Published in final edited form as:

Neuroscience. 2007 February 9; 144(3): 1087–1093.

Characterization of Green Fluorescent Protein-Expressing Retinal Cells in CD 44-Transgenic Mice

Vijay Sarthy¹, Hideo Hoshi², Stephen Mills², and V. Joseph Dudley¹

¹ Department of Ophthalmology, Northwestern University, Chicago, IL

² Department of Ophthalmology and Visual Science, University of Texas Houston Medical Center, Houston, TX

Abstract

Sensory information in the retina is transferred from rod and cone photoreceptors to higher visual centers via numerous parallel circuits that sample the photoreceptor mosaic independently. Each circuit consists of a unique combination of ganglion cell, bipolar and amacrine cell types. The morphology and physiological responses of many amacrine cells have been characterized. However, the synaptic connections and retinal circuits in which they participate are only rarely understood. A major problem that has prevented fuller characterization of retinal circuitry is the need for specific cellular markers for the more than 50 inner retinal cell types. One potential strategy for labeling cells is to use transgenic expression of a reporter gene in a specific cell type. In a recent study of CD44-EGFP transgenic mice, we observed that the green fluorescent protein was expressed in a population of amacrine and ganglion cells in the INL and the GCL. To characterize the morphology of the GFP-labeled cells, whole mount preparations of the retina were used for targeted iontophoretic injections of Lucifer Yellow and Neurobiotin. Furthermore, immunocytochemistry was used to characterize the antigenic properties of the cells. We found that many GFP-expressing cells were GABAergic and also expressed calretinin. In addition to the somatic staining, there was a strong GFP⁺-band located about 50–60% depth in the IPL. Double labeling with an antibody to choline acetyltransferase (ChAT) revealed that the GFP-band was located at strata 3 inner retina. The best-labeled GFP-expressing cell type in the INL was a wide-field amacrine cell that ramified in stratum 3. The GFP-expressing cells in the GCL resemble the type B1, or possibly A2 ganglion cells. The CD44-EGFP mice should provide a valuable resource for electrophysiological and connectivity studies of amacrine cells in the mouse retina.

Keywords

CD44; retina; amacrine cell; ganglion cell; transgenic mice

Introduction

The processing of visual information from the environment begins at the output of the photoreceptor synapse, where approximately a dozen bipolar cell subtypes distribute

Correspondence to: Vijay Sarthy, Department of Ophthalmology, Northwestern University Feinberg School of Medicine, Tarry Building, 5-715, 303 E. Chicago Avenue, Chicago, IL 60611, (312) 503-1940 (office); (312) 503-1120 (FAX), E-mail address: vjsarthy@northwestern.edu.

Section Editor: Dr. Charles R. Gerfen

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

photoreceptor output into an even greater diversity of retinal circuits. Amacrine cells, which are the most diverse cell type in the vertebrate retina make up ~ 40% of all neurons in the inner nuclear layer (INL) and participate in 64–87% of all synapses in the inner plexiform layer (IPL) (Dubin, 1970; Raviola & Raviola, 1982; Strettoi & Masland, 1995; Jeon et al., 1998). Amacrine cells vary considerably in their morphology; it has been estimated that there are 20–30 different amacrine cell types in the mammalian retina (MacNeil et al., 1999). The major variables are the dendritic field size, level of stratification in the IPL, and ramification pattern (unistratified, bistratified, or diffuse). Their functions are likely equally varied, but functional characterization has been made for only a handful of amacrine cell types, at best, most notably the starburst, reciprocal rod amacrine and AII amacrine cells of mammalian retinæ.

The central element of each type of individual retinal circuit is a specific type of ganglion cell. Each of these approximately 15 types (Rockhill *et al*, 2002.; Marc and Jones, 2002) receives input from 1 or more bipolar cell types and is influenced in its response characteristics by its inputs from the diversity of amacrine cells to which it is synaptically coupled.

