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Parvalbumin-immunoreactive neurons in the inner nuclear layer of zebrafish retina

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ABSTRACT

The purpose of this investigation is to characterize parvalbumin-immunoreactive (IR) neurons in the inner nuclear layer (INL) of zebrafish retina through immunocytochemistry, quantitative analysis, and confocal microscopy. In the INL, parvalbumin-IR neurons were located in the inner marginal portion of the INL. On the basis of dendritic stratification in the inner plexiform layer (IPL), at least two types of amacrine cells were IR for parvalbumin. The first one formed distinctive laminar tiers within s4 (PVs4) of the IPL, and the second within s5 (PVs5). The average number of PVs4 cells was 8263 cells per retina ($n = 3$), and the mean density was 1671 cells/mm^2. The average number of PVs5 cells was 1037 cells per retina ($n = 3$), and the mean density was 210 cells/mm^2. Quantitatively, 88.9% of anti-parvalbumin labeled neurons were PVs4 cells and 11.1% were PVs5 cells. Their density was highest in the midcentral region of the ventrotemporal retina and lowest in the periphery of the dorsonasal retina. The average regularity index of the PVs4 cell mosaic was 4.09, while the average regularity index of the PVs5 cell mosaic was 3.46. No parvalbumin-IR cells expressed calretinin or disabled-1, markers for AII amacrine cells, in several animals. These results indicate that parvalbumin-IR neurons in zebrafish are limited to specific subpopulations of amacrine cells and the expressional pattern of parvalbumin may not correspond to AII amacrine cells in several other animals. Their distribution suggests that parvalbumin-IR neurons are mainly involved in ON pathway information flow.

1. Introduction

Zebrafish is a powerful vertebrate model organism for biomedical sciences in various fields. It has the advantages of genetic and experimental accessibilities and optical transparency (Fadool and Dowling, 2008). The similarities of its central nervous system, especially its visual system, to those of other vertebrates also make this animal a popular model in retinal biology (Yazulla and Studholme, 2001; Mangrum et al., 2002; Connaughton et al., 2004; Song et al., 2008).

Calcium-binding proteins are thought to be important modulators in calcium cell signaling pathways by binding to Ca^{2+} (Heizmann and Braun, 1995). Among many calcium-binding proteins, parvalbumin is the first calcium-binding protein that was isolated from the skeletal muscle of lower vertebrates (Heizmann, 1984). Parvalbumin was subsequently found to be widely distributed in specific subpopulations of neurons in the central nervous system (Sanna et al., 1993). In particular, parvalbumin has been described as a good marker of AII amacrine cells in cat (Gábriel and Straznicky, 1992), rat (Wässle et al., 1993), rabbit (Casini et al., 1995), and bat retinas (Jeon et al., 2007). However, AII amacrine cells are not immunoreactive (IR) for parvalbumin in the mouse (Haverkamp and Wässle, 2000) and monkey retina (Kolb et al., 2002).

The retina contains more than 60 distinctive subtypes of cells (Masland, 2004). Amacrine cells constitute almost 30 subtypes of cells in the retina, representing approximately 40% of all neurons in the inner nuclear layer (INL) (Jeon et al., 1998; MacNeil et al., 1999; Masland, 2004). Amacrine cells influence retinal signal processing in the inner plexiform layer (IPL), integrating and modulating the spatial and temporal properties of the visual stimuli presented from bipolar cells to ganglion cells (Massey and Redburn, 1987).

Recently, 3 horizontal, 7 amacrine, 17 bipolar, and 11 ganglion cells were morphologically identified in the zebrafish retina (Mangrum et al., 2002; Connaughton et al., 2004; Song et al., 2008). Although, parvalbumin has been described as a good marker of AII amacrine cells in cat (Gábriel and Straznicky, 1992), rat (Wässle et al., 1993), rabbit (Casini et al., 1995), and bat retinas (Jeon et al., 2007), parvalbumin-IR cells have not been reported in zebrafish retina. Thus, the goal of this study is to examine the morphological types of parvalbumin-IR cells in the INL of the retina of zebrafish

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through immunocytochemistry, quantitative analysis, and confocal microscopy.

2. Materials and methods

2.1. Animals and tissue preparation

Adult zebrafish (*Danino rerio*) were used in this study. The animals were obtained from the Korea Zebrafish Organogenesis Mutant Bank, Daegu, Korea. Zebrafish were anesthetized with a 0.03% tricaine methanesulphonate solution (Sigma, St. Louis, MO, USA) for 3–4 min and their spinal cords transected behind the head with a razor blade. Immediately after enucleation, the eye was immersed in the fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) with 0.002% $CaCl_2$ added. After removing the cornea and lens, retinas were carefully isolated from the eye cup in the fixative solution. The isolated retinas were fixed for 2 h at 4–5 °C. Retinal tissues were processed as whole mounts and cut into 50 μm vertical thick sections using a vibratome. All protocols conform to National Institute of Health animal care guidelines.

2.2. Immunocytochemistry

A monoclonal antibody against parvalbumin was obtained from Sigma Chemical (clone PARV-19, isotype: mouse IgG1, St. Louis, MO, USA). This antibody is known to label both alpha and beta isoforms of the parvalbumin protein. Polyclonal antibodies against calretinin and disabled-1 were obtained from Chemicon (Temecula, CA, USA). The primary antiserum was diluted in a ratio of 1:1000. Standard immunocytochemical methods that have been described in detail in our previous studies (Jeon and Jeon, 1998; Jeon et al., 1998). For detection by immunofluorescence, the secondary antibodies were fluorescein-(FITC) conjugated anti-rabbit IgG (1:100, Vector Lab., Burlingame, CA, USA), and Cy3- or Cy5-conjugated anti-mouse IgG (1:100, Jackson ImmunoResearch, West Grove, PA, USA). Immunofluorescence images were obtained on a Bio-Rad MRC 1024 laser scanning confocal microscope. To identify layers, tissues were also examined and photographed on a Zeiss Axioplan microscope using differential interference contrast (DIC) optics. As a control, some tissues were incubated in the same solution without the addition of the primary antibody. These control tissues showed no parvalbumin immunoreactivity.

