THE JOURNAL OF COMPARATIVE NEUROLOGY 470:372–381 (2004)

AII Amacrine Cells in the Mammalian Retina Show Disabled-1 Immunoreactivity

EUN-JIN LEE,[1] HYUN-JU KIM,[1] EUN-JIN LIM,[1] IN-BEOM KIM,[1] WHA-SUN KANG,[2] SU-JA OH,[1] DENNIS W. RICKMAN,[3] JIN-WOONG CHUNG, AND MYUNG-HOON CHUN[1]*

[1]Department of Anatomy, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea

[2]Department of Natural Sciences, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea

[3]Departments of Ophthalmology and Neurobiology, Duke University, Durham, North Carolina 27710

ABSTRACT

Disabled 1 (Dab1) is an adapter molecule in a signaling pathway, stimulated by Reelin, which controls cell positioning in the developing brain. It has been localized to AII amacrine cells in the mouse and guinea pig retinas. This study was conducted to identify whether Dab1 is commonly localized to AII amacrine cells in the retinas of other mammals. We investigated Dab1-labeled cells in human, rat, rabbit, and cat retinas in detail by immunocytochemistry with antisera against Dab1. Dab1 immunoreactivity was found in certain populations of amacrine cells, with lobular appendages in the outer half of the inner plexiform layer (IPL) and a bushy, smooth dendritic tree in the inner half of the IPL. Double-labeling experiments demonstrated that all Dab1-immunoreactive amacrine cells were immunoreactive to antisera against calretinin or parvalbumin (i.e., other markers for AII amacrine cells in the mammalian retina) and that they made contacts with the axon terminals of the rod bipolar cells in the IPL close to the ganglion cell layer. Furthermore, all Dab1-labeled amacrine cells showed glycine transporter-1 immunoreactivity, indicating that they are glycinergic. The peak density was relatively high in the human and rat retinas, moderate in the cat retina, and low in the rabbit retina. Together, these morphological and histochemical observations clearly indicate that Dab1 is commonly localized to AII amacrine cells and that antiserum against Dab1 is a reliable and specific marker for AII amacrine cells of diverse mammals. J. Comp. Neurol. 470:372–381, 2004.

Indexing terms: parvalbumin; calretinin; glycine transporter-1; protein kinase C; rod bipolar cells; immunocytochemistry

Amacrine cells constitute the most diverse group of cell types in the retina in terms of morphology, size, and distribution. They appear to make up approximately 40% of all the neurons of the inner nuclear layer (INL). By using several techniques, such as Golgi staining (Dowling and Boycott, 1969; Kolb and Nelson, 1981; Mariani, 1990), intracellular injection with Lucifer yellow (Tauchi and Masland, 1984; Vaney, 1984, 1986; Sandell and Masland, 1986; Menger et al., 1998), and photofilling (MacNeil and Masland, 1998; MacNeil et al., 1999), 20–30 distinct types of amacrine cells have been identified in the mammalian retina (Masland, 1988; Vaney, 1990; Wässle and Boycott, 1991; Strettoi and Masland, 1996; MacNeil and Masland, 1998; MacNeil et al., 1999). Among these cells, one of the best characterized amacrine cells is the AII amacrine cell, which is a bistratified rod amacrine cell that has been described previously (Famiglietti and Kolb, 1975; Kolb,

Grant sponsor: Korea Research Foundation; Grant number: 2000-FP0001; Grant sponsor: National Institutes of Health; Grant number: EY11389.

*Correspondence to: Myung-Hoon Chun, Department of Anatomy, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Socho-gu, Seoul 137-701, Korea. E-mail: mhchun@catholic.ac.kr

Received 25 July 2003; Revised 23 October 2003; Accepted 30 October 2003.

DOI 10.1002/cne.20010

Published online the week of February 2, 2004 in Wiley InterScience (www.interscience.wiley.com).

1979). The AII amacrine cells form one part of a postsynaptic dyad at the ribbon synapse of rod bipolar cells in the inner plexiform layer (IPL), close to the ganglion cell layer (GCL; Kolb and Famiglietti, 1974; McGuire et al., 1984). They also make conventional chemical synapses with OFF-cone bipolar and OFF-ganglion cells and contact ON-cone bipolar cells via large gap junctions (Kolb and Famiglietti, 1974; Strettoi et al., 1992; Chun et al., 1993; Grünert and Wässle, 1996). AII amacrine cells are depolarized in response to light (Dacheux and Raviola, 1986; Boos et al., 1993), and stimulation of AII amacrine cells results in the hyperpolarization of OFF-cone bipolar and OFF-ganglion cells via inhibitory chemical synapses and the depolarization of ON-cone bipolar cells via gap junctions. In this way, AII amacrine cells play crucial roles in the rod-driven circuit in the retinas of various mammals.

Different methods have been used to identify AII amacrine cells in mammals. For example, in the cat and rabbit retinas, AII amacrine have been labeled by selective staining with a fluorescent dye (Vaney, 1985; Mills and Massey, 1991; Vaney et al., 1991). In immunocytochemical methods, different markers have been used across species. An antibody against parvalbumin has been used to label AII amacrine cells in the rat, cat, and rabbit retinas (Gabriel and Straznicky, 1992; Chun et al., 1993; Wässle et al., 1993; Casini et al., 1995). Antisera against calretinin also label AII amacrine cells in the rabbit (Mills and Massey, 1991; Massey and Mills, 1999), cat (Pasteels et al., 1990). and macaque monkey (Wässle et al., 1995; Mills and Massey, 1999; Kolb et al., 2002) retinas. However, in addition to AII amacrine cells, additional types of amacrine cells were labeled by an antibody against parvalbuimin or calretinin in these mammalian retina. Recently, antiserum against Disabled 1 (Dab1), an adapter molecule in a signaling pathway stimulated by Reelin, which controls cell positioning in the developing brain (Rice and Curran, 1999), has been shown to label AII amacrine cells selectively in the mouse (Rice and Curran, 2000) and guinea pig (Lee et al., 2003) retinas.

In the present study, we describe Dab1 immunoreactivity patterns and characterize Dab1-immunoreactive amacrine cells both morphologically and quantitatively in different species. The Dab1 staining patterns and densities in human, rat, rabbit, and cat retinas were compared with previous markers for AII amacrine cell in various mammals to establish whether Dab1 antisera is a common, specific, and reliable marker for AII amacrine cells in these species.

MATERIALS AND METHODS

Tissue preparation

In total 12 mammalian retinas were examined, from Sprague-Dawley rat (six retinas), New Zealand white rabbit (two retinas), cat (two retinas), and human (two retinas). The animals were treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea, Seoul, which conform to all NIH guidelines. The animals were deeply anesthetized by intraperitoneal injection of 4% chloral hydrate (1 ml/100 g body weight), and the eyes were enucleated. The anterior segments were then removed, and the eyecups were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 2–3 hours. Human eyes were obtained through the eye bank of St. Mary's Hospital of the Catholic University of Korea. The eyes were enucleated and fixed in 4% paraformaldehyde in PB and sent to our laboratory within 24 hours after the death of the donor. After fixation, the retinas were carefully dissected and transferred to 30% sucrose in PB for 24 hours at 4°C. They were then frozen in liquid nitrogen, thawed, and rinsed in 0.01 M phosphate-buffered saline (PBS; pH 7.4).

Immunocytochemistry

For fluorescence immunocytochemistry, 50-μm-thick vibratome sections were incubated in 10% normal goat serum (NGS) and 1% Triton X-100 in PBS, for 1 hour at room temperature, to block nonspecific binding sites. The sections were then incubated overnight with a rabbit polyclonal antibody directed against Dab1 [the anti-Dab1 antiserum was developed in rabbit by using the peptide C-terminal (CGEPPSGGDNISPQDGS) that correspond to the mDab555 sequence beginning at residue 542 (Howell et al., 1997); kindly provided by Dr. B. Howell, NIH] at a dilution of 1:1,000 in PBS containing 0.5% Triton X-100 at 4°C. Retinas were washed in PBS for 45 minutes (3 × 15 minutes) and incubated for 2 hours in carboxymethylindocyanine (Cy3)-conjugated affinity-purified anti-rabbit IgG (1:100; Jackson, West Grove, PA) at room temperature. Sections were then washed for 30 minutes with 0.1 M PB and coverslipped with 10% glycerol in 0.1 M PB. For whole-mount immunostaining, the same immunocytochemical procedures as described above were used, but with longer incubation times. Images were imported into Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA), and, for presentation, all manipulations (brightness and contrast only) were carried out equally.

