SHORT COMMUNICATION
Immunocytochemical identification and analysis of the diffuse bipolar cell type DB6 in macaque monkey retina

Tricia L. Chan, Paul R. Martin and Ulrike Grünert
Department of Physiology and Institute for Biomedical Research, The University of Sydney, NSW 2006, Australia

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Abstract
The distribution and morphology of CD15-immunoreactive bipolar cells were studied in the retina of macaque monkey. Labelled cells have a large dendritic tree contacting several cones and a narrowly stratified axon terminal that ends deep in the inner plexiform layer, close to the ganglion cell layer. The morphology of the labelled cells corresponds to that of the diffuse bipolar cell type named DB6 by Boycott & Wäsle (1991; Eur. J. Neurosci., 3,1069). We conclude that CD15 is a marker for DB6 bipolar cells, enabling the quantitative analysis of the distribution and connectivity of this diffuse bipolar cell type.

Introduction
Parallel pathways in the visual system can be identified from the first synapse in the retina, where cone photoreceptors make divergent synaptic contact with multiple, distinct morphological types of bipolar cells. In primate retina nine types of cone bipolar cell, and one type of rod bipolar cell, have been distinguished by Golgi-impregnation (Polyak, 1941; Boycott & Dowling, 1969; Boycott & Wäsle, 1991). The different cone bipolar cell types are thought to provide input to different ganglion cell classes with distinct functional properties. A critical methodological tool for studying the connectivity of bipolar cells is the use of immunocytochemical markers. Immunocytochemical labelling has allowed the quantitative analysis of several of the bipolar cell populations. These include blue cone bipolar cells [labelled by cholecystokinin-immunoreactivity (IR)]; both types of midget bipolar cells (by recoverin- and cholecystokinin-IR); the diffuse bipolar (DB) cell types DB2 (by antibodies to glutamate transporters); DB3 (by calbindin-IR), and DB4 (by protein kinase C-IR), and rod bipolar cells (by protein kinase C-IR) (Grürt & Martin, 1991; Kouyama & Marshak, 1992; Milam et al., 1993; Grünt et al., 1994; Wäsle et al., 1994; Luo et al., 1999; Jacoby & Marshak, 2000; Jacoby et al. 2000). While the cone synapses of all diffuse bipolar cell types have been analysed from Golgi-impregnated material (reviewed in Hopkins & Boycott, 1997), the immunocytochemical identification of three of the diffuse bipolar cell types (DB1, DB5, and DB6) distinguished by Boycott & Wäsle (1991) is still lacking. Accordingly, the connectivity of these bipolar cells with amacrine and ganglion cells in the inner plexiform layer remains poorly understood.

Recently, it was shown that an antibody against the carbohydrate epitope CD15 labels two populations of bipolar cells, an ON- and an OFF-type in a New World monkey, the common marmoset (Andressen & Mai, 1997). In rabbit retina, CD15-labelled ON-bipolar cells costratify with the direction-selective circuitry (Brown & Masland, 1999). Here we show that in macaque monkey retina the antibody against CD15 labels a single population of ON-bipolar cells, which correspond to DB6 cells.

Materials and methods
We used four retinae from three macaque monkeys. Three retinae (used for whole-mounts) were from two Macaca fascicularis, whereas one retina (used for vertical sections) was from one M. nemestrina. The animals were killed for experiments unrelated to those described here. All procedures conformed to the provisions of the Australian National Health and Medical Research Council code of practice of the care and use of animals.

Animals were given a lethal dose of pentobarbitone and perfused with physiological saline prior to perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The eyes were removed, cut open and the posterior eyecup was immersion-fixed in the same fixative for 30 min to 4 h. After rinsing in PB the retinae were dissected from the sclera and either kept in PB containing 0.1% sodium azide or immersed in 30% sucrose in PB and subsequently stored at −20 °C.

Immunolabelling was carried out using the avidin-biotin-peroxidase complex method as described previously (Grünt et al., 1994; Luo et al., 1999). Whole retinae were incubated in the primary antibody against CD15 (kindly provided by Professor Mai, University of Düsseldorf, Germany, Andressen & Mai, 1997) for 11–18 days at 4 °C. Vertical cryostat sections were incubated in the primary antibody for 16–20 h at room temperature. The dilution of the primary antibody was 1 : 6. Subsequently, retinal pieces and sections were processed with biotinylated horse antimouse IgG (Vector, Burlingame, CA, USA) and the ABC elite kit (Vector). After immunolabelling, retinal pieces were mounted in glycerol containing Mowiol (Hoehst, Sydney, Australia) (Harlow & Lane, 1988). Shrinkage in Mowiol mounted whole-mounts is <10% (Ghosh et al., 1996). The quantitative data are not corrected for this...
shrinkage. Maps of whole-mounts were drawn at a final magnification of 12.9 × with a drawing tube attached to an Olympus microscope (BH2). Labelled cells were drawn at a final magnification of 1875 × using a × 100 objective. In some patches, cone inner segments could also be seen and were drawn together with the labelled bipolar cell somata. For the density graphs, labelled somata in one whole-mount were counted using a computer-assisted camera lucida system (Halasz & Martin, 1984).

