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CHARACTERIZATION OF GREEN FLUORESCENT PROTEIN– EXPRESSING RETINAL CONE BIPOLAR CELLS IN A 5- HYDROXYTRYPTAMINE RECEPTOR 2a TRANSGENIC MOUSE LINE

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Abstract

Retinal bipolar cells relay visual information from photoreceptors to third-order retinal neurons. Bipolar cells, comprising multiple types, play an essential role in segregating visual information into multiple parallel pathways in the retina. The identification of molecular markers that can label specific retinal bipolar cells could facilitate the investigation of bipolar cell functions in the retina. Transgenic mice with specific cell type(s) labeled with green fluorescent protein (GFP) have become a powerful tool for morphological and functional studies of neurons in the CNS, including the retina. In this study, we report a 5-hydroxytryptamine receptor 2a (5-HTR2a) transgenic mouse line in which expression of GFP was observed in two populations of bipolar cells in the retina. Based on the terminal stratification and immunostaining, all the strongly GFP-labeled bipolar cells were found to be type 4 cone bipolar cells. A small population of weakly labeled bipolar cells was also observed, which may represent type 8 or 9 cone bipolar cells. GFP expression in retinal cone bipolar cells was seen as early as postnatal day 5. In addition, despite severe retinal degeneration due to an *rd1* (or *Pde6brd1*) gene in this transgenic line, the density of GFP-labeled cone bipolar cells remained stable up to at least 6 months of the age. This transgenic mouse line will be a useful tool for the study of type 4 cone bipolar cells in the retina under both normal and disease conditions.

Keywords

Green fluorescent protein; type 4 cone bipolar cell; retina; 5-HTR2a-EGFP; transgenic mouse; immunostaining

Retinal bipolar cells, the second-order neurons in the retina, relay visual information from photoreceptors to third-order retinal neurons. Bipolar cells comprise multiple types and are essential for segregating visual information into multiple parallel pathways in the retina (Wu et al., 2000; Wässle, 2004). Bipolar cells are subdivided into ON- and OFF-types based on their light response polarity and are subdivided into rod and cone bipolar cells based on their synaptic inputs. In mammals, a single type of rod bipolar cell (Boycoott & Dowling, 1969;

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Boycott & Kolb, 1973; Dacheux & Raviola, 1986) and at least nine or ten types of cone bipolar cells have been described, primarily based on their terminal stratification in the inner plexiform layer (IPL; Famiglietti, 1981; Kolb *et al.*, 1981; Pourcho & Goebel, 1987; Euler & Wässle, 1995; Ghosh *et al.*, 2004; Pignatelli and Strettoi, 2004; Wässle *et al.*, 2009). In addition to their distinct morphological properties and unique synaptic connections with photoreceptors and third order retinal neurons, increasing evidence also suggests that bipolar cells of each type express a distinct array of membrane channels and receptors (DeVries, 2000; Pan, 2000; Pan and Hu, 2000; Hu and Pan, 2001; Müller *et al.*, 2003; Ma *et al.*, 2005; Ivanova and Müller, 2006; Fyk-Kolodziey and Pourcho, 2007). Thus, detailed characterization of the anatomical and physiological properties of individual bipolar cell types could provide important insights into the functional roles of retinal bipolar cells in retinal processing. The identification of bipolar cell-specific markers could facilitate such studies.

Transgenic mice with specific cell type(s) labeled with green fluorescent protein (GFP) have become a powerful tool for morphological and functional studies of neurons in the CNS (Gong *et al.*, 2003). Several transgenic mouse lines in which specific retinal bipolar cell type(s) are labeled with fluorescent proteins have previously been described; including ON bipolar cells (Morgan *et al.*, 2006; Dhingra *et al.*, 2008), type 7 cone bipolar cells (Wong *et al.*, 1999; Huang *et al.*, 2003), type 9 cone bipolar cells (Haverkamp *et al.*, 2005), and, more recently, type 5 cone bipolar cells (Wässle *et al.*, 2009). In this study, we report a 5-hydroxytryptamine receptor 2a (5-HTR2a) transgenic mouse line in which strong GFP expression was found in type 4 cone bipolar cells.