For double-label studies, sections were incubated overnight in a mixture of anti-Dab1 antibody (1:1,000) with the following antibodies: monoclonal antiparvalbumin (1:500 dilution; Sigma, St. Louis, MO), monoclonal anticalretinin (1:1,000 dilution; Chemicon, Temecula, CA), monoclonal antiprotein kinase C (PKC; 1:500 dilution; Sigma), and goat polyclonal antiglycine transporter-1 antibody (1:10,000 dilution; Chemicon) with 0.5% Triton X-100 in 0.1 M PBS at 4°C.

Sections were rinsed for 30 minutes with 0.1 M PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated affinity-purified anti-mouse IgG or anti-goat IgG (1:100 dilution; Jackson) or with Cy3-conjugated anti-rabbit IgG (1:100 dilution; Jackson) for 1–2 hours at room temperature. Sections were washed for 30 minutes with 0.1 M PB and coverslipped with 10% glycerol in 0.1 M PB. To confirm that the secondary antibody did not cross-react with an inappropriate primary antibody, some sections were incubated in rabbit and goat polyclonal primary antibody, followed by anti-mouse secondary antibody, and other sections were incubated in mouse primary antibody, followed by anti-rabbit secondary antibody. These sections showed no immunostaining.

Confocal laser scanning microscopy

Sections were analyzed with a Bio-Rad Radiance Plus (Bio-Rad, Hemel Hempstead, United Kingdom) confocal scanning microscope installed on a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan). FITC and Cy3 signals were always detected separately. FITC labeling was excited using the 488-nm line of an argon ion laser and detected after passing an HQ513/30 (Bio-Rad) emis-

sion filter. For the detection of the Cy3 signal, the 543-nm line of a green HeNe laser was used in combination with the 605/32 (Bio-Rad) emission filter. Images were imported into Adobe Photoshop 5.5 (Adobe Systems) and photographed on slide film (Kodak Ektachrome 100; Eastman Kodak). For presentation, all manipulations (brightness and contrast only) were carried out equally.

Topography and quantitation

The topography of the Dab1-immunoreactive cell populations was analyzed, and these populations were quantified in well-stained retinas of human, rat, rabbit, and cat. The data for the density maps were plotted with conventional microscopy. For the density maps, a field of 200 × 200 μm^2 was sampled in 1-mm steps in the retina from superior to inferior. Nearest-neighbor analysis (Wässle and Riemann, 1978) was performed on the cells located in the midperipheral regions of the retina. The results were not corrected for shrinkage of the tissue during the mounting process, because this was negligible.

RESULTS

Dab1 immunoreactivity in the mammalian retina

In all mammalian retinas investigated, Dab1 immunoreactivity was found in a distinct subpopulation of amacrine cells (Fig. 1A–D). The somata of Dab1-immunoreactive cells were strongly immunostained and were found in the INL adjacent to the IPL border (Fig. 1A–D). These amacrine cells typically have a single primary dendrite that descends into the IPL and then gives off several side branches that run toward stratum 5 near the GCL. In sublamina a, short immunoreactive processes with large, irregular endings originate from the primary process. These processes resemble the lobular appendages previously described for AII amacrine cells of rabbit, cat, rat, monkey, mouse, and guinea pig retinas (Famiglietti and Kolb, 1975; Vaney, 1985; Dacheux and Raviola, 1986; Wong et al., 1986; Young and Vaney, 1990; Mills and Massey, 1991; Vaney et al., 1991; Strettoi et al., 1992; Wässle et al., 1993, 1995; Rice and Curran, 2000; Lee et al., 2003). Therefore, Dab1 immunoreactivity appeared in the same amacrine cell population in all species examined in the present study. However, in the human and cat retinas, Dab1 immunoreactivity was visible in other types of neurons in addition to amacrine cells. In the human retina (Fig. 1A), Dab1 immunoreactivity was visible in the outer segments of photoreceptor cells, in the horizontal cells that form a fiber plexus in the outer plexiform layer (OPL), and in some bipolar cells with processes directed both to the IPL and to the OPL. In the cat retina (Fig. 1D), Dab1 immunoreactivity was seen in certain neurons in the GCL.

When we focused on the INL in whole-mount retinal preparations of each species, numerous Dab1-immunoreactive amacrine cell somata were distributed throughout the retina (Fig. 2A–D). These somata were round or pyriform in shape. In Figure 2A–D, the lobules are clearly visible in sublamina a of the IPL. A different focal plane at the inner aspect of the IPL shows a few processes ramifying in stratum 5 of the IPL. The terminal branches of labeled cells were difficult to trace, insofar as they intermingled with other labeled processes originating from other Dab1-labeled cells (data not shown).

Double immunofluorescence for Dab1 and AII amacrine cell markers

A few immunocytochemical markers that recognize AII amacrine cells, such as antisera against parvalbumin (Wässle et al., 1995) and calretinin (Wässle et al., 1995; Massey and Mills, 1999; Mills and Massey, 1999), were applied to the retinas of cat, rabbit, human, and rat. To identify whether Dab1 and calretinin or parvalbumin immunoreactivities are expressed within the same amacrine cells in different species, double-labeling experiments were performed. Figure 3 shows an example of vibratome sections doubly labeled with antisera against Dab1 (Fig. 3A,D,G,J) and calretinin (Fig. 3B) or parvalbumin (Fig. 3E,H,K) in human (Fig. 3A–C), rat (Fig. 3D–F), rabbit (Fig. 3G–I), and cat (Fig. 3J–L) retinas. In merged images (Fig. 3C,F,I,L), all Dab1-immunoreactive amacrine cells show calretinin (Fig. 3C) or parvalbumin (Fig. 3F,I,L) immunoreactivity. In the human, rat, and rabbit retinas, amacrine cells showing no colocalization of Dab1 and calretinin (Fig. 3C) or parvalbumin immunoreactivities were observed (Fig. 3F, I). The parvalbumin-labeled amacrine cells in the rat and rabbit retinas have been reported to belong to a class of wide-field amacrine cells (Wässle et al., 1993; Casini et al., 1995). Therefore, our results clearly demonstrate that antisera directed against Dab1 specifically labeled AII amacrine cells with lobular appendages and narrow-field dendrites in the mammalian retinas examined in the present study.

Dab1-labeled amacrine cells show Glyt-1 immunoreactivity

In the mammalian retina, most amacrine cells contain either glycine or γ-aminobutyric acid (GABA; Marc, 1989; Vaney, 1990; Marc et al., 1995; Vardi and Auerbach, 1995). Moreover, the Dab1 is known to label AII amacrine cells in the mammalian retina (Rice and Curran, 2000; Lee et al., 2003), which are glycinergic (Marc and Liu, 1985; Pourcho and Goebel, 1985; Grünert and Wässle, 1993; Goebel and Pourcho, 1997; Wright et al., 1997; Menger et al., 1998; Rice and Curran, 2000). Therefore, to confirm whether Dab1-labeled amacrine cells are glycinergic, we further characterized the Dab1-labeled amacrine cells neurochemically by using double labeling with antisera directed against Dab1 and glycine transporter 1 (Glyt-1). Figure 4 shows vibratome sections doubly labeled with antibodies against Dab1 (Fig. 4A) and Glyt-1 (Fig. 4B) in the human retina. All Dab1-labeled amacrine cells in human retina showed Glyt-1 immunoreactivity. Therefore, Dab1-labeled amacrine cells constitute a subpopulation of glycinergic amacrine cells.