Results and discussion

Six diffuse bipolar cell types have been classified by Boycott & Wässle (1991) in Golgi-impregnated macaque retina using the stratification and branching pattern of the axon terminal as the main criterion. DB6 cells have a relatively large axon terminal, which stratifies close to the ganglion cell layer.

Figure 1 shows a vertical cryostat section through central macaque retina immunolabelled with the antibody against CD15. A single population of bipolar cells is labelled. The cells have a relatively large dendritic tree contacting several cones. The somata of the cells are located in the outer half of the inner nuclear layer. The axon terminals are narrowly stratified close to the ganglion cell layer. The morphology of the CD15-labelled cells corresponds to that of the DB6 cells described by Boycott & Wässle (1991).

The population of CD15-labelled bipolar cells was studied in whole-mounts. Figures 2 and 3 show CD15-labelled bipolar cells at an eccentricity between 6 and 7 mm in temporal retina. They have a rather large dendritic tree (between 30 and 50 μm in diameter) and a relatively large axon terminal. In Fig. 3A the labelled bipolar somata are drawn together with the overlying cone mosaic. The location of the cone pedicles could not be determined. However, when the pattern of the cone inner segments (Fig. 3A) was superimposed onto the drawing of the labelled bipolar cells shown in Fig. 3B, and allowance was made for the displacement of Henle fibres (see also Boycott & Wässle, 1991), it became evident that the dendrites of CD15-labelled cells probably contact all the cones in their dendritic field. Consistently, the EM analysis of a Golgi-impregnated DB6 cell showed that all cones in its dendritic field were contacted (Hopkins & Boycott, 1996). Thus, DB6 cells are probably not colour selective. However, given that short-wavelength sensitive cones make up <10% of all cones in macaque retina (Martin & Grünert, 1999), we cannot determine whether DB6 cells show a bias of cone connectivity, as shown for the connections of the two horizontal cell types with short-wavelength sensitive cones (Dacey et al., 1996; Goodchild et al., 1996).

Counts of the number of cones and the number of CD15-labelled bipolar cells in Fig. 3A give a ratio (numerical convergence) of 8 cones for each CD15-labelled bipolar cell at about 6.3 mm eccentricity. Two DB6 cells analysed in Golgi-impregnated retina had a similar number of cone contacts: one DB6 cell, from 6 to 7 mm eccentricity, investigated by light microscopy, contacted 10 cones (Boycott & Wässle, 1991); another DB6 cell from 3.5 mm eccentricity, analysed by electron microscopy, contacted 7 cones (Hopkins & Boycott, 1996).

The cell bodies of CD15-labelled bipolar cells form a regular mosaic (Fig. 3C). Their dendrites and axon terminals occupy distinct domains with little or no overlap (Fig. 3B and D). The density of...
CD15-labelled bipolar cells at 6.7 mm eccentricity, as determined from Fig. 3C, is 578 cells/mm² and the bipolar : cone ratio is 0.123. These numbers compare well with an estimated density of 870 DB6 cells/mm² and a DB6 : cone ratio of 0.17 by Boycott & Wässle (1991). Thus, we conclude that CD15 is a marker for DB6 cells in macaque retina, and can be used for the quantitative analysis of this cell type.

The density of DB6 cells was estimated along the horizontal meridian of one macaque retina by counting CD15-labelled somata (Fig. 4). The maximum density was 1901 cells/mm² at 0.64 mm eccentricity in temporal retina, decreasing to approximately 270 cells/mm² in peripheral retina. Across the retina, the density of DB6 cells is significantly lower than that of midget bipolar, blue cone bipolar and DB4 cells (Kouyama & Marshak, 1992; Grünert et al., 1994; Wässle et al., 1994), but similar to the density of DB3 cells (Boycott & Wässle, 1991; Martin & Grünert, 1992; Grünert et al., 1994; Jacoby & Marshak, 2000).

The cell type(s) postsynaptic to DB6 cells are not known, and no ganglion cell types stratifying in the same region have been described yet. Judged on the basis of their stratification depth, DB6 axon terminals must be intermingled with rod bipolar and blue cone bipolar axon terminals. Rod bipolar cells make contact with AII amacrine and other amacrine cells (Grünert & Martin, 1991), whereas blue cone bipolar cells contact small bistratified ganglion cells and amacrine cells (Calkins et al., 1998; Ghosh & Grünert, 1999). Thus, it is possible that DB6 cells are presynaptic to the same amacrine and/or...
ganglion cell types as rod bipolar cells and blue cone bipolar cells. The robust and highly specific label of the CD15 antibody gives promise that the synaptic connectivity of the DB6 bipolar cell can be analysed by EM immunocytochemistry.

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Abbreviations

DB, diffuse bipolar; EM, electron microscopic; IR, immunoreactivity; PB, phosphate buffer.

References

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