Experimental procedures

Animals

5-HTR2a-EGFP transgenic mice were obtained from the Mutant Mouse Regional Resource Centers (MMRRC; Line: DQ118). The transgenic mice were generated by homologous recombination with a bacterial artificial chromosome (BAC) containing the promoter of the 5-HTR2a receptor with the 5-HTR2a receptor coding sequence being replaced with a sequence encoding the EGFP reporter gene (Gong *et al.*, 2003). The 5-HTR2a-EGFP transgenic mice were generated in an FVB/N-Swiss Webster hybrid background. All animal handling procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The experiments were carried out on mice at one month of age unless otherwise indicated.

Immunocytochemical staining

Mice were deeply anesthetized with CO₂ and decapitated. The retinas were fixed in the eyecups with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 minutes. GFP fluorescence without enhancement with antibody was sufficient to visualize the GFP-expressing cells. The expression of GFP in the retina was examined in retinal whole-mounts and vertical sections. For whole-mounts, after fixation the retina was dissected free in PB solution, flat mounted on slides, and cover slipped. For retinal vertical sections, the retinas were cryoprotected in a sucrose gradient (10%, 20%, and 30% w/v in PB), and cryostat sections were cut at 20 μ m.

To identify the GFP-expressing cell types, GFP fluorescence was enhanced by applying antibodies against GFP, while the GFP labeled cells were immunostained for other specific retinal cell markers. The following antibodies were used in this study: rabbit anti-GFP (1:2000; Molecular Probes); mouse anti-GFP (1:2000; Neuromab) goat anti-choline acetyl transferase (ChAT, 1:2000; Chemicon); rabbit anti-HCN4 (1:500; Alomone Labs); mouse anti-protein kinase A (PKA) RII β (1:80000; BD); mouse anti-calsenilin (1:2000; W. Wasco, Harvard

Medical School); mouse anti-synaptotagmin II (Syt-2; 1:600; Zebrafish International Resource Center).

For immunostaining, retinal whole-mounts or sections were blocked for 1 h in a solution containing 5% Chemiblocker (membrane-blocking agent, Chemicon), 0.5% Triton X-100 and 0.05% sodium azide (Sigma). The primary antibodies were diluted in the same solution and applied overnight, followed by incubation (1 h) in the secondary antibodies, which were conjugated to Alexa 555 (1:600; red fluorescence, Molecular Probes) or Alexa 488 (1:600, green fluorescence, Molecular Probes). All steps were carried at room temperature (RT).

All images were made using a Zeiss Axioplan 2 microscope with the Apotome oscillating grating to reduce out-of-focus stray light. Individual cells were selected, and Z-stack images were captured using a Zeiss Apotome microscope. Image projections were made by collapsing individual z-stacks of optical sections into a single plain, unless otherwise indicated. The brightness and contrast were adjusted using Adobe Photoshop CS4.

Results

GFP expression was observed in the retina of the GFP-transgenic mouse line under the control of the 5-HTR2a receptor promoter. In retinal whole-mounts, GFP-labeled cell somas were observed throughout the entire retina with the focal plane at the inner nuclear layer (INL) (Fig. 1A). Most of the cell somas were brightly labeled, but some weakly labeled cells were also observed. When examined in retinal vertical sections, the labeled cells located in the INL were found to be bipolar cells based on their morphological characteristics (Fig. 1B). The labeled cell bodies were located in the middle of the INL, with their dendrites and axon terminals extending into the outer plexiform layer (OPL) and the inner plexiform layer (IPL), respectively. The vast majority of the labeled axon terminals ramified in the distal portion of the IPL. Some axon terminals were found to ramify in the inner portion of the IPL, but they were barely visible without antibody enhancement (see below). In addition, some weakly labeled cells located in the retinal ganglion cell layer were observed (see Fig. 1B).

It should be noted that since the 5-HTR2a-EGFP transgenic mice were generated in an FVB/N-Swiss Webster hybrid background, which carries an *rd1* (or *Pde6brd1*) gene (Bowes et al., 1990), these mice displayed rapid retinal degeneration. Only a single layer of photoreceptor cell bodies remained at one month of age (see the Nomarski micrograph in the left panel of Fig. 1B).