Double immunofluorescence for Dab1 and PKC

The AII amacrine cell is one of two types of postsynaptic amacrine cells at rod bipolar ribbon synapses (Kolb and Famiglietti, 1974; Famiglietti and Kolb, 1975; Kolb, 1979; Pourcho, 1982; McGuire et al., 1984; Dacheux and Raviola, 1986; Raviola and Dacheux, 1987; Voigt and Wässle, 1987; Sterling et al., 1988; Kolb et al., 1990, 1991; Strettoi et al., 1990, 1992; Chun et al., 1993; Wässle et al., 1995). PKC is expressed primarily by rod bipolar cells in the mammalian retina (Negishi et al., 1988; Greferath et al., 1990), so we used PKC to identify rod bipolar cells in the mammalian retina. Figure 5 shows vertical sections

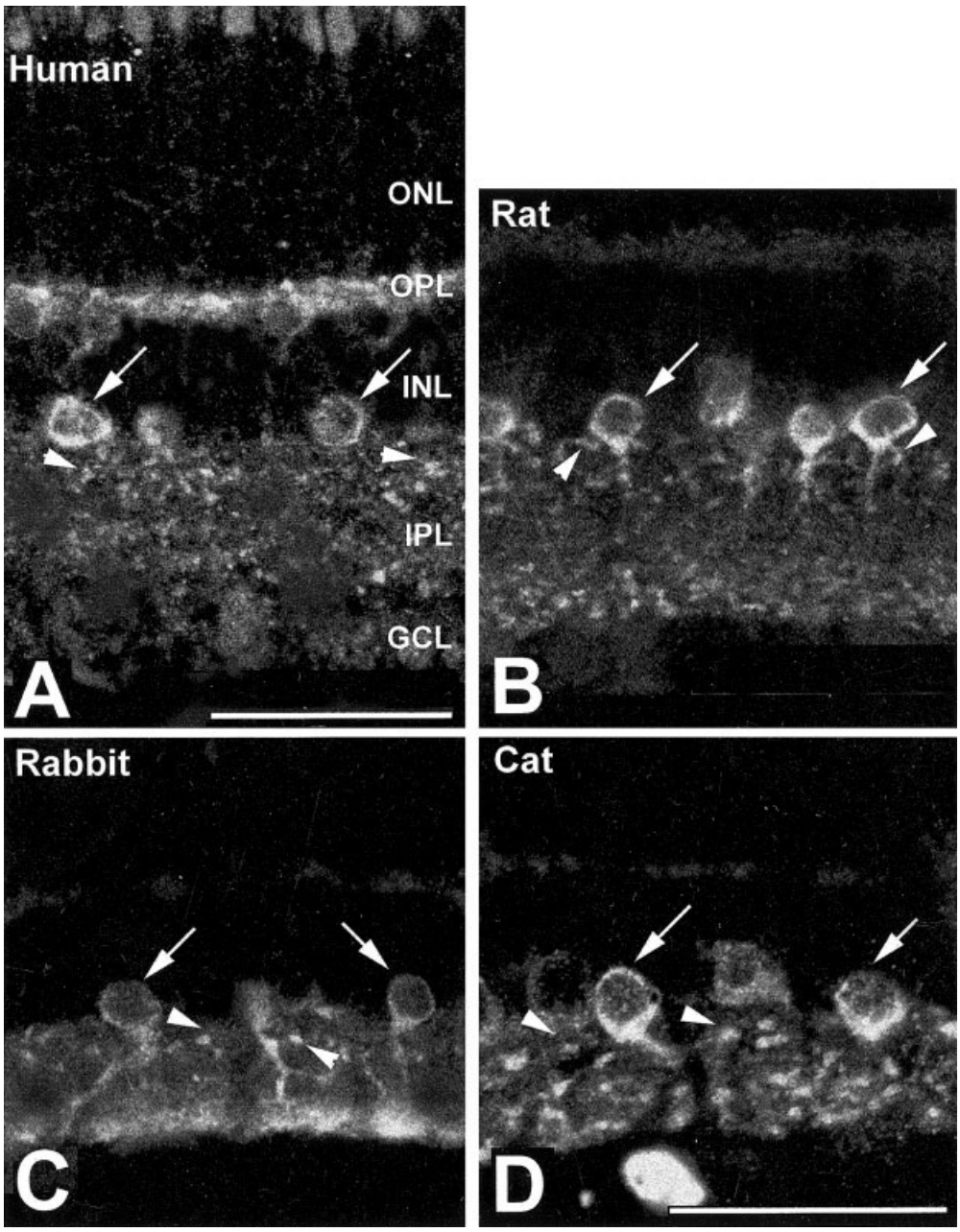

Fig. 1. Light micrographs taken from 50-μm-thick vertical vibratome sections processed for Dab1 immunoreactivity. In the human (**A**), rat (**B**), rabbit (**C**), and cat (**D**) retinas, Dab1 immunoreactivity is present in amacrine cell bodies (arrows) located in the proximal inner nuclear layer (INL) and processes located in the entire thickness of the inner plexiform layer (IPL). A single primary dendrite emerging from a cell body and lobular appendages (arrowheads) are visible in sublamina a of the IPL. In sublamina b, labeled processes form a diffuse network of dendrites. In the human retina (A), outer segments of photoreceptors, horizontal cells, and some bipolar cells are also labeled. In the cat retina (D), a Dab1-immunoreactive cell is visible in the ganglion cell layer (GCL). ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bars = 50 μm.

doubly labeled with antisera against Dab1 (Fig. 5A) or parvalbumin (Fig. 5D) and antisera against PKC (Fig. 5B,E) in rat retina. PKC immunoreactivity was found in cell bodies located in the outer part of the INL and in their axons, which terminated as two or three boutons in the IPL close to the GCL. In the merged image (Fig. 5C), it is apparent that the axon terminals of the rod bipolar cells make contacts with AII amacrine cell processes. However, parvalbumin-immunoreactive wide-field amacrine cell processes are not closely associated with the axon terminals of the rod bipolar cells, as shown in the Figure 5F. As expected from previous studies, there are contacts between the axon terminals of the rod bipolar cells and Dab1-immunoreactive cell processes. These results further confirm that the Dab1-labeled amacrine cells are indeed AII amacrine cells in the mammalian retina.