Advances in characterization of these second and third order neurons have been hindered by the lack of cell-specific ‘markers’ for the morphologically distinct cell types (Masland & Raviola, 2000). One potential strategy for labeling cells is to use transgenic expression of a reporter gene. In the mouse, the availability of transgenic technology makes it possible to express reporter molecules such as β -galactosidase (β -gal), human placental alkaline phosphatase (PALP) or Green fluorescent protein (GFP) under the control of a cell-specific promoter and thus, mark a specific cell type (Feigenspan et al., 1998; Masland & Raviola, 2000; Sinclair & Nirenberg, 2001; Fei & Hughes, 2001).

In a recent study, we generated transgenic mice by injecting a construct in which the CD44 promoter drove the enhanced GFP (EGFP) gene. The CD44 protein is known to be present in Müller cells, but not in retinal neurons (Chaitin & Wortham, 1994; Chaitin et al., 1994). We were therefore surprised to find in three different lines GFP was expressed in amacrine and ganglion cells. In the present study, we report the morphological and immunocytochemical characterization of these GFP-expressing neurons in the mouse retina.

Methods

Generation and screening transgenic mice

The CD44 promoter/report construct was made by the fusion of a 1.3-kb fragment of the CD44 promoter to the EGFP reporter gene followed by SV40 DNA supplying an intron and signal for polyadenylation (Fig. 1). The mouse CD44 promoter (–1262/+109) was obtained from Dr. Perrella (Harvard Medical School). The CD44-EGFP construct was first tested in cell cultures by transfection.

Transgenic mice were generated at the Northwestern University Transgenic Facility. Genomic DNAs obtained from ear punch were tested by PCR using the following primers which span the junction between the CD44 promoter and EGFP genes: 5'-CAG GAC GCG CTT CTC ATA GGC TGG-3' (CD44) and 5'-TCG ATG CGG TTC ACC AGG GTG TCG-3'(EGFP). Mice identified as positive for the transgene were mated to wild-type mice to determine the level of expression, pattern of integration, and inheritance of the transgene. Heterozygous animals were used in the experiments.

Morphology

Mouse retinas were isolated from the sclera and mounted vitread side up on filter paper. They were placed in a chamber and continuously perfused with oxygenated Ames medium (Ames & Nesbitt, 1981). The GFP fluorescence was visible through a standard blue filter set of an

Olympus BW50WI mercury epifluorescence microscope. Individual cells were impaled with sharp electrodes containing 3.5% Neurobiotin + 0.5% Lucifer Yellow in 0.05M phosphate buffer. Neurobiotin was introduced into the cells by iontophoretic current (+0.5 nA current at 6 Hz) for 5 min. After several cells were injected, the tissue was fixed in 4% paraformaldehyde for 1 hr, then rinsed and incubated overnight in 1:200 streptavidin-Cy3 (Jackson ImmunoResearch, West Grove, PA) in PBS containing 0.5% Triton X-100. Cells were imaged on a Zeiss LSM510 confocal microscope. Stratification depth will be referred by terms of strata 1–5, each consisting of 20% thickness of the IPL and numbered from bordering the INL (1) to the border of the GCL (5).

Immunocytochemistry

Animals were euthanized and the eyeballs from transgenic and non-transgenic mice were fixed for 2 hr in 0.1 M phosphate buffer (pH 7.4) (PBS) containing 4% formaldehyde. The tissues were then incubated in 10% sucrose in PBS overnight. The eyecups were then mounted in Tissue-Tek O.C.T. (Miles Laboratories, Naperville, IL), sectioned on a cryostat (−20 °C) at 20 µm thick and collected on microscope slides. The sections were pretreated with a blocking solution containing 0.1% Triton X-100 and 10% normal goat serum in PBS for 1 hr at room temperature to saturate non-specific binding sites. The sections were then incubated overnight at 4°C with the primary antibody. Several antibodies were used in this study- ChAT (1:300; Chemicon) staining, GAD67 (1:100; gift from Dr. N. Brecha, UCLA), and Calretinin (Chemicon, 1:200). Sections were then rinsed for 5 min with PBS and incubated with 1:100 biotinylated FITC secondary antibody for 30 min at room temperature. The sections were washed three times (10 min each time) in PBS, coverslipped with Vectashield (Vector Laboratory Inc., Burlingame, CA), and photographed in a Zeiss universal microscope equipped for incident-light fluorescence.

