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SHORT COMMUNICATION

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Localization of CD15 immunoreactivity in the rat retina

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Abstract Using immunocytochemistry, we have investigated the localization of CD15 in the rat retina. In the present study, two types of amacrine cell in the inner nuclear layer (INL) and some cells in the ganglion cell layer were labeled with anti-CD15 antisera. Type 1 amacrine cells have large somata located in the INL, with long and branched processes ramifying mainly in stratum 3 of the inner plexiform layer (IPL). Type 2 cells have a smaller soma and processes branching in stratum 1 of the IPL. A third population showing CD15 immunoreactivity was a class of displaced amacrine cells in the ganglion cell layer. The densities of type 1 and type 2 amacrine cells were 166/mm^2 and 190/mm^2 in the central retina, respectively. The density of displaced amacrine cells was 195/mm^2. Colocalization experiments demonstrated that these CD15-immunoreactive cells exhibit γ-aminobutyric acid and neuronal nitric oxide synthase (nNOS) immunoreactivities. Thus, the same cells of the rat retina are labeled by anti-CD15 and anti-nNOS antisera and these cells constitute a subpopulation of GABAergic amacrine cells.

Keywords CD15 · GABA · nNOS · Colocalization · Retina · Rat (Sprague Dawley)

Introduction

CD15, the CD protein cluster designation for the epitope represented by 3[a1–3]-fucosyl-*N*-acetyl-lactosamine, is a glycoconjugate. The epitope has been reported to be involved in cellular adhesion by homophilic reactions (Bird and Kimber 1984; Fenderson et al. 1984, 1990) and by heterophilic reactions with selectin (Brandley et al. 1990; Springer and Laskey 1991; Kerr and Stocks 1992). In addition, it plays important roles in the differentiation of dendrites and in synapse formation (Mai and Schoenlau 1992; Schoenlau and Mai 1995; Mai et al. 1999).

The distribution of CD15-immunoreactive neurons has been studied in the retinas of several mammals, including monkeys (Andressen and Mai 1997; Chan et al. 2001a, b), mice (Andressen and Mai 1997), rats (Andressen and Mai 1997), tree squirrels (Andressen and Mai 1997), guinea pigs (Andressen and Mai 1997; Kim et al. 2001), and rabbits (Brown and Masland 1999). Although there are variations across species, CD15 is consistently expressed in morphologically defined subpopulations of amacrine cells, bipolar cells, and ganglion cells in all the mammalian retinas examined so far. In particular, subpopulations of amacrine cells have been found in all mammalian species examined, with the exception of the marmoset monkey (Andressen and Mai 1997).

This study was conducted to characterize the morphologically defined types of CD15-immunoreactive neurons in the rat retina using immunocytochemistry with antisera against CD15.

Materials and methods

Tissue preparation

Five adult Sprague-Dawley rats of either sex were used. The animals were treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea, Seoul, which conform to all National Institutes for Health guidelines. The animals were deeply anesthetized by intraperitoneal injection of 4% chloral hydrate (1 ml/100 g body weight). The eyes were enucleated, and the animals were killed by an overdose of 4% chloral hydrate. Afterwards, the anterior segments of the eyes were removed. For Western blot analysis, retinal tissues were quickly dissected on an ice-cold plate, frozen on dry ice, and stored at –70°C.

Eun-Jin Lee and Wook-Hyun Shon contributed equally to this study

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For immunocytochemistry, the eyecups were fixed by immersion in fixative [4% paraformaldehyde/0.2% picric acid in 0.1 M phosphate buffer (PB), pH 7.4] for 2–3 h. Following fixation, the retinas were carefully dissected and transferred to 30% sucrose in PB for 24 h at 4°C. They were then frozen in liquid nitrogen, thawed, and rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.4).