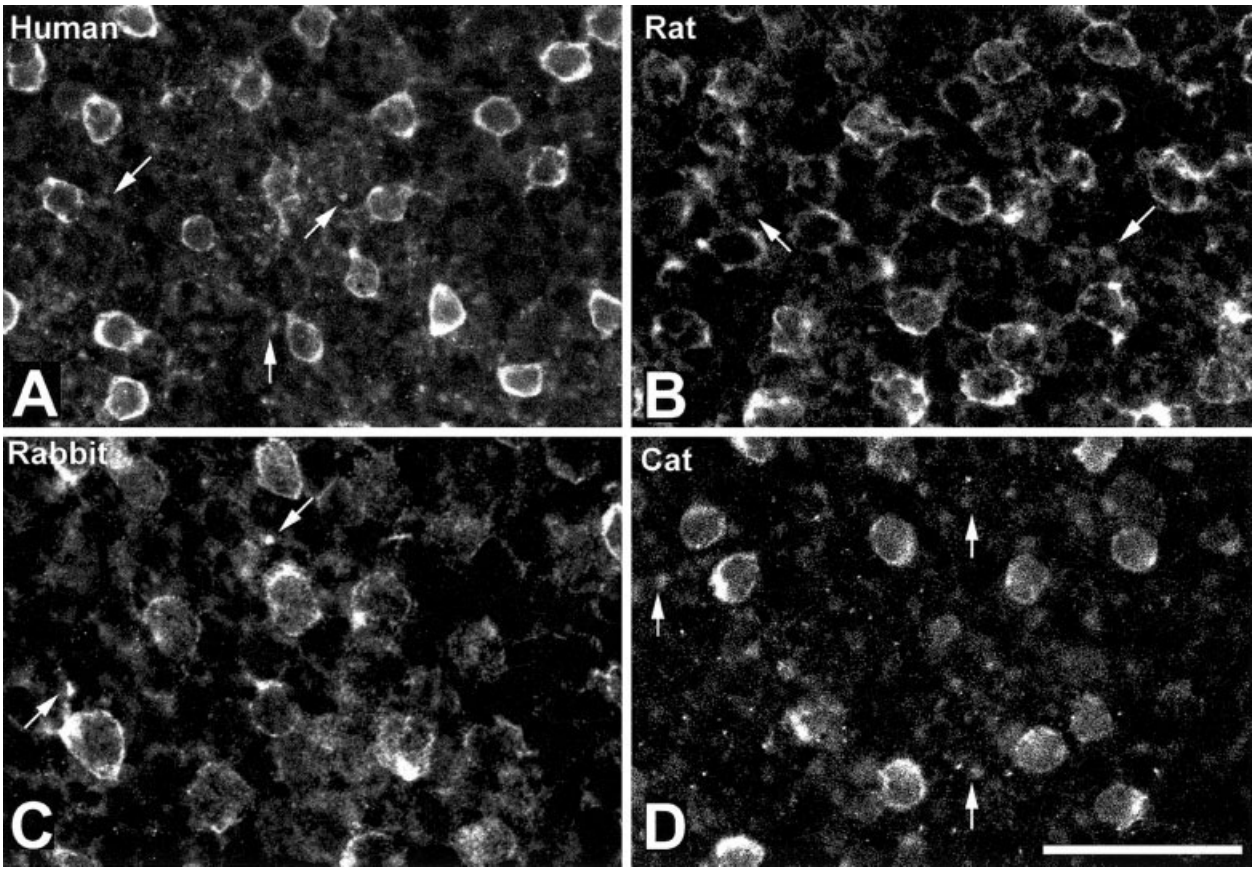

Fig. 2. Light micrographs of whole-mount human (**A**), rat (**B**), rabbit (**C**), and cat (**D**) retinas processed for Dab1 immunoreactivity. The focus is on the IPL close to the INL. Dab1-labeled amacrine cell bodies and lobular appendages (arrows) are visible. Scale bar = 50 μm.

Quantitative analysis

Quantitative studies have characterized the Dab1-immunoreactive amacrine cell population. The density of Dab1-immunoreactive amacrine cells was measured from the superior to inferior retina of a whole mount (Fig. 6). The peak density was 4,400 ± 169 cells/mm^2 at 3-mm eccentricity in the human retina, 5,200 ± 139 cells/mm^2 in the central region around the optic disc in the rat retina, 3,250 ± 119 cells/mm^2 at 3-mm eccentricity in the rabbit retina, and 4,675 ± 120 cells/mm^2 at 3-mm eccentricity in the cat retina. The lowest density was at the retinal periphery of the inferior retina, with 1,275 ± 219 cells/mm^2 in the human retina, 1,000 ± 154 cells/mm^2 in the rat retina, 900 ± 191 cells/mm^2 in rabbit retina, and 1,000 ± 112 cells/mm^2 in the cat retina.

To assess quantitatively the distribution of Dab1-immunoreactive amacrine cells, we used nearest-neighbor analysis (Wässle and Riemann, 1978). The distance from each soma to its nearest neighbor was measured in 0.25 × 0.25 mm^2 areas of the midperipheral retina. The resulting histogram for the midperipheral retinal region is shown in Figure 7. The histogram fits a Gaussian distribution fairly well in terms of mean distances and standard deviations (solid lines), indicating a statistically regular mosaic of labeled cells in this region.

DISCUSSION

In the present study, we demonstrated that antiserum directed against Dab1 can act as a specific marker for AII amacrine cells by comparing the morphologies of Dab1-labeled amacrine cells with those of AII amacrine cells labeled with other markers in human, rat, rabbit, and cat retinas. Dab1 encodes a cytoplasmic protein containing a motif known as a *protein interaction/phosphotyrosine-binding domain*. This domain was originally identified in the adaptor protein, Shc, as a region required for binding to the epidermal growth factor receptor, the insulin receptor, and other tyrosine-phosphorylated proteins (Margolis, 1996). Mammalian Dab1 was originally identified as an Src-binding protein in a yeast two-hybrid screen (Howell et al., 1997). It is expressed at high levels in the developing central nervous system and is phosphorylated on tyrosine residues during brain development. Tyrosine phosphorylation of Dab1 promotes its interaction with several nonreceptor tyrosine kinases, including Src, Fyn, and Ab1, through their SH_2 domains. This implies that Dab1 functions in kinase signaling cascades during development and plays an important role in the final positioning of migrating neurons in the mammalian central nervous system (Howell et al., 1997). In the present study, a certain population of amacrine cells was labeled by antisera di-