Results

GFP-labeled cells in the retina

Several independent lines were established with a single integration site. We examined the EGFP expression in retinal whole mounts from all the transgenic lines, ranging in age from postnatal day 15 (P15) to P 60. EGFP was found in three different lines (3234, 3266, and 3258) and Line 3234 showed the highest level GFP expression.

Immunocytochemical localization

In retinal whole mounts, the GFP-expressing cells were widely distributed across the retina, and could be readily identified by their strong fluorescence (Fig. 2A). Fluorescent somas were seen in both the proximal inner nuclear layer and ganglion cell layer. The labeled cells appeared heterogenous. Because we initially thought that GFP⁺ cells consisted of only one or two types in each layer, nearest neighbor analysis (Wässle & Riemann, 1978) was used to determine if the labeled cells in the INL comprised a regularly spaced cell population. The distance between each cell and its nearest neighbor was measured. The mean distance between cells was found to be 6.6 µm with a standard deviation of 4.1 µm. The regularity index was calculated by dividing the mean distance between nearest cells by the standard deviation. The mean/SD ratio for the GFP⁺ cells was 1.6, which again suggested a heterogeneous distribution of cell types.

When a retinal cross section was examined under a confocal microscope, the GFP⁺ somata were seen located in the INL, close to the border of the INL/IPL as well as in the GCL (Fig. 2B). This strongly suggested that the former population were amacrine cells, while those in the GCL could be either ganglion cells or displaced amacrine cells. The size of the somas in the INL made it unlikely that they were displaced ganglion cells, and indeed, in subsequent dye injections, none were found to have axons.

Immunocytochemical studies were next carried out to characterize the INL somata that expressed GFP. Antibodies to Choline acetyl transferase (ChAT; Fig. 2C), glutamic acid decarboxylase (GAD₆₇; Fig. 2D–F) and Calretinin (Fig. 2G–I) were used. GABAergic amacrine cells constitute a large fraction of all INL cells in the mammalian retina (Yazulla, 1986). As shown in Fig. 2D–F, many of the GFP-expressing cells were positive for glutamate decarboxylase (GAD₆₇)-immunoreactivity showing that they are GABAergic (Fig. 2D–F). In order to further characterize the GFP⁺ cells, we carried out co-localization of calretinin and GFP. Results of the study are presented in Fig. 2G–I, and show that many of the GFP-expressing amacrine cells also contained calretinin. It is likely that some of the GABAergic cells are calretinin-positive. Finally, we did not find co-localization of ChAT and GFP in any cell body in the INL or GCL (Fig. 2C).

In addition to the somatic staining, there was a strong GFP⁺-band located about 50–60% depth in the IPL. To better localize the GFP⁺-band to a specific sublamina, retinal sections were double-labeled with an antibody to choline acetyltransferase (ChAT), which is known to label strata 2 and 4 in the mouse retina (Haverkamp & Wässle, 2000). In immunostained retinal sections, the GFP⁺-band was found located midway between the two ChAT bands suggesting its localization to stratum 3 (Fig. 2C). Labeling with an antibody against the glutamate transporter, GLT-1, showed that the GFP-band co-stratified with the GLT-1 positive cone bipolar cell terminals (Haverkamp & Wässle, 2000), indicating that these cells may be post-synaptic to the GLT-1 cone bipolar cell (Data not shown).

Morphological characterization

In order to characterize the morphology of the GFP-labeled cells, whole mount preparations of the retina were used for targeted iontophoretic injections of Lucifer Yellow and Neurobiotin. Both the GCL (Figure 3) and INL (Figure 2) were dominated by two types each, one bright and one of intermediate brightness. Dimmer somas were often seen, especially in the GCL, but less reliably and we did not examine them in detail.

The brightest somas in both the INL and GCL could be reliably discriminated. We injected Lucifer yellow or Neurobiotin into about 200 somas in a number of different retinas and found reproducible dendritic morphologies. In addition, we filled many of the cells of intermediate brightness in each layer. A few cell types of reproducible morphology also resulted from these injections. All injections were in the mid-periphery to periphery.