Western blot analysis

Western blot analysis was performed on the retinal extracts which were homogenized in 10 vol 20 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% sodium azide, 1 mM PMSF, and 5 µg/ml leupeptin. Protein concentration in each sample was assayed by the Lowry method (Lowry et al. 1951; Peterson 1979) in duplicate and the result averaged. Duplicate sets of protein standards containing 0, 1, 3, 5, 10, 20, 40, or 60 µg bovine serum albumin were assayed by the same method and results averaged and graphed to give a linear equation that was used to estimate the protein contents in retinal extracts. Optical density of each sample was measured at 660 nm using a spectrometer (Spectronic 20; Bausch and Lomb, Rochester, N.Y, USA). Aliquots of tissue samples corresponding to 25 µg total protein were heated at 100°C for 10 min with an equivalent volume of 2× sample buffer (containing 4% SDS and 10% mercaptoethanol) and loaded onto 10% polyacrylamide gels. The proteins were electrotransferred to a nitrocellulose membrane in TRIS-glycine-methanol buffer. The membrane was blocked for 1 h at room temperature in a blocking solution containing 5% non-fat dry milk, 0.05% Tween 20, and PBS (pH 7.4). The membrane was then incubated for 15 h at 4°C with a mouse monoclonal antibody directed against CD15 (diluted at 1:50; Zymed Laboratories, San Francisco, Calif., USA) in the blocking solution. The membrane was rinsed with 0.05% Tween 20 in PBS for three washes of 10 min and incubated for 1 h at room temperature in a 1:2,000 dilution of peroxidase-conjugated goat anti-mouse IgG antibody (Vector Laboratories, Burlingame, Calif., USA). The blot was washed for 10 min three times and then processed for analysis using an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, Ill., USA).

Immunostaining

For CD15 immunocytochemistry, immunostaining was performed using the avidin-biotin-peroxidase complex (ABC) method (Hsu et al. 1981). Forty-micrometer-thick vertical and horizontal vibratome sections were incubated in 10% normal goat serum and 1% Triton X-100 in PBS, for 1 h at room temperature, to block nonspecific binding sites. They were then incubated with a mouse monoclonal antibody directed against CD15 (Zymed Laboratories), diluted 1:50 in PBS containing 0.5% Triton X-100 for 3 days at 4°C. Retinas were washed in PBS for 45 min (3×15 min), incubated for 12 h in biotinylated goat anti-mouse IgG (Vector Laboratories) with 0.5% Triton X-100 at 4°C, rinsed in PBS, and subsequently incubated in ABC (Vector Laboratories) in PBS for 12 h at 4°C. Retinas were rinsed in two changes of PBS and three changes of 0.05 M TRIS-HCl buffer (TB), pH 7.6, for 5 min each at room temperature, and incubated in 0.05% 3,3′-diaminobenzidine tetrahydrochloride in TB for 10 min. Hydrogen peroxide was added to the incubation medium to a final concentration of 0.01%, and the container was gently shaken as the reaction proceeded. The reaction was stopped with several washes of TB and PB after 1–2 min, as determined by the degree of staining. The retinas were mounted on gelatin-coated slides with the ganglion cell layer facing upward, and coverslips were applied with glycerol. For the specificity of immunostaining, immunostaining was performed with the primary antibody that had been preabsorbed with 10^{-5} M synthetic CD15 (Sigma, St. Louis, Mo., USA).

For double-label studies of CD15 and γ-aminobutyric acid (GABA) or neuronal nitric oxide synthase (nNOS), 50-µm-thick vibratome sections were incubated overnight in a mixture of anti-CD antibody (1:50) and rabbit polyclonal anti-GABA antibody (Sigma; dilution rate 1:6,000) or rabbit polyclonal anti-nNOS antibody (Sigma; dilution rate 1:2,000) with 0.5% Triton X-100 in 0.1 M PB at 4°C. Sections were rinsed for 30 min with 0.1 M PBS and incubated in fluorescein-conjugated affinity-purified goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa., USA; dilution rate 1:50) and Cy3-conjugated goat anti-rabbit IgG (Jackson; dilution rate 1:500) for 1–2 h at room temperature. Sections were washed for 30 min with 0.1 M PB and coverslipped with 10% glycerol in 0.1 M PB. To ensure that the secondary antibody had not cross-reacted with the inappropriate primary antibody, some sections were incubated in rabbit polyclonal primary antibody followed by anti-mouse secondary antibody, while other sections were incubated in mouse primary antibody followed by anti-rabbit secondary antibody. These sections did not show any immunostaining. Sections were analyzed using a BioRad Radiance Plus confocal scanning microscope (BioRad, Hemel Hempstead, UK), installed on a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan). Fluorescein and Cy3 signals were always detected separately. Fluorescein labeling was excited using the 488 nm wavelength line of the argon ion laser and detected after passing a HQ513/30 emission filter (BioRad). For detection of the Cy3 signal, the 543-nm line of the green HeNe laser was used in combination with the 605/32-emission filter (BioRad).