To examine the labeled bipolar cells in more detail, GFP fluorescence was enhanced using an antibody against GFP. Again, cell somas with strong and weak GFP expression were observed in retinal whole-mounts with the focal plane at the INL (Fig. 2A). Figure 2B shows double labeling with choline acetyltransferase (ChAT) in a retinal vertical section. Clearly, the strongly GFP-labeled axon terminals ramified in sublaminae 1 and 2 of the IPL. The weakly GFP-labeled axon terminals stratified in sublaminae 4 and 5 (an axon is indicated by an arrowhead, the axon terminals are indicated by an arrow in Fig. 2B). Figure 2C shows a stacked image of a retinal whole-mount that captured both cell somas and dendrites. Each cell soma sent out several dendritic branches with distal apical terminals, presumably forming contacts with photoreceptor cells. Figure 2D shows a stacked image focusing on the distal portion of the IPL that was taken at the same field as in figure 2C. The terminal arborizations of individual bipolar cells are visible.

Based on the previously reported classification of retinal bipolar cells (Ghosh et al., 2004), the strongly GFP-labeled bipolar cells with their axon terminals ramifying in sublaminae 1 and 2 should be OFF type cone bipolar cells, whereas the weakly GFP-labeled bipolar cells with their axon terminals ramifying in sublaminae 4 and 5 should be ON type bipolar cells. To identify

the bipolar cell types, we double labeled GFP-positive cells with several bipolar cell-specific antibodies. The GFP-labeled bipolar cells were not immunostained with an antibody to recoverin (Fig. 3A-C) that labels type 2 cone bipolar cells in the mouse retina (Haverkamp et al., 2003). The GFP-labeled bipolar cells were also not double labeled with an antibody to HCN4 (Fig. 3D-F) or PKARII β (Fig. 3G-I), with the exception of a few cells for the latter (arrow in Fig. 3G and H). HCN4 and PKARII β antibodies were reported to label two subtypes of type 3 cone bipolar cells (Mataruga et al., 2007). Furthermore, the GFP-positive bipolar cells were not double labeled with an antibody to synaptotagmin II (Syt-2) (Fig. J-L). The enlarged images in the inserts of figure J-L show the lack of co-localization of the Syt-2 positive axon terminals and the weakly GFP labeled axon terminals in laminae 4 and 5. The anti-Syt 2 antibody was previously reported to label type 2 (Fox and Sanes, 2007) and type 6 bipolar cells (Wässle et al., 2009). These results suggest that the GFP expressing bipolar cells are not type 2, 3, or 6.

On the other hand, we found that the majority of the GFP-labeled bipolar cells were double labeled with an antibody to calnenilin. The calnenilin antibody has been reported to label type 4 cone bipolar cells (Haverkamp et al. 2008). Figure 4A-C shows co-labeling with GFP and calnenilin antibodies in a retinal whole-mount with the focal plane at the INL. The majority of the GFP-labeled cells, including all of the strongly labeled cell somas and a small portion of the weakly labeled cell somas, were positive for calnenilin. The co-localization of GFP and calnenilin positive bipolar cells (cell somas) was also apparent in vertical sections (Fig. 4D-F). These results indicate that the majority of the GFP-labeled bipolar cells, including all strongly GFP-labeled bipolar cells, are type 4 cone bipolar cells. Furthermore, in both whole-mounts and retinal vertical sections, all calnenilin-positive cells were found to express GFP, suggesting that all type 4 cells express GFP in this transgenic mouse line. The cell density of the calnenilin positive and negative cells at the ages of one month and 6 months was counted, and the values are shown in table 1. The cell density of the calnenilin-positive (type 4) and -negative cells was not found to be significantly different between the ages of 1 and 6 months.

To examine the developmental and age-dependent expression of GFP in this transgenic line, animals were examined from postnatal day 5 to 6 months of age. As shown in figure 5A, GFP expression in the INL was already observed at postnatal day 5, although only a few GFP-labeled cells were observed at this time point. At P7, many GFP-labeled cone bipolar cells were found (Fig. 5B). Of note, the photoreceptor cells were present at these early postnatal ages. Figure 5D-F shows GFP expression in the retina 2 weeks, 1 month, and 6 months. This expression persisted until at least 6 months of age, the latest time point examined in this study.