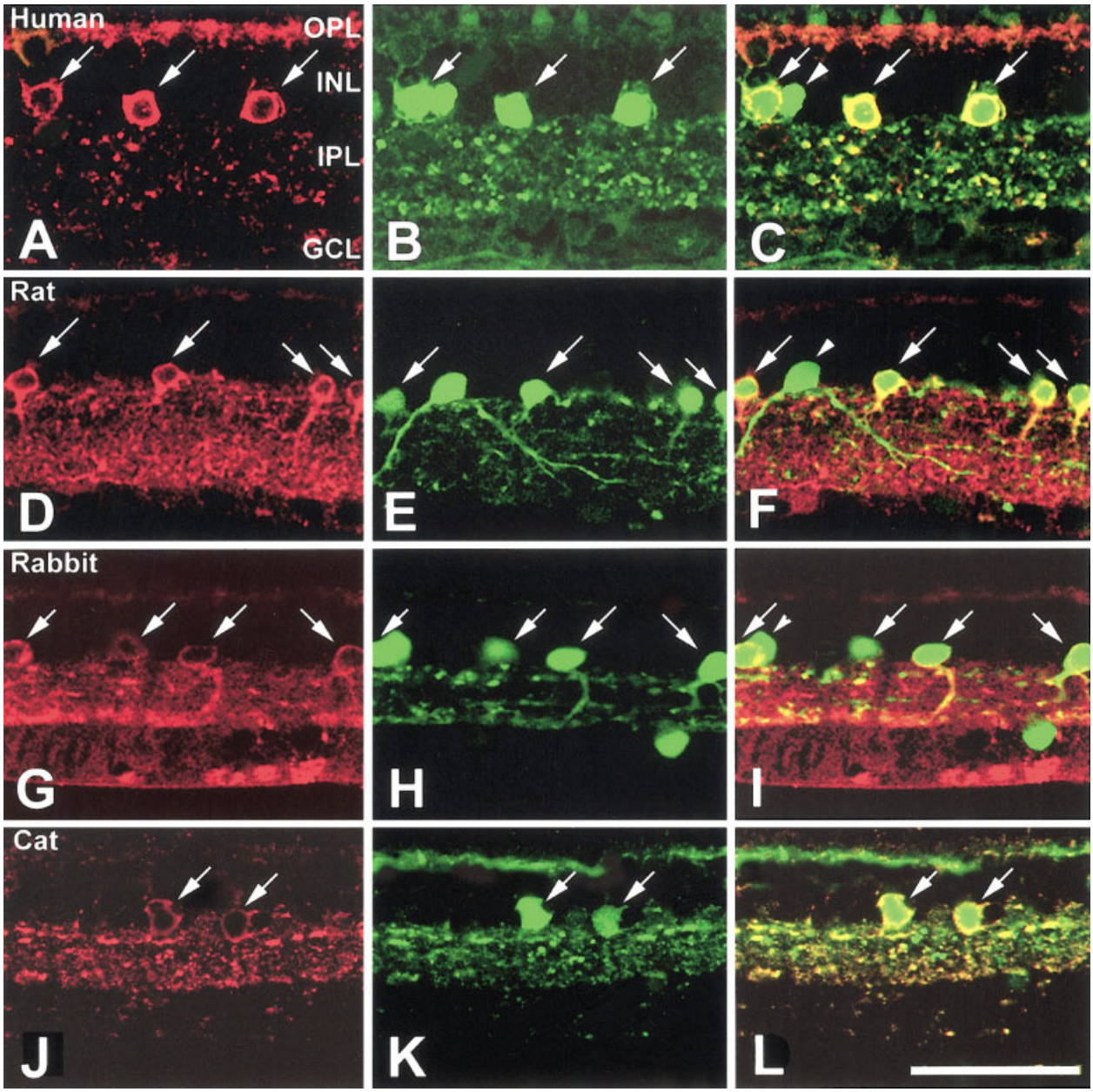

Fig. 3. Confocal micrographs of a 50-μm-thick vertical vibratome section processed for Dab1 (A,D,G,J) and calretinin (B) or parvalbumin (E,H,K) immunoreactivity in human (**A–C**), rat (**D–F**), rabbit (**G–I**), and cat (**J–L**) retinas. Dab1 immunoreactivity was visualized by using a Cy3-conjugated secondary antibody. Calretinin and parvalbumin immunoreactivities were visualized by using an FITC-conjugated secondary antibody. Double exposure (C,F,I,L) shows colocalization (yellow) of Dab1 and calretinin or parvalbumin within the same amacrine cell bodies located in the proximal row of the INL (arrows). Note that Dab1 immunoreactivity is not visible in a parvalbumin-immunoreactive wide-field amacrine cell in F and I (arrowheads). Scale bar = 50 μm.

rected against Dab1, in good agreement with previous reports on mouse and guinea pig retinas (Rice and Curran, 2000; Lee et al., 2003). These Dab1-labeled amacrine cells were located in a single row in the proximal INL, had thick primary processes with branched lobular appendages in sublamina a of the IPL, and ramified as bushy arbors in sublamina b of the IPL. They also showed positive immunoreactivity to antisera against parvalbumin or calretinin (i.e., markers for AII amacrine cells in the mammalian retinas examined so far), and their processes made close contacts with the axon terminals of the rod bipolar cells. These results clearly indicate that Dab1-labeled amacrine cells are AII amacrine cells in mammalian retinas. Different antisera have been used to identify AII amacrine cells in the mammalian retina, according to species. For instance, antisera against parvalbumin have been used in cat, rat, and rabbit retinas (Gabriel and Straznicky, 1992; Chun et al., 1993; Wässle et al., 1993; Casini et al., 1995)

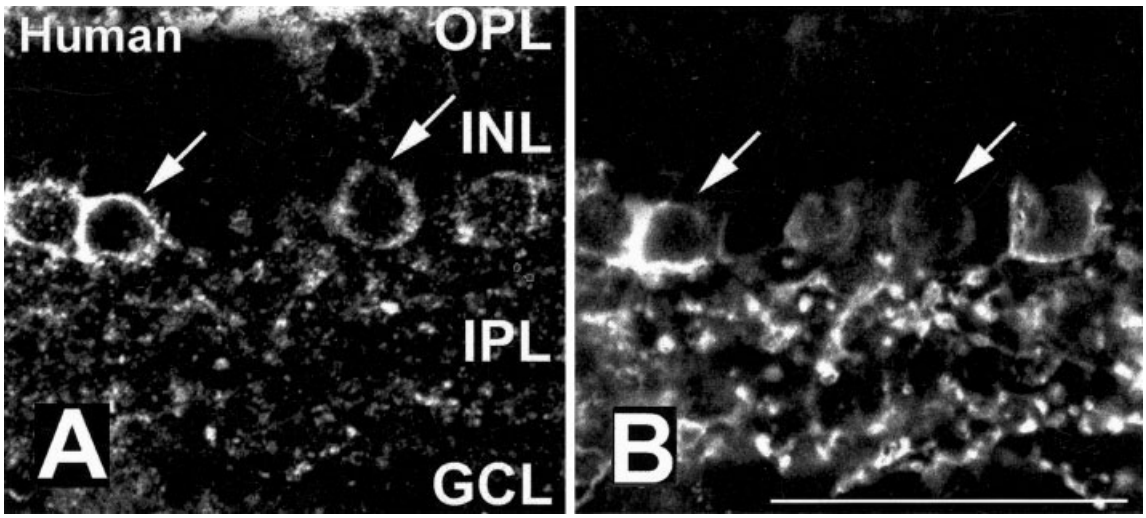

Fig. 4. Confocal micrographs of 50-μm-thick vertical vibratome sections processed for Dab1 (**A**) and Glyt-1 (**B**) immunoreactivity in human retina. Dab1 immunoreactivity was visualized by using a Cy3-conjugated secondary antibody. Glyt-1 immunoreactivity was visualized by using an FITC-conjugated secondary antibody. Dab1-labeled cell bodies are located in the proximal row of the INL (arrows). Numerous Glyt-1-immunoreactive cell bodies are visible in the INL (arrows). Comparison of the two figures clearly demonstrates that all Dab1-labeled cells display Glyt-1 immunoreactivity. Scale bar = 50 μm.

and antisera against calretinin in the primate retina (Wässle et al., 1995; Mills and Massey, 1999; Kolb et al., 2002). Why AII amacrine cells contain different calcium-binding proteins according to species is not known. However, the distribution of some markers in the vertebrate retinas tends to vary according to phylogenetic proximity (Marc, 1986; Mandell et al., 1990). In the present study, all AII amacrine cells were labeled by an antiserum directed against Dab1 regardless of species, clearly suggesting that Dab1 plays a role in AII amacrine cells of the mammalian retina. It has been suggested that Dab1 plays a role in the segregation of functionally distinct synapses in the IPL during development of the mouse retina (Rice and Curran, 2000). Reelin activates an intracellular signaling pathway by binding to lipoprotein receptors present on the surface of neurons in the developing brain and by stimulating tyrosine phosphorylation of Dab1 (D'Arcangelo et al., 1999; Hiesberger et al., 1999; Howell et al., 1999). Mutation of either Reelin or Dab1 results in abnormalities in laminar structures throughout the brain (Rice and Curran, 1999). These facts imply that AII amacrine cells and their processes express Dab1 so that they can be positioned properly in response to Reelin, which is produced by adjacent ganglion cells, as suggested by Rice and Curran (2000).

In the present study, all Dab1-immunoreactive cells in the human retina showed calretinin immunoreactivity, and those in rat, rabbit, and cat retinas showed parvalbumin immunoreactivity, whereas not all calretinin- or parvalbumin-labeled amacrine cells showed Dab1 immunoreactivity in human, rat, and rabbit retinas. These results are in good agreement with those of several investigators (Wässle et al., 1993; Casini et al., 1995, 1996; Massey and Mills, 1999; Kolb et al., 2002) who have shown that a class of wide-field amacrine cells expresses calretinin or parvalbumin immunoreactivity in the rat, monkey, and rabbit retinas. For example, W¨assle et al. (1993) have reported that 1% of all parvalbumin-immunoreactive amacrine cells belong to a class of wide-field amacrine cells in the rat retina. Casini et al. (1995) noted the presence of a small population (4%) of non-AII amacrine cells stained for parvalbumin, and Massey and Mills (1999) also showed that 15% of the parvalbumin-labeled amacrine cells were non-AII amacrine cells in the rabbit retina. Furthermore, Kolb et al. (2002) identified that calretinin-immunoreactive amacrine cells in the fovea are diffuse amacrine cell types and that those in the extrafoveal region are AII amcrine cells and A19 amacrine cells in the monkey retina. With these findings taken together, it can be inferred that an antibody against parvalbumin or calretinin could not be a reliable marker for AII amacrine cells in the mammalian retina. Therefore, our results further demonstrate that antiserum against Dab1 that labels the pure morphology of AII amacrine cell is a reliable and specific AII amacrine cell marker in the retinas of a wide variety of mammals.