Results and discussion

CD15 is a glycoconjugate that may be involved in cellular adhesion functions by means of homophilic reactions (Bird and Kimber 1984; Fenderson et al. 1984, 1990) and heterophilic reactions with selectin (Brandley et al. 1990; Springer and Laskey 1991; Kerr and Stocks 1992). In the adult brain of various species, CD15 has been consistently detected by immunocytochemistry in glial cells and certain neuronal cells (Niedieck and Löhler 1987; Mai and Reifenberger 1988; Gocht and Löhler 1993; Gocht et al. 1994, 1996), but its functions in the adult brain are unclear.

In order to characterize an antiserum against CD15, Western blot analysis and immunostaining with anti-CD15 antibody preincubated with antigen were performed. As shown in Fig. 1A, Western blot experiments revealed a distinct band of CD15 glycosylated proteins. The molecular weight of the immunoreactive band was 90 kDa, as shown in the chicken retina (Andressen et al. 1996). No immunostaining was seen in the section immunostained with anti-CD15 antibody preincubated with antigen (Fig. 1B).

In the present study, specific CD15 immunoreactivity was present within the inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) in all retinal regions. No CD15 immunoreactivity was observed in the outer nuclear layer, ganglion cell axon layer, or optic nerve head. CD15-labeled cell somata were sparsely distributed in the INL and GCL throughout the retina (Fig. 2). The labeled somata in the INL were round or oval. Two sorts of labeled amacrine cell, types 1 and 2, were distinguishable according to their morphological features. The type 1 cells had a larger soma and showed strong CD15 immunoreactivity, whereas the type 2 cells had a smaller soma and showed weak immunoreactivity (Fig. 2A). CD15-labeled somata in the

