles of neurons. Indeed, the functions of some RGCs with a specific dendritic morphology have been identified. For example, direction-selective RGCs have a uniform honeycomb-like appearance in which the dendrites curve back toward the cell bodies (Yang and Masland, 1994). A sparse dendritic

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tree is one of the unique morphological features of intrinsically photosensitive RGCs (Berson et al., 2002). Therefore, cells with a particular morphology may have a distinct physiological function (Masland, 2001a, b). In view of this, morphological analyses of RGCs have been extensively performed using a variety of methods in a number of species (Badea and Nathans, 2004; Coombs et al., 2006; Kolb et al., 1992, 1981; Kong et al., 2005; Rockhill et al., 2002; Sun et al., 2002; Sümbül et al., 2014; Völgyi et al., 2009). Up until now, the structural classification of RGCs has been widely studied in the mouse retina. Fourteen types of RGCs have been identified in the mouse retina by DiI particle bombardment (Sun et al., 2002); twelve types of RGCs have been quantitatively analyzed using an alkaline phosphatase reporter (Badea and Nathans, 2004); fourteen RGC clusters have been classified by Coombs et al. (2006) and eleven RGC clusters have been classified by Kong et al. (2005) using the particle-mediated transfer of DiI, microinjection of Lucifer yellow, or visualization of green fluorescent protein; and 22 types of RGCs have been classified by genetic labeling (Sümbül et al., 2014).

Calcium-binding proteins (CBPs) modulate many aspects of calcium functioning by regulating and controlling intracellular calcium homeostasis. They may act as calcium-buffer proteins that simply buffer and transport calcium, or as calcium-sensing proteins that participate in calcium-dependent signal transduction (Baimbridge et al., 1992; Ikura, 1996). Among the many CBPs, calbindin D28k (CB), calretinin (CR), and parvalbumin (PV) are wildly used as distinct markers of neuronal subpopulations in the central nervous systems of various species because of their extensive and specific distributions (Andressen et al., 1993; Baimbridge et al., 1992; Celio, 1990; Whitney et al., 2008).

CB has been considered a marker of horizontal cells in the mouse retina. Localization of CB in the retina has been studied in approximately 20 different species, and CB-immunoreactive (IR) cells have been identified as cone photoreceptors, horizontal cells, bipolar cells, amacrine cells, and ganglion cells, with variation between species (Dalil-Thiney et al., 1994; Fosser et al., 2013; Lee and Jeon, 2013; Morona et al., 2011, 2008, 2007; Pasteels et al., 1990; Pochet et al., 1991; Kim et al., 2010).

Although the precise functional role of CB in the RGCs is yet to be established, there is evidence that CB acts as a calcium buffer in neurons (Airaksinen et al., 1997; Chard et al., 1993), and other findings have also revealed some calcium-sensing properties (Berggård et al., 2002; Schwaller, 2009). In the past two decades, many studies have demonstrated that CB is involved in important signal transduction processes such as the regulation of the behavioral and molecular response to light (Stadler et al., 2010), and possibly the regulation of the circadian clock in the suprachiasmatic nuclei (SCN) (Arvanitogiannis et al., 2000; Kriegsfeld et al., 2008; LeSauter et al., 2009, 1999; Stadler et al., 2010). It has also been suggested that CB is involved in the visual sensitivity to contrast and orientation, and the transfer of rod signals to the ON cone bipolar cells in the IPL (Kim et al., 2010).

As different proteins confer different physiological properties, identifying the neurons expressing a particular protein will be extremely valuable. However, it is difficult to classify neuronal morphology in detail using traditional immunocytochemical techniques, as many proteins are expressed only by the cell body. The absence of a dendritic localization for specific proteins makes it difficult to classify neurons on the basis of their characteristic morphology. Single-cell injection after immunocytochemistry is a recently developed method used to classify neurons by the morphological structure and the expression of a particular protein (Kao and Sterling, 2003; Kim and Jeon, 2006; Kwon et al., 2014; Lee et al., 2013, 2010).

In previous studies, we have identified the morphological types and distribution of CR- and PV-IR RGCs in mouse and rabbit retinas by single-cell injection after immunocytochemistry (Kim and Jeon, 2006; Kwon et al., 2014; Lee et al., 2010, 2013). The aim of the present study is to identify the morphological types of CB-IR RGCs and compare them with results obtained previously, and to estimate the density of CB-IR RGCs in the mouse retina.

2. Materials and methods

2.1. Animals and tissue processing

Adult mice (C57BL/6) were anesthetized using a mixture of ketamine hydrochloride (30–40 mg/kg) and xylazine (3–6 mg/kg). To suppress blink reflexes, we applied proparacaine HCl (100–200 μl) to the mouse cornea. After the superior pole was marked with a reference point, the eyes were quickly enucleated and immersed in 0.1 M phosphate buffer (PB, pH 7.4). Then, the animals were sacrificed using an overdose of the same anesthetics. Immediately after enucleation, the retinas were isolated in 0.1 M PB and mounted onto a black, nonfluorescent filter membrane (HABP; Millipore, Bedford, MA) with the ganglion cell layer (GCL) uppermost. The isolated retinas were then immersed in a solution containing 4% paraformaldehyde for 30 min. The fixed retinas were rinsed three times with 0.1 M PB for 10 min each rinse. All investigations involving animals conformed to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was also approved (permission NO. 2015-0104) by the animal rights committee at Kyungpook National University, Deagu, South Korea.

2.2. Horseradish peroxidase immunocytochemistry

The tissues were incubated in a 1:500–1:1000 dilution of rabbit anti-CB primary antibody (Sigma, St. Louis, MO) in 0.1 M PB for 3 days at 4 °C. After three rinses in 0.1 M PB, the tissues were incubated in a 1:200 dilution of biotinylated anti-rabbit IgG secondary antibody (Vector Labs., Burlingame, CA) in 0.1 M PB for 1 day at 4 °C. The tissues were then rinsed three times and incubated in avidin-biotin complex (ABC) complex reagent for 1 day at 4 °C. After incubation in the ABC reagent, the tissues were rinsed three times in 0.25 M Tris buffer and treated with 3, 3′-diaminobenzidine (DAB) for 2–5 min. The methods are described in detail in our previous report (Jeon et al., 1998).

2.3. Fluorescence immunocytochemistry

The tissues were incubated in a 1:500 dilution of the rabbit anti-CB primary antibody (Sigma) in 0.1 M PB for 1 days at 25 °C, and then incubated in a 1:200 dilution of fluorescein (FITC)-conjugated goat anti-rabbit IgG secondary antibody (Vector Labs.) in 0.1 M PB for 2 h at 25 °C after three rinses in 0.1 M PB.

2.4. Cell injection

The procedure for single-cell injection following immunocytochemistry on the whole-mount retina has been described in detail in previous reports (Kao and Sterling, 2003; Kim and Jeon, 2006). The immunocytochemistry before single-cell injection was performed without Triton X-100, because this detergent may influence the diffusion of the dye in the neuronal membrane. The fixed tissues were incubated in a 1:200 dilution of rabbit anti-CB (Sigma) primary antibody in 0.1 M PB for 2 h at room temperature, and then incubated in a 1:50 dilution of fluorescein (FITC)-conjugated goat anti-rabbit IgG secondary antibody (Vector Labs.) in 0.1 M PB for 2 h at room temperature after three rinses with 0.1 M PB. The