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Localization of melanopsin-immunoreactive cells in the Mongolian gerbil retina

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ABSTRACT

Melanopsin-expressing intrinsically photosensitive retinal ganglion cells (ipRGCs) are involved in circadian rhythm and pupil responses. The purpose of this study was to reveal the organization of melanopsin-immunoreactive (IR) neurons in the Mongolian gerbil retina using immunocytochemistry. Melanopsin-IR cells were primarily located in the ganglion cell layer (GCL; M1c; 75.15%). Many melanopsin-IR cells were also observed in the inner nuclear layer (INL; M1d; 22.28%). The M1c and M1d cell types extended their dendritic processes into the OFF sublayer of the inner plexiform layer (IPL). We rarely observed bistratified cells (M3; 2.56%) with dendrites in both the ON and OFF sublayers of the IPL. Surprisingly, we did not observe M2 cells which are well observed in other rodents. Melanopsin-IR cell somas were small to medium in size and had large dendritic fields. They had 2–5 primary dendrites that branched sparingly and had varicosities. Melanopsin-IR cell density was very low: they comprised 0.50% of the total ganglion cell population. Moreover, none of the melanopsin-IR cells expressed calbindin-D28K, calretinin, or parvalbumin. These results suggest that in the Mongolian gerbil, melanopsin-IR cells are expressed in a very small RGC subpopulation, and are independent of calcium-binding proteins-containing RGCs.

1. Introduction

Intrinsically photosensitive retinal ganglion cells (ipRGCs) are sensitive to light, and are considered a third class of photoreceptors separate from classic rods and cones (Berson et al., 2002; Warren et al., 2003; Cui et al., 2015). These ipRGCs project to several brain areas, such as the suprachiasmatic nucleus (SCN) and olivary pretectal nucleus (OPN), where they are involved in non-image forming vision. ipRGCs are involved in both circadian rhythm regulation and the pupillary light reflex (Berson et al., 2002; Hattar et al., 2002; Hankins et al., 2008). A recent study showed that these cells are also responsible for image-forming vision (Schmidt et al., 2011).

ipRGCs have been investigated for more than a decade, and research in this field has recently grown exponentially. ipRGCs in the mammalian retina comprise about 1–2% of total RGCs (Hattar et al., 2002; Berson et al., 2010). Various sophisticated studies elucidated the architecture of ipRGCs. Based on the stratification patterns of dendrites and cell bodies, multiple ipRGC subtypes were observed in various mammalian retinas, including mouse, rat, marmoset, and macaque (Dacey et al., 2005; Ecker et al., 2010; Hughes et al., 2013; Reifler et al., 2015).

The M1 type of ipRGCs has cell bodies in the ganglion cell layer (GCL), and its large and sparsely branched dendrites monostratify at the top of the OFF sublayer of the inner plexiform layer (IPL) (Viney et al., 2007; Schmidt et al., 2008). Conversely, the M2 type of ipRGCs has cell bodies in the GCL, and its dendrites stratify at the bottom of the ON sublayer of the IPL (Hattar et al., 2006; Schmidt and Kofuji, 2009). ipRGCs with dendrites that stratify in both the ON and OFF sublayers of the IPL are classified as the M3 type (Schmidt et al., 2008; Schmidt and Kofuji, 2011). In addition, recent studies identified additional ipRGC types, termed M4 and M5, which had cell bodies in the GCL and dendrites that stratified in the ON sublayer of the IPL. These cell types also stratify in the ON sublayer of the IPL; however, each cell type has a unique morphology and can be differentiated from M2 cells. The M4 and M5 types differ in that M4 has generously branched and larger dendritic fields than M5, which has small, bushy dendritic fields (Ecker et al., 2010).

Calcium-binding proteins (CBPs) are crucial modulators of calcium cell signaling pathways, and act as markers that help to

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distinguish different neuronal subpopulations. The different localization of CBPs among different subpopulations also aids in the understanding of diverse neurological disorders that occur when the distribution of CBPs is modified (Heizmann and Braun, 1992; Leuba et al., 1998; Reynolds et al., 2001). Although the precise function of CBPs remains unclear, significant attention has been focused on investigating CBP expression in the retina. Among the many CBPs, at least three EF-hand CBPs (calbindin-D28K, calretinin, and parvalbumin) have been extensively studied in the retina. The anti-calbindin-D28K antibody labels horizontal cells, some bipolar cells, and some medium to large RGCs in the retina (Massey and Mills, 1996; Kwon et al., 2005). The anti-calretinin antibody labels amacrine cells, including AII cells, cone bipolar cells, and numerous cell bodies in the GCL (Massey and Mills, 1996; Jeon and Jeon, 1998; Kwon et al., 2005). The anti-parvalbumin antibody labels amacrine cells, including AII cells, horizontal cells, a subpopulation of RGCs, and displaced amacrine cells in the retina (Casini et al., 1995; Kwon et al., 2005). Our laboratory executed studies using single cell injection after immunocytochemistry, which revealed that different subtypes of RGCs express CBPs. Specifically, at least eight different types of RGCs expressed parvalbumin (Kim and Jeon, 2006; Lee et al., 2013), while at least 10 different types of RGCs expressed calretinin (Lee et al., 2010; Kwon et al., 2014).