2.3. Quantitative analysis

Cell density was expressed as the number of parvalbumin-IR cells/mm^2 of retinal surface. Confocal microscopy was used to count the different types of cells in the INL. In three fluorescence-reacted whole mount retinas, serial optical sections were taken every 0.5 μm (0.25 μm at s4 and s5) along the z-axis with a Bio-Rad MRC 1024 laser scanning confocal microscope. The different types of parvalbumin-IR cells in the INL were identified on the basis of a dendritic stratification pattern within the IPL in through focus images. Parvalbumin-IR cells were viewed using a Nikon Plan Fluor ×40 objective (Nikon, Tokyo, Japan) at 200 μm intervals along the central dorsoventral and nasotemporal axes. The sample area was 100 × 100 μm. In one fluorescence-reacted whole mount retina, parvalbumin-IR cells in the INL were viewed on the computer monitor using a Zeiss Plan-Apochromat ×20 objective and a Zeiss AxioCam HRc digital camera (Carl Zeiss, Göttingen, Germany). Parvalbumin-IR cells were counted in 440 sample areas (each 100 × 100 μm field) in the whole retina. A transparency sheet was placed on the computer monitor, and labeled cells were circled with a pen. The samples were taken at evenly distributed positions across the retina. Isodensity contours were fitted to the data and coded by a gray scale such that regions of highest density were black, with areas of decreasing density being increasingly lighter gray. The total number of parvalbumin-IR cells was determined in each sample area and expressed as the number of cells/mm^2. The cell density was multiplied by retinal area to determine the total number of parvalbumin-IR cells. A nearest-neighbor analysis was performed on the cells located in the INL from the one midcentral, midperipheral, and peripheral regions of the retina. The nearest-neighbor analysis to determine the regularity index (mean nearest- neighbor distance/standard deviation) has been previously reported (Wässle and Riemann, 1978). The size of the mosaic field for regularity index in the study was 300 × 300 μm wide or 500 × 500 μm wide. The average area and diameter of labeled cells were computed using a digital camera from the same regions where the nearest-neighbor analyses were determined. Analyses for area and diameter were done with ×40 Zeiss Plan-Apochromat objective (Carl Zeiss). A cursor was moved manually around the outer contour of each cell using Zeiss Axiovision (Ver. 4, Carl Zeiss).

3. Results

Fig. 1 shows 50 μm vertical sections through a parvalbumin-IR zebrafish retina. Parvalbumin-IR cell bodies are visible both at the INL and the ganglion cell layer (GCL). Parvalbumin-IR cells in the GCL are thought to be a mixture of ganglion cells and displaced amacrine cells (Haverkamp and Wässle, 2000; Kim and Jeon, 2006). As the distinction between displaced amacrine cells and ganglion cells in the GCL could not definitely be made, amacrine cells in the INL were the main focus of the present study. In the inner margin of the INL, in agreement with a previous report (Avanesov et al., 2005), parvalbumin immunoreactivity was found in many neurons. These cells formed a distinctive dendritic stratification exclusively in the lower region of the IPL, the physiological ON-center layer (Connaughton and Nelson, 2000). On the basis of a dendritic stratification pattern, at least two types of parvalbumin-IR amacrine cells could be identified in the INL of the zebrafish retina. We adopted the six IPL sublayers in zebrafish retina (Connaughton et al., 2004). Figs. 1B,C show cells that have a single apical dendritic process extending proximally from the soma into s4 (PVs4). Figs. 1D,E show a cell that has a single apical dendritic process extending proximally from the soma into s5 (PVs5). At the end of the primary dendrite, each type formed a laterally extending tier of processes. The parvalbumin-IR tier in s5 was slightly wider than that of the tier within s4. Cells in the ganglion cell layer also had processes branching into s5. Fig. 1F shows a cell in the GCL that has a process extending from the soma into s5. However, processes that extend into s4 were not found in the present study.

To determine whether all parvalbumin-IR neurons have dendritic processes extending into the lower IPL, a confocal z-series analysis was performed on whole mounts of zebrafish retina. Fig. 2 shows a well-labeled whole mount retina from the mid-periphery. In the fluorescence-reacted retina, the cell bodies in the INL create a regular mosaic (Fig. 2A). At the middle of the IPL, these cells show the primary dendrites extending from the cell bodies into the parvalbumin-IR tiers (Fig. 2B). Fig. 2C shows that cell bodies (green) directly match the primary dendrites (red) that extend into the lower IPL. At the lower IPL, parvalbumin-IR cells formed a dense plexus of arboreal dendrites (Fig. 2D). Parvalbumin immunoreactivity was found in many cell bodies in the GCL (Fig. 2E). We believe that many of these labeled cells are ganglion cells because of the well-labeled fibers in the nerve fiber layer.

The estimated total number of PVs4 cells varied from 7898 to 8817 cells among the three sampled retinas in this study. The estimated total number of PVs5 cells varied from 948 to 1102 cells. Table 1 and Fig. 3 show these results. There were 7898 PVs4 cells in retina #1L, 8075 PVs4 cells in retina #2R, and 8817 PVs4 cells in retina #3R. Therefore, the average number of PVs4 cells per retina