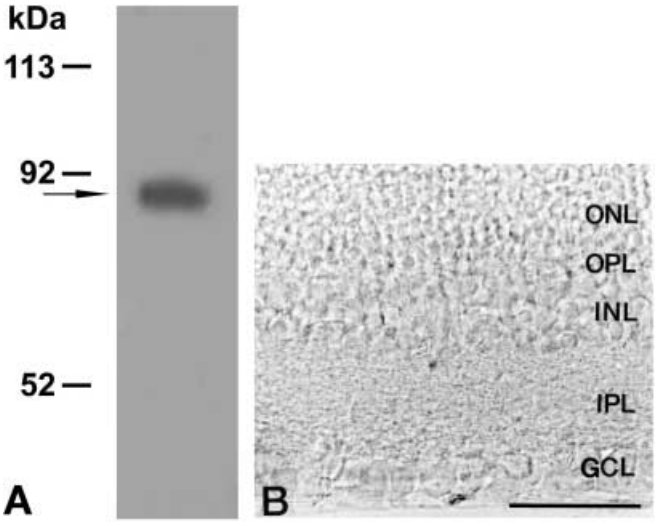
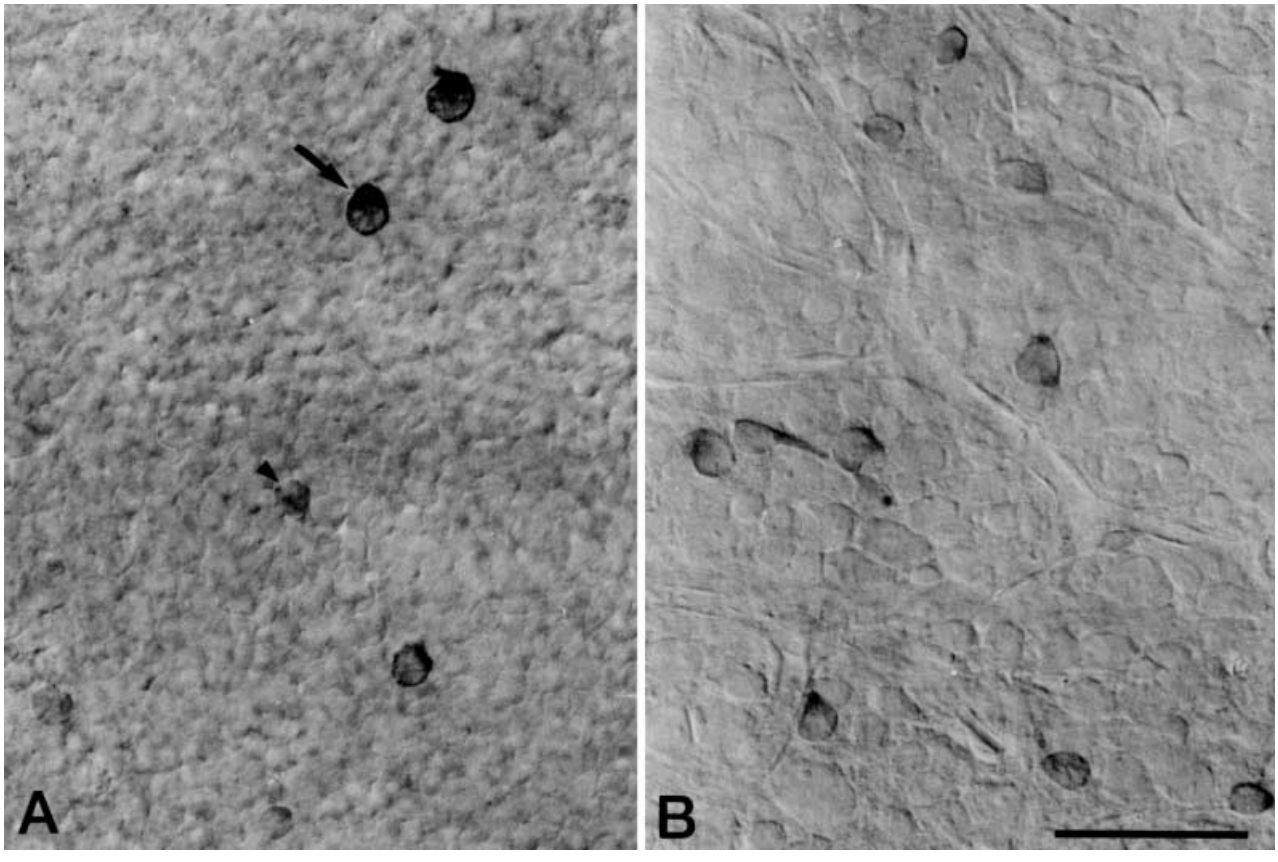


Fig. 1A, B Characterization of anti-CD15 antibody. **A** Western blot analysis of CD15 in the rat retina. Position of molecular weight (kDa) is indicated by an *arrow*. **B** A 40-µm-thick vibratome section was immunostained with anti-CD15 antibody preincubated with antigen. No immunostaining is seen (*ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer). *Scale bar* 50 µm

GCL were round or oval and showed weak to moderate immunoreactivity (Fig. 2B).