Staining of the brightest GFP-positive cell in the INL reliably revealed a large, radiate amacrine cell whose sinuous dendrites were studded with varicosities (Figure 4a). The view in whole mount is reminiscent in these respects of the A17 amacrine cell of cat retina. However, this GFP-positive cell was found to stratify between the starburst amacrine cell bands (4b), while the reciprocal rod amacrine (A17 in the cat) ramifies with the rod bipolar cell endfeet. Double-staining with a PKC α antibody produced normal staining of rod bipolars with their terminals at the proximal part of the INL, well below the processes of this GFP⁺ cell (not shown). We are confident, then, that this is not the reciprocal rod amacrine, general appearances aside. We were able to estimate a coverage factor and density for this amacrine cell by filling 2 nearest neighbors of one of these cells (Figure 5). The distance from the cell to its neighbors was 82 and 119 μm , for an average of 101.5 μm . This translates into a cell density of 112 cells/ mm^2 . These three cells had an average dendritic field area of 27,970 μm^2 (diameter = 189 μm), which would produce a coverage factor of 3.1.

The cell type represented by the brightest soma in the ganglion cell layer was similarly characterized ($n = 21$). The morphology of this cell is shown in Figure 6a. It stratified primarily below the OFF cholinergic band (Fig 6b). Filling of the cells of intermediate intensity in the ganglion and amacrine cell layer produced a variety of types (Figure 7). The ganglion cell in

Figure 7C ramifies just distal to the cholinergic band in sublamina a. Based upon its size and stratification, it may be the B1 or B3 ganglion cells (Sun et al., 2002). The densities of these cells could not be reliably determined. Some other cell types with less GFP-fluorescence were also filled, with a much lower frequency. Staining of these GFP⁺ somas routinely revealed axons running toward the optic nerve head. We believe none of the GFP⁺ somas in the GCL are displaced amacrine cells, but ganglion cell subtypes.

Discussion

The present study shows that the CD44-GFP transgenic mouse retina contains four readily-identifiable types of GFP-expressing amacrine cells with two types having their somas located in the INL (Figure 4, Figure 7B) and two with somas in the GCL (Figure 6, Figure 7C). The most distinctive GFP-positive cell in the INL has a morphology similar to that of the A17 amacrine cell of cat retina, but stratifies between the starburst amacrine cell bands, while the reciprocal rod amacrine (A17 in the cat) ramifies with the rod bipolar cell endfeet.

As to the identity of the other GFP-expressing amacrine cells, it is usually difficult to make correspondences with morphological types from other studies. This is particularly the case when different staining techniques are used, and comparisons are made across species. The amacrine cell populations of the mouse retina have not yet been well characterized, but we noted that some known amacrine cell types ramify similarly to the brightest GFP-positive amacrine cell type in the CD44-GFP mice. One of the two amacrine cell types reported in transgenic mice that express GFP from the tyrosine hydroxylase promoter (Zhang et al., 2004) has a morphology and stratification depth similar to that of a CD44-GFP amacrine cell type. However, it does appear to have a slightly wider diameter and perhaps fewer dendrites, but might fall within the overall range of variation we noted across the mouse retina. Another cell that stratifies similarly might be one of the nitric oxide synthase-containing amacrine cells of mouse retina (Kim et al., 1999), but a lack of information regarding the appropriate amacrine cell in flatmounts, especially without its neighbors, makes fuller comparison difficult. The rabbit retina is the best characterized with respect to its amacrine cell population (Macneil et al., 1999), but none of the types we noted appear well suited for possible homology.

The most common and brightest GFP-labeled cell in the GCL stratified primarily below the OFF cholinergic band. Based on the morphology, it is difficult to match this cell type with those previously published in the mouse retina (Sun et al., 2002). However, based on its size (avg. dendritic field diameter = 239 μ m), branching pattern, and level of stratification, we suggest that it might be a type B1, or possibly A2, while the ganglion cell in Figure 7B may be a B1 or B3 ganglion cell, based upon its size and stratification. Comparison of the stratification pattern of the injected cells with those of the vertical sections in Fig. 2 suggest that the most distal layer of the processes may be primarily contributed by GFP-positive ganglion cells, while the middle band may be primarily contributed by the brightest amacrine cell, which ramifies at this depth. We have not found by iontophoretic injection a regular cell type which contributes to the proximal GFP-positive band.