Discussion

This study identified GFP-expressing bipolar cells in a 5-HTR2a-EGFP transgenic mouse line. Our results show that all the strongly GFP labeled bipolar cells are type 4 cone bipolar cells, based on both their terminal stratification pattern and immunostaining. The axon terminals of the strongly GFP-labeled bipolar cells were found to ramify in sublaminae 1 and 2 of the IPL. Based on the classification of bipolar cells in rodents, type 1-4 bipolar cells have axon terminals that ramify in sublamina 1 and/or 2 (Ghosh et al., 2004). Among these, however, only type 4 cone bipolar cells have axon terminals that ramify in both sublaminae 1 and 2. The GFP-labeled cells were also immunoreactive for calnenilin, an antibody that has been shown to label type 4 cone bipolar cells in the mouse retina (Haverkamp et al. 2008). Furthermore, the GFP-labeled bipolar cells were not recognized by antibodies against recoverin and Syt-2. Both of these antibodies label type 2 cone bipolar cells in mice. The GFP-labeled bipolar cells also failed to co-localize with HCN4-positive cells and PKARII β -positive cells, with the exception of a few cells for the latter. HCN4 and PKARII β antibodies have been reported to label two subtypes of type 3 cone bipolar cells (Mataruga et al., 2007). The reason for the co-localization of a few

GFP-positive cells with PKARII β immunoreactive cells is not clear. It is possible that a few of the GFP-labeled bipolar cells were type 3 cone bipolar cells or that the anti-PKARII β antibody occasionally labels for type 4 cone bipolar cells.

In addition, we found that the axon terminals of a small number of GFP-labeled bipolar cells, albeit weakly labeled, ramified in sublaminae 4 and 5. Because of the diffuse nature of their axon terminals in the proximal portion of the IPL, they are unlikely to be type 7 cone bipolar cells, which are restricted in their axon terminal stratification to sublamina 4 (Huang et al., 2003), or rod bipolar cells, which have axon terminals stratifying in sublamina 5 close to the ganglion cell layer. Since neither the GFP-labeled bipolar cell somas nor the weakly labeled axon terminals were labeled with an anti Syt-2 antibody, which recognizes type 2 and 6 cone bipolar cells, the cone bipolar cells with weakly labeled axon terminals in the proximal portion of the IPL cannot be type 6 cone bipolar cells. Taken together, our results suggest that most of the weakly labeled bipolar cells are likely to be type 8 or 9 cone bipolar cells. Unfortunately, there are currently no available antibodies that can be used to label type 8 or 9 cone bipolar cells.

GFP expression in cone bipolar cells in this transgenic line was observed as early as postnatal day 5 and was stable at least until 6 months of age. The cell density for both calnenilin-positive and -negative cone bipolar cells at 1 and 6 months was not significantly changed. The value for the calnenilin-positive cells was $\sim 3600/\text{mm}^2$, which is slightly higher than that of type 4 cone bipolar cells reported in a recent study ($\sim 3,000/\text{mm}^2$; Wässle et al., 2009). This discrepancy could be due to the different mouse strains used in these studies (FVB/N-Swiss vs. C57BL/6J). The cell density of calnenilin-negative cells was $\sim 1,500/\text{mm}^2$, while the cell density of the type 9 cone bipolar cells was reported to be around $600/\text{mm}^2$ (Haverkamp et al., 2005). To date, the cell density of type 8 cone bipolar cells remains unknown.

It is worth noting that since the mouse strain used in this study carries an *rd1* (or *Pde6brd1*) gene, the unchanged cell density observed for both types of GFP-labeled bipolar cells between the ages of 1 and 6 month suggests that retinal remodeling did not cause significant cell death of these cone bipolar cells. Dramatic morphological remodeling of second order cells after the death of photoreceptors, however, has been previously reported (Strettoi & Pignatelli, 2000). Thus, it would be interesting to examine how the retinal degeneration affects synaptic circuits. This transgenic mouse line with GFP-labeled bipolar cells could be used for such a purpose.

To date, it remains unclear whether mammalian retinas express 5-HTR2a receptors. An early study reported 5-HTR2a immunoreactivity in the rabbit retina (Pootanakit et al., 1999). The labeling was reported to be in photoreceptor terminals and rod bipolar cells. A recent study showed 5-HTR2a immunoreactivity in glial cells and some amacrine cells in the bullfrog retina but not in the rat retina (Han et al., 2007). It remains to be determined whether the expression of GFP in this transgenic mouse line targets to the 5-HTR2a-expressing neurons. Nevertheless, the targeted expression of GFP to specific cone bipolar cells in this transgenic mouse line will be a useful tool for retinal research.