The processes emerging from the type 1 cells ramified mainly in stratum 3 of the IPL, whereas the processes originating from the type 2 cells and cells in the GCL ramified in strata 1 and 5 of the IPL, respectively (Figs. 3, 4). The terminal branches originating from type 2 amacrine cells and displaced amacrine cells are too fine to trace. The primary process originating from type 1 cells branches into three secondary processes, which could be followed up to about 250 µm from individual cell bodies (Fig. 4). Soma sizes were measured from 50 cells of each type of cell located in central regions of the retina. Each cell body was measured by an Image Analyzer. The mean diameter (means ± SD) of the type 1 cells was 10.4±0.8 µm and that of the type 2 cells was 7.0±0.6 µm. The soma size of the displaced amacrine cells was similar to that of the type 1 cells (11.5±1.1 µm). The densities (means ± SD) of the CD15-immunoreactive cells were also calculated for the central region. The mean densities were 165.5±13.7/mm^2 for the type 1 cells, 189.7±18.6/mm^2 for the type 2 cells, and 195.3±11.2/mm^2 for the displaced amacrine cells.

Fig. 2A, B Light micrographs taken from 40-µm-thick horizontal vibratome sections processed for CD15 immunoreactivity. The field is taken from the midperipheral nasal region of the retina. **A** The focal plane is at the inner nuclear layer, and several round or oval cell bodies showing CD15 immunoreactivity are visible. According to the soma size and label intensity, two types, type 1 (*arrow*) and type 2 (*arrowhead*), of labeled amacrine cells are clearly distinguishable. **B** The focal plane is at the ganglion cell layer (GCL). Several somata showing weak to moderate immunoreactivity are visible. *Scale bar* 50 µm



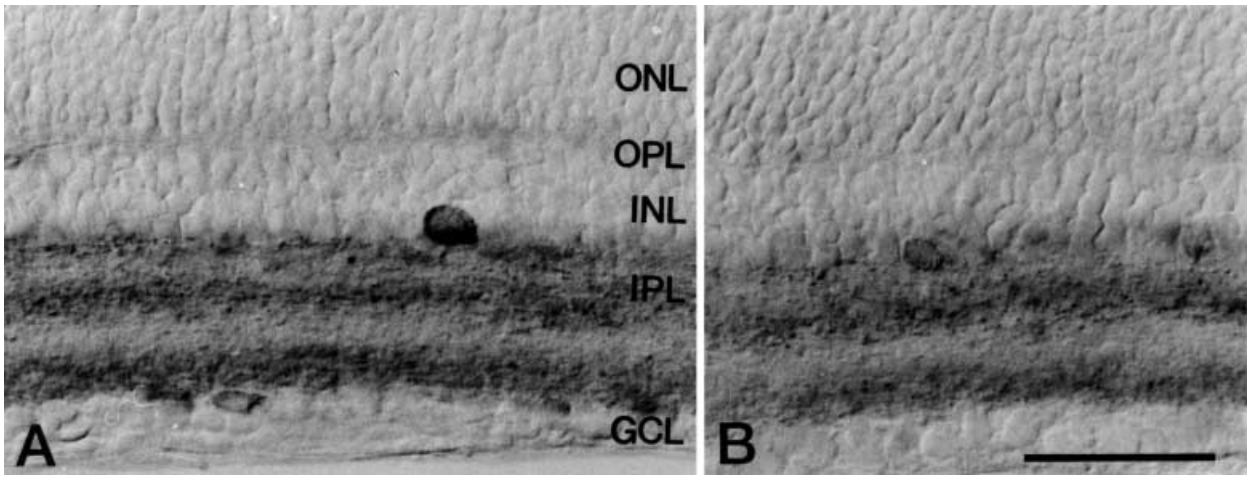


Fig. 3A, B Light micrographs taken from 40-µm-thick vertical vibratome sections processed for CD15 immunoreactivity. **A** CD15 immunoreactivity is present in an amacrine cell body (type 1) located in the inner nuclear layer (*INL*), and also in a cell in the ganglion cell layer (*GCL*). **B** Type 2 amacrine cell body showing CD15 immunoreactivity is visible in the INL. Three bands of labeled processes are visible in the inner plexiform layer (*IPL*); one is in stratum 1 and the other is in strata 3 and 5. *ONL* outer nuclear layer, *OPL* outer plexiform layer. *Scale bar* 50 µm