It is well known that AII amacrine cells accumulate glycine and that their synaptic function is blocked by strychnine (Pourcho and Goebel, 1985; Müller et al., 1998). Therefore, the Dab1-immunoreactive amacrine cells were further characterized by double-label immunocytochemistry with an antiserum directed against Glyt-1, which is known to label functionally glycinergic amacrine cells (Zafra et al., 1995; Vaney et al., 1998). Double immunocytochemical labeling with antisera directed against Dab1 and Glyt-1 showed that all Dab1-immunoreactive amacrine cells expressed Glyt-1, demonstrating that Dab1-labeled amacrine cells are a subtype of glycinergic amacrine cells, which is consistent with previous reports (Menger et al., 1998; Rice and Curran, 2000; Lee et al., 2003). Therefore, AII amacrine cells of the retinas of mammals use glycine as an inhibitory neurotransmitter in visual processing.

In the present study, the density of Dab1-labeled amacrine cells varied greatly according to species. The peak density was relatively high (5,200–5,400 cells/mm^2) in the

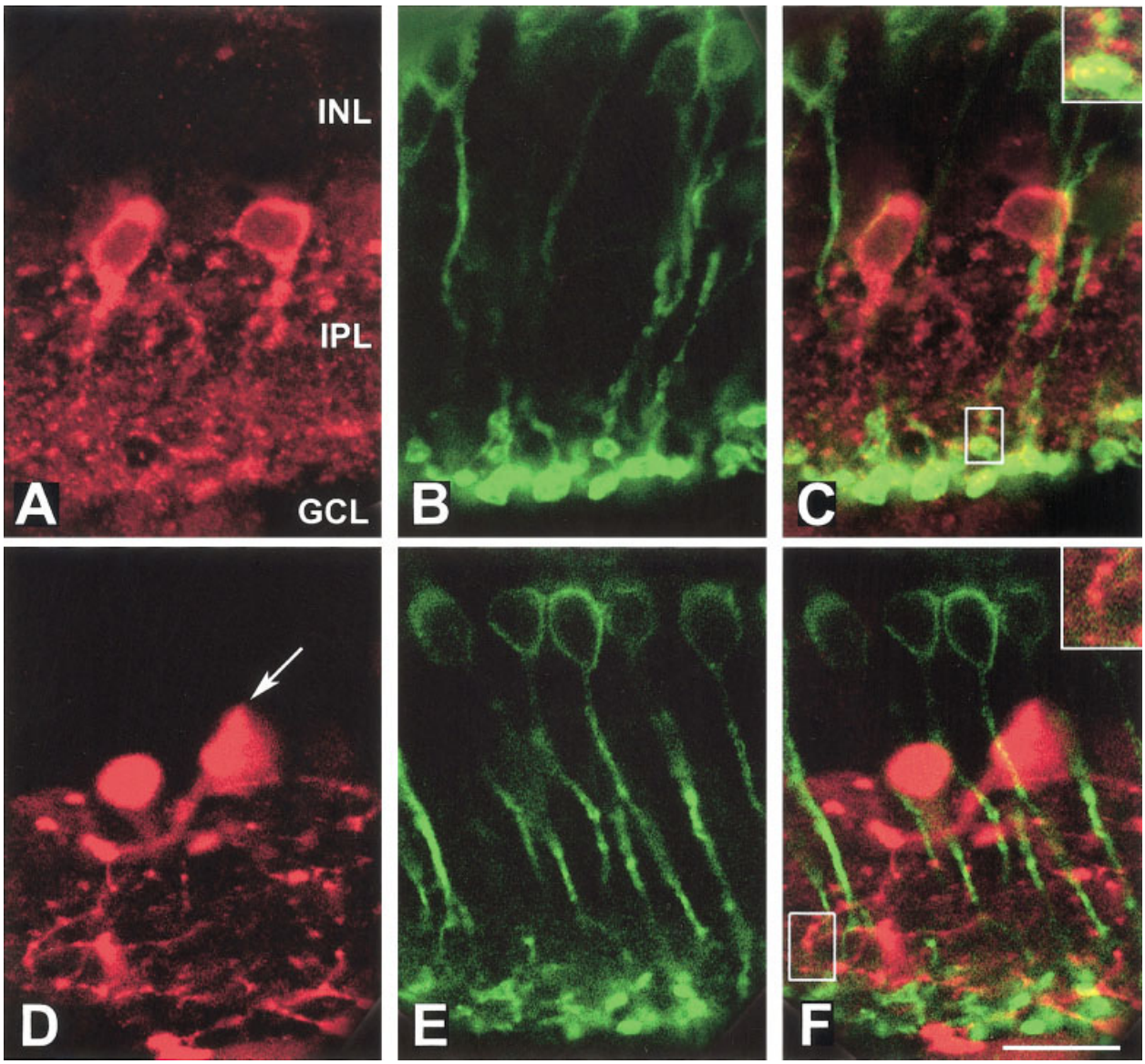

Fig. 5. Confocal micrographs of 50-μm-thick vertical vibratome sections processed for Dab1 (A) or parvalbumin (D) and PKC (B,E) in rat retinas. Dab1 and parvalbumin immunoreactivities were visualized by using a Cy3-conjugated secondary antibody. Protein kinase C (PKC) immunoreactivity was visualized by using an FITC-conjugated secondary antibody. **A:** Two Dab1-immunoreactive cell bodies are visible. **B,E:** PKC-immunoreactive cell bodies and terminals are visible. **C:** Double exposure of A and B shows that Dab1 processes are localized adjacent to the rod bipolar terminals (yellow). **D:** Two parvalbumin-immunoreactive cell bodies, an AII amacrine cell, and a wide-field amacrine cell (arrow) are visible. **F:** Double exposure of D and E shows that processes of the wide-field amacrine cell make no contact with rod bipolar terminals. The **insets** show higher magnification views of the boxed areas. Scale bar = 10 μm.

human and rat retinas, moderate (4,600 cells/mm^2) in the cat retina, and low (3,200–3,600 cells/mm^2) in the rabbit retina. AII amacrine cells are important interneurons that transfer signals from rod bipolar cells to ON-type cone bipolar cells. The peak density of rod bipolar cells is 15,000–20,000 cells/mm^2 in the macaque monkey retina (Grünert and Martin, 1991), 5,450 cells/mm^2 in the rabbit retina (Strettoi and Masland, 1995), and 4,700 cells/mm^2 in the cat retina (Freed et al., 1987). The ratio of AII amacrine cells to rod bipolar cells is 0.6 in the rabbit retina and 1.0 in the cat retina. If we assume that the density of rod bipolar cells in the monkey retina is similar to that in the human retina, the human ratio of AII amacrine cells to rod bipolar cells will be 0.35. Therefore, the different densities of AII amacrine cells may be attributed to the different ratios of AII amacrine cells to rod bipolar cells in these species (Sterling et al., 1988).

To quantify the regularity of the Dab1-immunoreactive cell mosaic, a nearest-neighbor analysis was performed (Wässle and Riemann, 1978). Dab1-labeled amacrine cells form a nonrandom mosaic, as shown by the distribution of their nearest-neighbor distances. The regularity of a cell mosaic is expressed by the regularity index (r), which is the ratio between the mean of the nearest-neighbor dis-

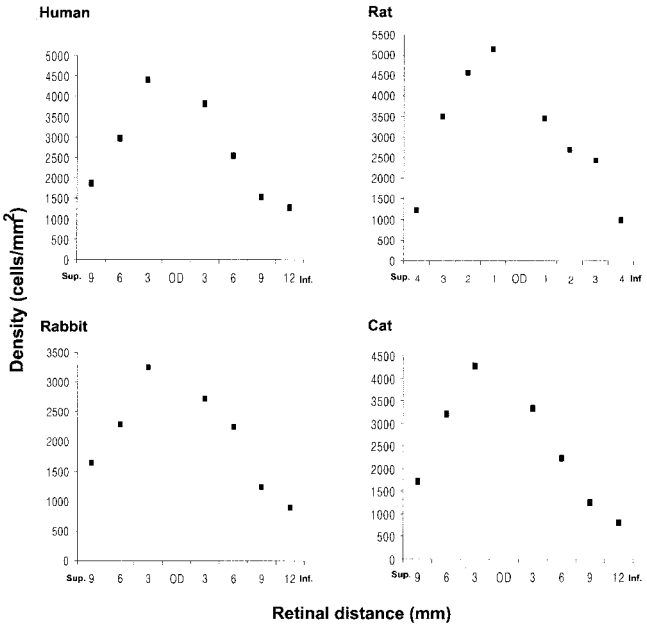

Fig. 6. Density of Dab1-immunoreactive cells in whole mounts of human, rat, rabbit, and cat retinas. Dab1-immunoreactive amacrine cell densities measured in the proximal INL of different species. Sup, superior; Inf, inferior.