As noted, the presence of GFP-expressing amacrine cells in the CD44-transgenic mice was a surprise because the transgene construct designed to direct GFP expression in retinal Muller cells. CD44 protein is known to be present in Müller cells, but not in retinal neurons (Chaitin & Wortham, 1994; Chaitin et al., 1994). Also, in the mammalian retina, CD44 is found on the Muller cell apical microvilli where it might mediate the attachment of the neural retina to the interphotoreceptor matrix (IPM).

We do not know the reason for the ectopic GFP expression in cells of the neural retina. It is likely, however, that this expression is due to a position effect, i.e., the CD44-EGFP construct

has integrated into the genome at a site that is under the control of a promoter active in the amacrine cell. Copy number of the integrated transgenes may also have contributed to this effect. A similar variability in patterns of XFP (variants of GFP) expression among mice generated from the same construct has been reported before (Feng et al., 2000). Nevertheless, GFP labels the amacrine cells in a 'Golgi-like' fashion, thus facilitating novel analyses of the synaptic connectivity and neurotransmitter receptor distribution in the retina. Furthermore, GFP-expressing cells are also found in the specific regions of the brain and the spinal cord in the CD44-GFP transgenic mice (unpublished data). These cells remain to be characterized and it is hoped that the CD44-GFP mice provide a valuable resource for electrophysiological and connectivity studies in the CNS, as the cell types we have reported can be targeted with a high degree of reliability.

Acknowledgements

This work was supported by grants from the National Eye Institute (EY13125, VS; EY 10121 and EY10608, SLM) and by unrestricted grants from the Research to Prevent Blindness, Inc.

References

- Ames A III, Nesbitt FB. In vitro retina as an experimental model of the central nervous system. *Journal of Neurochemistry* 1981;37:867–877. [PubMed: 7320727]
- Chaitin MH, Wortham HS, Brun-Zinkernagel AM. Immunocytochemical localization of CD44 in the mouse retina. *Experimental Eye Research* 1994;58:359–366. [PubMed: 7513650]
- Chaitin MH, Brun-Zinkernagel AM. Immunolocalization of CD44 in the dystrophic rat retina. *Experimental Eye Research* 1998;67:283–292. [PubMed: 9778409]
- Dowling, JE. *An Approachable Part of the Brain*. Belknap Press; Cambridge, MA: 1987. *The Retina*.
- Dubin MW. The inner plexiform layer of the vertebrate retina: a quantitative and comparative electron microscopic analysis. *Journal of Comparative Neurology* 1970;140:479–506. [PubMed: 4098652]
- Fei Y, Hughes TE. Transgenic expression of the jellyfish green fluorescent protein in the cone photoreceptors of the mouse. *Visual Neuroscience* 2001;18:615–623. [PubMed: 11829307]
- Feigenspan A, Gustincich S, Bean BP, Raviola E. Spontaneous activity of solitary dopaminergic cells of the retina. *Journal of Neuroscience* 1998;18:6776–6789. [PubMed: 9712649]
- Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JN, Lichtman JW, Sanes JR. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron* 2000;28:41–51. [PubMed: 11086982]
- Haverkamp S, Wässle H. Immunocytochemical analysis of the mouse retina. *Journal of Comparative Neurology* 2000;424:1–23. [PubMed: 10888735]
- Jeon C-J, Strettoi E, Masland RH. The major cell populations of the mouse retina. *Journal of Neuroscience* 1998;18:8936–8946. [PubMed: 9786999]
- Kim IB, Lee EJ, Kim KY, Ju WK, Oh SJ, Joo CK, Chun MH. Immunocytochemical localization of nitric oxide synthase in the mammalian retina. *Neurosci Lett* 1999;267:193–196. [PubMed: 10381009]
- MacNeil MA, Heussy JK, Dacheux RF, Raviola E, Masland RH. The shape and numbers of amacrine cells: Matching of photofilled with Golgi-stained cells in the rabbit retina and comparison with other mammalian species. *Journal of Comparative Neurology* 1999;41(3):305–326. [PubMed: 10524341]
- Masland RH, Raviola E. Confronting complexity: Strategies for understanding the microcircuitry of the retina. *Annual Review of Neuroscience* 2000;23:249–284.
- Masland RH. The fundamental plan of the retina. *Nature Neuroscience* 2001;4:877–886.
- Raviola E, Raviola G. Structure of the synaptic membranes in the inner plexiform layer of the retina: a freeze-fracture study in monkeys and rabbits. *Journal of Comparative Neurology* 1982;209:233–248. [PubMed: 7130454]
- Rodieck, RW. *The First Steps in Seeing*. Sinauer Associates; Sunderland, MA: 1998.
- Roska B, Werblin F. Vertical interactions across ten parallel, stacked representations in the mammalian retina. *Nature* 2001;410:583–587. [PubMed: 11279496]