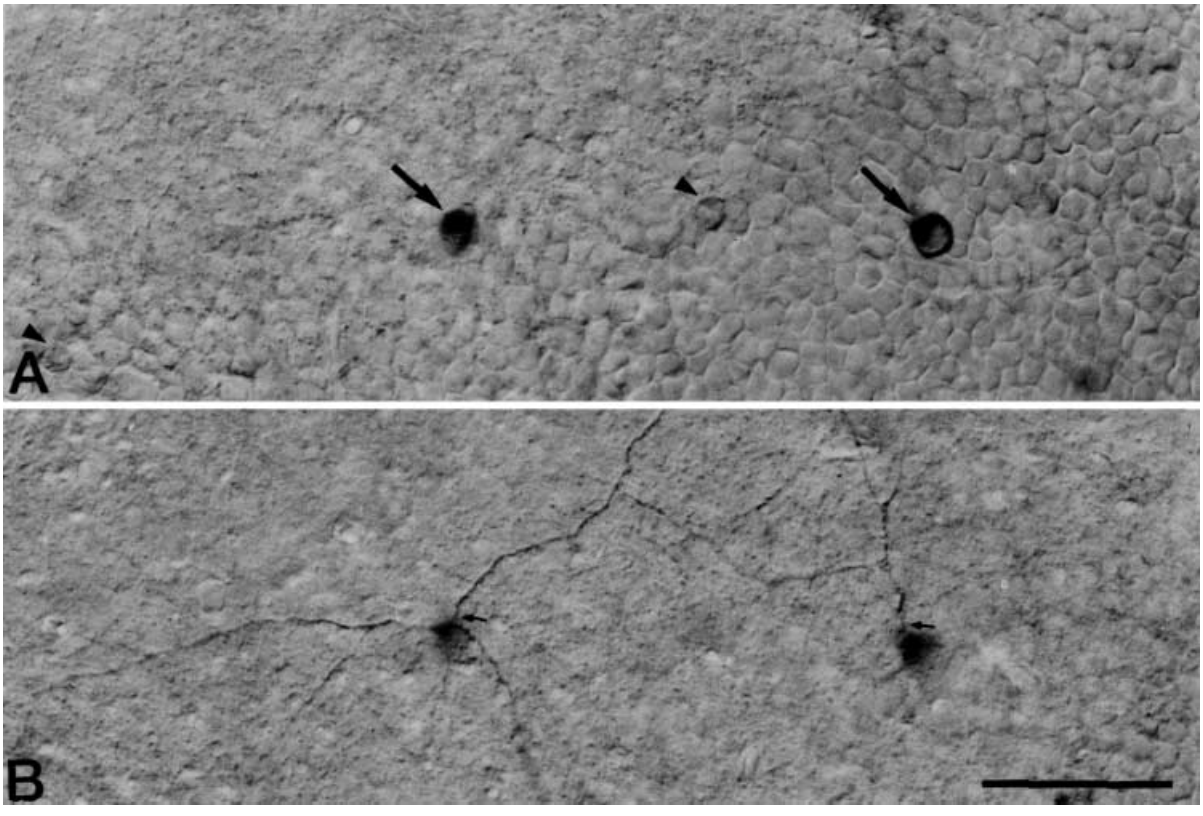


Fig. 4A, B Light micrographs taken at different focal planes in the same field of a 40-µm-thick horizontal vibratome section processed for CD15 immunoreactivity. **A** The focal plane is at the inner nuclear layer. CD15 immunoreactivity is seen to be localized to type 1 (*arrows*) and type 2 (*arrowheads*) amacrine cell somata. **B** The focal plane is at the middle of the inner plexiform layer. Primary dendrite originating from type 1 cell is seen to branch into three secondary processes. At this level, the somata of the labeled amacrine cells are out of focus. *Scale bar* 50 µm

To identify whether CD15 and GABA immunoreactivities are expressed within the same amacrine cells, double-labeling experiments using antisera to CD15 and GABA were performed. Immunostaining using GABA antibodies is in good agreement with earlier reports on the rat retina (Agardh et al. 1986; Mosinger et al. 1986; Versaux-Botteri et al. 1989; Lake 1994; Fletcher and Kalloniatis 1997), with numerous GABA-immunoreac-