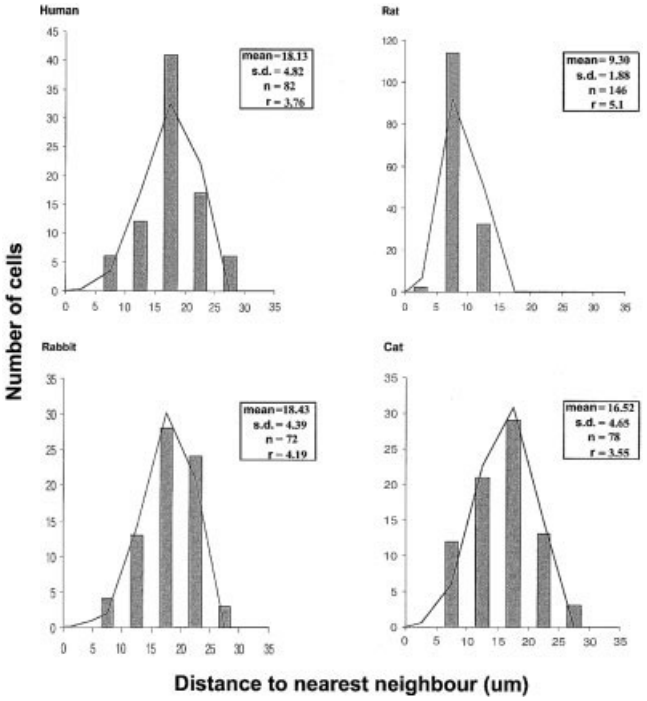

Fig. 7. Nearest-neighbor analysis of Dab1-immunoreactive amacrine cells in the inner nuclear layer of human, rat, rabbit, and cat retinas. Nearest-neighbor distance histograms for Dab1-immunoreactive amacrine cells in the midperipheral regions are shown. The histograms for Dab1-immunoreactive amacrine cells are relatively well matched by a Gaussian curve (solid line), which describes a regular cell distribution. Each histogram shows the number of cells in the sample (n), the mean distance between cells (m), the standard deviation (SD), and the regularity index (r).

tances and its standard deviation. A ratio of 1.0 indicates a random distribution and, the higher this ratio, the more regular the distribution (Eberhardt, 1967; Wässle and Riemann, 1978). In this study, the regularity index was 3.76 in the human retina, 5.1 in rat retina, 4.19 in the rabbit retina, and 3.55 in the cat retina. These are in good agreement with the AII amacrine cell mosaic of the cat, rabbit, and rat retinas (Vaney, 1985; Mills and Massey, 1991; Vaney et al., 1991; Wässle et al., 1993).

In conclusion, Dab1 immunoreactivity in different species is localized to a population of bistratified amacrine cells identical to the AII amacrine cells defined in other mammals by reactivity to calretinin or parvalbumin antibodies. Therefore, there is strong evidence, based on their morphology, distribution, and neurochemical content, that the Dab1-immunoreactive amacrine cells in the proximal INL of all the species examined here are indeed AII amacrine cells. Dab1 is therefore a reliable marker for the visualization of the AII amacrine cell population in mammalian retinas.

LITERATURE CITED

Boos R, Schneider H, Wässle H. 1993. Voltage- and transmitter-gated currents of AII-amacrine cells in a slice preparation of the rat retina. J Neurosci 13:2874–2888.

Casini G, Rickman DW, Brecha NC. 1995. AII amacrine cell population in the rabbit retina: identification by parvalbumin immunoreactivity. J Comp Neurol 356:132–142.

Casini G, Grassi A, Trasarti L, Bagnoli P. 1996. Developmental expression of protein kinase C immunoreactivity in rod bipolar cells of the rabbit retina. Vis Neurosci 13:817–831.

Chun MH, Han SH, Chung JW, Wässle H. 1993. Electron microscopic analysis of the rod pathway of the rat retina. J Comp Neurol 332:421–432.

Dacheux RF, Raviola E. 1986. The rod pathway in the rabbit retina: A depolarizing bipolar and amacrine cell. J Neurosci 6:331–345.

D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T. 1999. Reelin is a ligand for lipoprotein receptors. Neuron 24:471–479.

Dowling JE, Boycott BB. 1969. Retinal ganglion cells: a correlation of anatomical and physiological approaches. UCLA Forum Med Sci 8:145–161.

Eberhardt LL. 1967. Some developments in "distance sampling." Biometrics 23:207–216.

Famiglietti EV Jr, Kolb H. 1975. A bistratified amacrine cell and synaptic circuitry in the inner plexiform layer of the retina. Brain Res 84:293–300.

Freed MA, Smith RG, Sterling P. 1987. Rod bipolar array in the cat retina: pattern of input from rods and GABA-accumulating amacrine cells. J Comp Neurol 266:445–455.

Gabriel R, Straznicky C. 1992. Immunocytochemical localization of parvalbumin and neurofilament triplet protein immunoreactivity in the cat retina: colocalization in a subpopulation of AII amacrine cells. Brain Res 595:133–136.

Goebel DJ, Pourcho RG. 1997. Calretinin in the cat retina: colocalizations with other calcium-binding proteins, GABA and glycine. Vis Neurosci 14:311–322.

Greferath U, Grünert U, Wässle H. 1990. Rod bipolar cells in the mammalian retina show protein kinase C-like immunoreactivity. J Comp Neurol 301:433–442.

Grünert U, Martin PR. 1991. Rod bipolar cells in the macaque monkey retina: immunoreactivity and connectivity. J Neurosci 11:2742–2758.

Grünert U, Wässle H. 1993. Immunocytochemical localization of glycine receptors in the mammalian retina. J Comp Neurol 335:523–537.

Grünert U, Wässle H. 1996. Glycine receptors in the rod pathway of the macaque monkey retina. Vis Neurosci 13:101–115.

Hiesberger T, Trommsdorff M, Howell BW, Goffinet A, Mumby MC, Cooper JA, Herz J. 1999. Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. Neuron 24:481–489.

Howell BW, Gertler FB, Cooper JA. 1997. Mouse disabled (mDab1): an Src

binding protein implicated in neuronal development. EMBO J 16:121–132.

Howell BW, Herrick TM, Cooper JA. 1999. Reelin-induced tryosine phosphorylation of disabled 1 during neuronal positioning. Genes Dev 13: 643–648.

Kolb H. 1979. The inner plexiform layer in the retina of the cat: electron microscopic observations. J Neurocytol 8:295–329.

Kolb H, Famiglietti EV. 1974. Rod and cone pathways in the inner plexiform layer of cat retina. Science 186:47–49.

Kolb H, Nelson R. 1981. Amacrine cells of the cat retina. Vis Res 21:1625–1633.

Kolb H, Cuenca N, Wang HH, Dekorver L. 1990. The synaptic organization of the dopaminergic amacrine cell in the cat retina. J Neurocytol 19:343–366.

Kolb H, Cuenca N, Wang HH, Dekorver L. 1991. Postembedding immunocytochemistry for GABA and glycine reveals the synaptic relationships of the dopaminergic amacrine cell of the cat retina. J Comp Neurol 310:267–284.

Kolb H, Zhang LI, Dekorver L, Cuenca N. 2002. A new look at calretinin-immunoreactive amacrine cell types in the monkey retina. J Comp Neurol 453:168–184.