Transgenic mouse lines expressing GFP in ON bipolar cells (Morgan et al., 2006; Dhingra et al., 2008) and type 5, 7, and 9 cone bipolar cells (Wong et al., 1999; Huang et al., 2003; Haverkamp et al., 2005; 2009; Wässle et al., 2009) have been previously reported. These bipolar cell-specific GFP transgenic mouse lines have been used in the study of bipolar cell development (Morgan et al., 2006), synaptic circuits (Han and Massey, 2005; Lin et al., 2005; Lin and Masland, 2005; Wässle et al., 2009), physiological properties (Duebel et al., 2006), and molecular biology (Huang et al., 2003; Dhingra et al., 2008). The identification of GFP expression in type 4 cone bipolar cells and possibly also type 8 or 9 cone bipolar cells in this transgenic line further expands this valuable tool. In particular, the expression of GFP in

type 4 bipolar cell bodies is sufficient bright without the enhancement of antibody; therefore, this line can be used for *in vitro* electrophysiological recordings of type 4 cone bipolar cells in retinal slices or dissociated preparations. It should be noted that although type 4 cone bipolar cells in the mouse can be labeled using an anti-calsenilin antibody (Haverkamp et al. 2008), this antibody also labels the processes of third-order neurons in the IPL, which precludes the use of this antibody to label axon terminals of the type 4 cone bipolar cells. Thus, this mouse line will also be useful for examining synaptic circuits of type 4 cone bipolar cells with third-order retinal neurons. As shown in this study, the expression of GFP in bipolar cells is detectable in both early postnatal and aged retinas. Therefore, this transgenic mouse line could be used to study the development and aging of retinal bipolar cells. Finally, crossing this mouse with wild-type and other transgenic lines with retinal diseases will allow studies of the effects on retinal bipolar cells during disease processes.

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Abbreviations

GFP	green fluorescent protein
5-HTR2a	5-hydroxytryptamine receptor 2a
IPL	inner plexiform layer
INL	inner nucleus layer
OPL	outer plexiform layer
ChAT	choline acetyl transferase
Syt-2	synaptotagmin II

Figure 1. GFP-labeled cells in the 5-HTR2a-EGFP transgenic mouse line. A, GFP-labeled cells viewed in a retinal whole-mount. B, GFP-labeled cells viewed in a retinal vertical section. Left panel in B shows the Nomarski micrograph. INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 25 μ m.

Figure 2.

GFP-labeled retinal bipolar cells after enhancement using an anti-GFP antibody. A, GFP-labeled cells viewed in a retinal whole-mount with the focal plane at the INL. B, GFP-labeled cells were co-labeled with an anti-ChAT antibody (red), viewed in a retinal vertical section. The weakly GFP-labeled axon terminals located in the proximal portion of the IPL are indicated by an arrow; the axon is indicated by an arrowhead. C, Whole-mount view of the dendritic trees of the GFP-labeled cone bipolar cells with a stacked image that captures both cell somas and dendrites. D, Whole-mount view of the axon terminals of the GFP-labeled cone bipolar cells with the focal plane at the IPL. The images in C and D were taken in the same field. OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 25 μ m.

Figure 3.

Double labeling using GFP and retinal bipolar cell-specific antibodies. A-C, A retinal vertical section was immunostained for GFP (A) and recoverin (B). The overlay of A and B (C). Double immunostaining for GFP and HCN4 (D-F), for GFP and PKARIIβ (G-I), and for GFP and Syt-2 (J-L). A cell double labeled for GFP and PKARIIb is pointed by an arrow in G and H. The enlarged inserts in the bottom-right of J-L are single z-section images. Scale bars: 25 μm.

Figure 4.

Double immunostaining for GFP and calsenilin. A-C, Co-labeling of GFP (A) and calsenilin (B) viewed in a retinal whole-mount. The overlay of A and B (C). D-F, Co-labeling of GFP (D) and calsenilin (E) viewed in a retinal vertical section. The overlay of D and E (F). Scale bars: 25 μm.

Figure 5.

Age-dependent expression of GFP in retinal bipolar cells. The expression of GFP viewed in retinal whole-mounts and vertical sections at postnatal day 5 (A), 7 (B), 11 (C), 2 weeks (D), 1 month (E), and 6 months (F). Scale bars: 25 μ m.

Table 1

Comparison of calsenilin-positive and -negative cone bipolar cells labeled with GFP at 1 and 6 months of age

	1 month	6 months
Calsenilin-positive CBCs	$3,561 \pm 333 / \text{mm}^2$	$3,659 \pm 780 / \text{mm}^2$
Calsenilin-negative CBCs	$1,564 \pm 619 / \text{mm}^2$	$1,543 \pm 757 / \text{mm}^2$