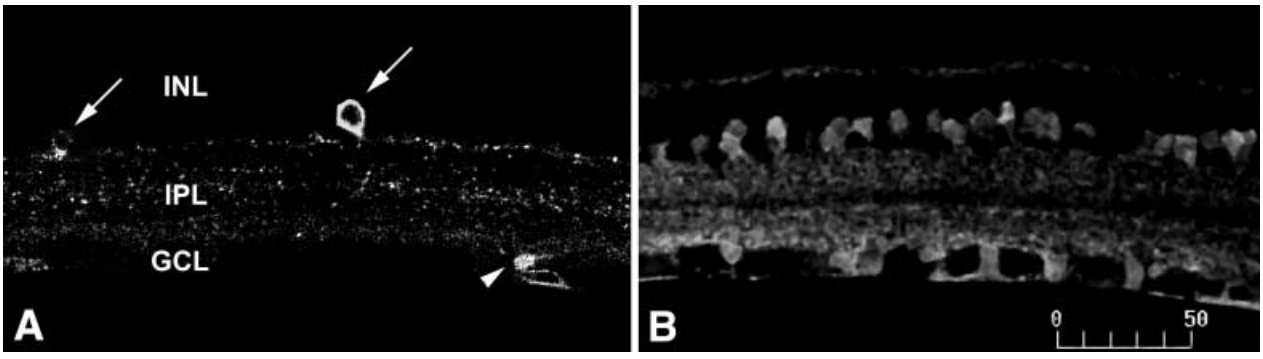


Fig. 5 Confocal micrographs taken from a vertical vibratome section processed for CD15 (**A**) and γ-aminobutyric acid (GABA; **B**) immunoreactivities. CD15 immunoreactivity was visualized using a fluorescein-conjugated secondary antibody, and GABA immunoreactivity was visualized using a Cy3-conjugated secondary antibody. **A** Two amacrine cell somata (*arrows*) and one displaced amacrine cell body (*arrowhead*) showing CD15 immunoreactivity are visible in the inner margin of the inner nuclear layer (*INL*) and in the ganglion cell layer (*GCL*). **B** Numerous GABA-immunoreactive cell bodies are visible in the INL and GCL. The IPL contains a dense network of labeled processes. Comparison of the two figures clearly demonstrates that CD15-labeled cells display GABA immunoreactivity. *Scale bar* 50 µm