- Sinclair JR, Nirenberg S. Characterization of Neuropeptide Y-expressing cells in the mouse retina using immunohistochemical and transgenic techniques. *Journal of Comparative Neurology* 2001;432:296–306. [PubMed: 11246209]
- Strettoi E, Masland RH. The organization of the inner nuclear layer of the rabbit retina. *Journal of Neuroscience* 1995;15:875–888. [PubMed: 7823187]
- Strettoi E, Masland RH. The organization of the inner nuclear layer of the rabbit retina. *Journal of Neuroscience* 1995;15:875–888. [PubMed: 7823187]
- Sun W, Li N, He S. Large-scale morphological survey of mouse retinal ganglion cells. *Journal of Comparative Neurology* 2002;451:115–126. [PubMed: 12209831]
- Wässle H, Riemann HJ. The mosaic of nerve cells in the mammalian retina. *Proceedings of the Royal Society of London, B. Biological Science* 1978;200:441–461.
- Yazulla S. GABAergic mechanisms in the retina. *Progress in Retina Research* 1986;5:1–51.
- Zhang DQ, Stone JF, Zhou T, Ohta H, McMahon DG. Characterization of genetically labeled catecholamine neurons in the mouse retina. *Neuroreport* 2004;15:1761–1765. [PubMed: 15257143]

Figure 1.
Construct for generating CD44-EGFP transgenic mice.

Figure 2.

Characterization of GFP-expressing cells in line 3234. **A.** Whole mount -Z-series with focus on the inner nuclear layer (INL); **B.** A transverse section showing labeled cells in the INL and a band in the IPL. Labeled cells are also seen in the GCL **C.** retinal section stained with anti-ChAT. Note the GFP band (green) lying in the middle of the ChAT bands (red). ChAT-positive cell bodies (red) can be seen in the INL and the GCL; **D–F.** Section stained with anti-GAD₆₇, **D.** anti-GAD, **E.** GFP and **F.** Combined. The arrows mark cell bodies with GFP and GAD co-localization. **G–I.** Section stained with anti-calretinin, **G.** anti-calretinin, **H.** GFP and **I.** Combined. Arrows show doubled-labeled cells. Also note co-localization in the middle band in the IPL.

Figure 3.
The GFP⁺ fluorescent somas in the GCL contain multiple cell types recognizable by their relative fluorescent intensities.

Figure 4.

(a) Injection of Neurobiotin into the brightest GFP-fluorescent soma in the inner nuclear layer reliably labeled an amacrine cell of the above morphology. (b) This cell stratified in about the middle of the inner plexiform layer.

Figure 5. Injection of three neighboring cells of the amacrine cell shown in Figure 2 reveals the consistent morphology and dendritic field size of this type of cell, as well as their dendritic overlap.

Figure 6. (a) Injection of Neurobiotin in the brightest GFP-fluorescent soma in the ganglion cell layer reliably led to cells with the morphology shown. (b) This ganglion cell type (red) ramified between the ChAT-immunoreactive bands (green), but predominantly in the OFF sublamina.

Figure 7. Injection of Neurobiotin into other GFP-fluorescent somas labeled two other types of somas in each layer. These were two ganglion cell types (left) and two amacrine cell types (right).