ipRGC architecture is well studied in rodents, such as mice and rats (Ecker et al., 2010; Müller et al., 2010; Hu et al., 2013). Although mice and gerbils both belong to the Muridae family, gerbils are physiologically, anatomically, and behaviorally different from mice. For example, mice and rats are nocturnal animals, while Mongolian gerbils are diurnal. Gerbils are broadly used in biomedical research (Levine and Payan, 1966; Wikler et al., 1989; Ren et al., 2014); therefore, the purpose of the present study was to reveal the organization of ipRGCs in gerbil retina through immunocytochemistry, quantitative analysis, and conventional/confocal microscopy. Although calbindin-D28K, calretinin, and parvalbumin are localized in different types of RGCs, it was unclear whether gerbil retina ipRGCs contained these CBPs. Therefore, we also determined whether calbindin-D28K, calretinin, or parvalbumin was specifically expressed in ipRGCs.

2. Materials and methods

2.1. Animals and tissue preparation

Mongolian gerbils (*Meriones unguiculatus*) were used in this study. The animals (2–3 months of age, 70–90 g, $n = 21$) were obtained from a local vendor. The Mongolian gerbils 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 the blink reflex. The eyes were immediately enucleated and the retinas were isolated. The isolated retinas were fixed for 2 h at 4 °C with a solution containing 4% paraformaldehyde in phosphate buffer (pH 7.4). The retinas were processed as whole mounts, and cut into 50-μm thick sections using a vibratome. All investigations involving animals conformed to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This research was also approved (permission no. 2014161) by the animal rights committee at Kyungpook National University, Daegu, South Korea.

2.2. Horseradish peroxidase (HRP) immunocytochemistry

A polyclonal antibody against melanopsin was obtained from Thermo Scientific (Cat#PA1-781, Rockford, IL, USA). This antibody was raised against a C-terminal peptide from rat melanopsin (aa E(455) Q K S K T P K T K R H L P S L D R R M(474)) and detects melanopsin from retinal ganglion cells. The specificity of this antibody for the detection of melanopsin-IR cells was shown in many previous studies in mouse (Baver et al., 2008; Müller et al., 2010; Karnas et al., 2012), in rat (Karnas et al., 2013) and even in human (Lesnik Oberstein et al., 2011). The primary antiserum was diluted (1:200). Retinal tissues were processed as whole mounts and cut into 50-μm thick vertical sections using a vibratome. We used standard immunocytochemical techniques and methods described in detail in our previous reports (Jeon et al., 1998). The tissue was examined and photographed on a Zeiss Axioplan microscope, using conventional or differential interference contrast (DIC) optics.

2.3. Fluorescence immunocytochemistry

Monoclonal antibodies against calbindin-D28K and parvalbumin were obtained from Sigma Chemicals (St. Louis, MO, USA). A monoclonal antibody against calretinin was purchased from Millipore (Millipore, Bedford, MA, USA). The primary antibodies used and their concentrations were as follows: rabbit anti-melanopsin (1:200), mouse anti-calbindin-D28K (1:500), mouse anti-calretinin (1:500), and mouse anti-parvalbumin (1:250). For immunofluorescence detection, we used secondary antibodies: fluorescein (FITC)/Cy3-conjugated goat anti-rabbit immunoglobulin IgG (1:150, Vector Laboratories, Burlingame, CA, USA), or Cy3-conjugated goat anti-mouse IgG (1:150, Jackson ImmunoResearch, West Grove, PA, USA). The tissue was coverslipped using Vectashield® mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were obtained using a Bio-Rad MRC 1024 laser scanning confocal microscope.

To identify the specificity of rabbit anti-melanopsin antibody, we performed two experiments: a negative control test and a preabsorption experiment. For the negative control test, whole progress was under the same condition except for the primary antibody which was not treated in the negative control test. For the preabsorption experiment, blocking peptide of rabbit anti-melanopsin (Peptron, Daejeon, South Korea) was mixed with primary antibody at 10:1 ratio to inactive the primary antibody. And then, the mixture was pre-incubated for 1 h at room temperature. Tissues were incubated with the preabsorbed antibody in place of the primary antibody. According to the negative control test and preabsorption experiment, the Mongolian gerbil retina showed no labeling in the results (Fig. 1).

2.4. Camera lucida drawings

Camera lucida drawings of melanopsin-IR neurons were generated with the aid of a drawing tube attached to a Zeiss Axioplan microscope (Carl Zeiss) at 400× magnification. Based on stratification, dendrites were drawn in pink (the OFF sublayer of the IPL) and blue (the ON sublayer of the IPL), and cell bodies were drawn in black (GCL) and in green (INL) on acetate sheets. The final color picture was produced by superimposing the acetate sheets on the drawing paper.

2.5. Quantitative analysis

Average soma and dendritic field diameters were computed using a digital camera, Zeiss AxioCam HRc (AxioVison 4; Carl Zeiss Meditec, Inc., Dublin, CA, USA). The fields were located in the mid-peripheral retina. In the three retinas with the best labeling, we measured the soma diameter of 180 cells and the dendritic field diameter of 60 cells. Melanopsin-IR cell soma diameters were analyzed through a 40× Zeiss Plan-Apochromat objective (Carl Zeiss) by circling the soma with a pen on the monitor. The dendritic field diameters were also analyzed through a 40× objective by connecting the distal-most tips of the dendrites.