Lee EJ, Kim HJ, Kim IB, Park JH, Oh SJ, Rickman DW, Chun MH. 2003. Morphological analysis of Disabled-1-immunoreactive amacrine cells in the guinea pig retina. J Comp Neurol 466:240–250.

MacNeil MA, Masland RH. 1998. Extreme diversity among amacrine cells: Implications for function. Neuron 20:971–982.

MacNeil MA, Heussy JK, Dacheux RF, Raviola E, Masland RH. 1999. The shapes and numbers of amacrine cells: matching of photofilled with Golgi-stained cells in the rabbit retina and comparison with other mammalian species. J Comp Neurol 413:305–326.

Mandell JW, Townes-Anderson E, Czernik AJ, Cameron R, Greengard P, De Camilli P. 1990. Synapsins in the vertebrate retina: absence from ribbon synapses and heterogeneous distribution among conventional synapses. Neuron 5:19–33.

Marc RE. 1986. Neurochemical stratification in the inner plexiform layer of the vertebrate retina. Vis Res 26:223–238.

Marc RE. 1989. The role of glycine in the mammalian retina. Prog Ret Res 8:67–107.

Marc RE, Liu WL. 1985. [^{3}H]glycine-accumulating neurons of the human retina. J Comp Neurol 232:241–260.

Marc RE, Murry RF, Basinger SF. 1995. Pattern recognition of amino acid signatures in retinal neurons. J Neurosci 15:5106–5129.

Margolis B. 1996. The PI/PTB domain: a new protein interaction domain involved in growth factor receptor signaling. J Lab Clin Med 128:235–241.

Mariani AP. 1990. Amacrine cells of the rhesus monkey retina. J Comp Neurol 301:382–400.

Masland RH. 1988. Amacrine cells. Trends Neurosci 11:405–410.

Massey SC, Mills SL. 1999. Antibody to calretinin stains AII amacrine cells in the rabbit retina: double-label and confocal analyses. J Comp Neurol 411:3–18.

McGuire GA, Stevens JK, Sterling P. 1984. Microcircuitry of bipolar cells in cat retina. J Neurosci 4:2920–2938.

Menger N, Pow DV, Wässle H. 1998. Glycinergic amacrine cells of the rat retina. J Comp Neurol 401:34–46.

Mills SL, Massey SC. 1991. Labeling and distribution of AII amacrine cells in the rabbit retina. J Comp Neurol 304:491–501.

Mills SL, Massey SC. 1999. AII amacrine cells limit scotopic acuity in central macaque retina: a confocal analysis of calretinin labeling. J Comp Neurol 411:19–34.

Müller F, Wässle H, Voigt T. 1988. Pharmacological modulation of the rod pathway in the cat retina. J Neurophysiol 59:1657–1672.

Negishi K, Kato S, Teranishi T. 1988. Dopamine cells and rod bipolar cells contain protein kinase C-like immunoreactivity in some vertebrate retinas. Neurosci Lett 94:247–252.

Pasteels B, Rogers J, Blachier F, Pochet R. 1990. Calbindin and calretinin localization in retinas from different species. Vis Neurosci 5:1–16.

Pourcho RG. 1982. Dopaminergic amacrine cells in the cat retina. Brain Res 252:101–109.

Pourcho RG, Goebel DJ. 1985. A combined Golgi and autoradiographic study of [^{3}H]glycine-accumulating amacrine cells in the cat retina. J Comp Neurol 233:473–480.

Raviola E, Dacheux RF. 1987. Excitatory dyad synapse in rabbit retina. Proc Natl Acad Sci USA 84:7324–7328.

Rice DS, Curran T. 1999. Mutant mice with scrambled brains: understanding the signaling pathways that control cell positioning in the CNS. Genes Dev 13:2758–2773.

Rice DS, Curran T. 2000. Disabled-1 is expressed in type AII amacrine cells in the mouse retina. J Comp Neurol 424:327–338.

Sandell JH, Masland RH. 1986. A system of indoleamine-accumulating neurons in the rabbit retina. J Neurosci 6:3331–3347.

Sterling P, Freed MA, Smith RG. 1988. Architecture of rod and cone circuits of the on-beta ganglion cell. J Neurosci 8:623–642.

Strettoi E, Dacheux RF. 1992. Synaptic connections of the narrow-field, bistratified rod amacrine cell (AII) in the rabbit retina. J Comp Neurol 325:152–168.

Strettoi E, Masland RH. 1995. The organization of the inner nuclear layer of the rabbit retina. J Neurosci 15:875–888.

Strettoi E, Masland RH. 1996. The number of unidentified amacrine cells in the mammalian retina. Proc Natl Acad Sci USA 93:14906–14911.

Strettoi E, Dacheux RF, Raviola E. 1990. Synaptic connections of rod bipolar cells in the inner plexiform layer of the rabbit retina. J Comp Neurol 295:449–466.

Strettoi E, Raviola E, Dacheux RF. 1992. Synaptic connections of the narrow-field, bistratified rod amacrine cell (AII) in the rabbit retina. J Comp Neurol 325:152–168.

Tauchi M, Masland RH. 1984. The shape and arrangement of the cholinergic neurons in the rabbit retina. Proc R Soc Lond B Biol Sci 223:101–119.

Vaney DI. 1984. &“Coronate” amacrine cells in the rabbit retina have the “starburst” dendritic morphology. Proc R Soc Lond B Biol Sci 220:501–508.

Vaney DI. 1985. The morphology and topographic distribution of AII amacrine cells in the cat retina. Proc R Soc Lond B Biol Sci 224:475–488.

Vaney DI. 1986. Morphological identification of serotonin-accumulating neurons in the living retina. Science 233:444–446.

Vaney DI. 1990. The mosaic of amacrine cells in the mammalian retina. Prog Ret Res 9:49–100.

Vaney DI. 1991. Many diverse types of retinal neurons show tracer coupling when injected with biocytin or neurobiotin. Neurosci Lett 29:187–190.

Vaney DI, Gynther IC, Young HM. 1991. Rod-signal interneurons in the rabbit retina: 2. AII amacrine cells. J Comp Neurol 310:154–169.

Vaney DI, Nelson JC, Pow DV. 1998. Neurotransmitter coupling through gap junctions in the retina. J Neurosci 18:10594–10602.

Vardi N, Auerbach P. 1995. Specific cell types in cat retina express different forms of glutamic acid decarboxylase. J Comp Neurol 351:374–384.

Voigt T, Wässle H. 1987. Dopaminergic innervation of AII amacrine cells in mammalian retina. J Neurosci 7:4115–4128.

Wässle H, Boycott BB. 1991. Functional architecture of the mammalian retina. Physiol Rev 71:447–480.

Wässle H, Riemann HJ. 1978. The mosaic of nerve cells in the mammalian retina. Proc R Soc Lond B Biol Sci 200:441–461.

Wässle H, Grünert U, Röhrenbeck J. 1993. Immunocytochemical staining of AII-amacrine cells in the rat retina with antibodies against parvalbumin. J Comp Neurol 332:407–420.

Wässle H, Grünert U, Chun MH, Boycott BB. 1995. The rod pathway of the macaque monkey retina: identification of AII-amacrine cells with antibodies against calretinin. J Comp Neurol 361:537–551.

Wong ROL, Henry GH, Medveczky CJ. 1986. Bistratified amacrine cells in the retina of the Tammar wallaby—*Macropus eugenii*. Exp Brain Res 63:102–105.

Wright LL, Macqueen CL, Elston GN, Young HM, Pow DV, Vaney DI. 1997. DAPI-3 amacrine cells of the rabbit retina. Vis Neurosci 14:473–492.

Young HM, Vaney DI. 1990. The retinae of prototherian mammals possess neuronal types that are characteristic of non-mammalian retinae. Vis Neurosci 5:61–66.

Zafra F, Aragon C, Olivares L, Danbolt NC, Gimenez C, Storm-Mathisen J. 1995. Glycine transporters are differentially expressed among CNS cells. J Neurosci 15:3952–3969.