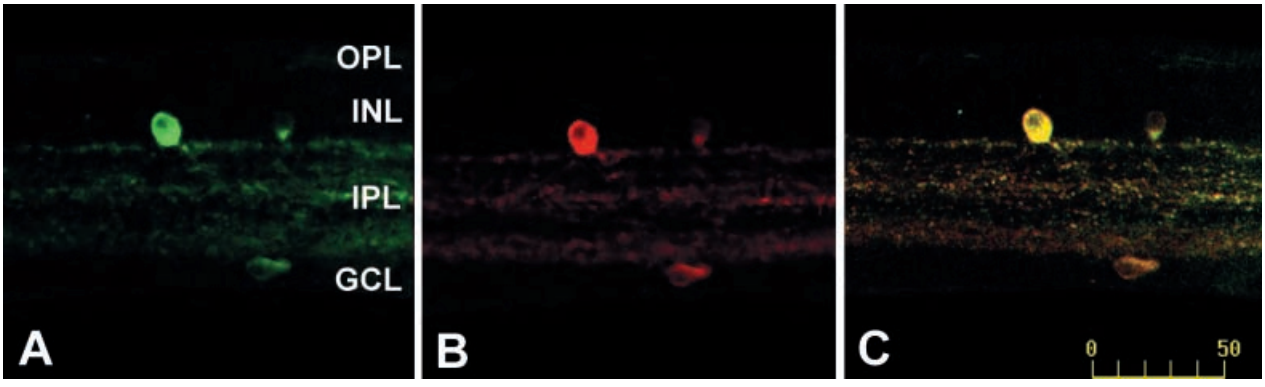


Fig. 6 Confocal micrographs taken from a vertical vibratome section processed for CD15 (**A**) and neuronal nitric oxide synthase (nNOS; **B**) immunoreactivities. CD15 immunoreactivity was visualized using a fluorescein-conjugated secondary antibody (*green*), and nNOS immunoreactivity was visualized using a Cy3-conjugated secondary antibody (*red*). **A** Three CD15-immunoreactive cell bodies are visible. **B** Three nNOS-immunoreactive cell bodies are seen. **C** Colocalization (*yellow*) of CD15 and nNOS within the same cells is clearly noted. *Scale bar* 50 µm

tive amacrine and displaced amacrine cells and dense processes in the IPL. Figure 5 shows an example of a vibratome section double-immunostained with anti-CD15 (Fig. 5A) and anti-GABA (Fig. 5B) antisera. Figure 5A, B shows CD15 and GABA immunoreactivities are colocalized within the same cells. All CD15-labeled cells (n=187) investigated in this study showed GABA immunoreactivity. Therefore, we conclude that all CD15-immunoreactive cells also express GABA immunoreactivity and thus the CD-labeled cells constitute a subpopulation of GABAergic cells in the rat retina, as previously shown in the guinea pig (Kim et al. 2001). As the morphological features of CD15-labeled cells in the rat retina were similar to those of nNOS-labeled cells previously reported in the rat retina (Oh et al. 1998; Chun et al. 1999; Kim et al. 2000), we used double-labeling experiments to examine whether CD15 and nNOS immunoreactivities are expressed within the same amacrine cells. All the CD15-labeled amacrine cells (n=85) investigated in the present study also showed nNOS immunoreactivity (Fig. 6). Therefore, we conclude that the same cells of the rat retina are labeled by anti-CD15 and anti-nNOS antisera.

In the present study, CD15 immunoreactivity was clearly localized to a certain population of amacrine cells and displaced amacrine cells, in general agreement with the findings from a previous study of the rat retina (Andressen and Mai 1997). CD15-labeled amacrine cells could be subdivided into types 1 and 2, according to their morphological features. However, our findings, together with previous reports, demonstrate great variation in the type or types of CD15-immunoreactive neurons in different mammalian retinas immunostained with the same antisera. For instance, in the rabbit retina, a population of cone bipolar cells with axon terminals providing synaptic input to the ON-starbust amacrine cells and/or to the ON-plexus of the ON-OFF direction-selective ganglion cells has been reported to show CD15 immunoreactivity (Brown and Masland 1999). In the monkey retina, the antibody against CD15 labels a single population of ON bipolar cells, which correspond to dif-

fuse bipolar (DB) type 6 cells (Chan et al. 2001a, b). In the guinea pig retina, two types of amacrine cells, type 1 and type 2 cells, and some displaced amacrine cells are CD15 immunoreactive (Kim et al. 2001). However, processes originating from type 1 cells of the guinea pig retina stratify in strata 4 and 5 of the IPL, unlike those of rat retina. This diversity of morphological types of CD15-immunoreactive cells in mammalian retinas suggests that these cells have different roles in the retinas of different species or that the expression of CD15 may depend on the visual behavior of a given species, as suggested by Andressen and Mai (1997).

In the present study, all CD15-labeled cells showed nNOS and GABA immunoreactivities, demonstrating that CD15, nNOS, and GABA are coexpressed in the same neuronal types in the rat retina. It has been reported that nNOS-immunoreactive amacrine cells comprise a subpopulation of GABAergic amacrine cells in both the rabbit and rat retinas (Oh et al. 1998). Taken together, in the rat retina, the same cells express both CD15 and nNOS immunoreactivities and these cells comprise a subpopulation of GABAergic cells. Although the functional significance of such a colocalization of CD15 and GABA or nNOS within the same neurons of the rat retina remains unknown, anti-CD15 antisera could be used as a useful marker to identify particular morphological cell types in the mammalian retina.

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