Immunohistochemical localization of a macrophage-specific antigen in developing mouse retina

Immunohistochemical Localization of a Macrophage-specific Antigen in Developing Mouse Retina: Phagocytosis of Dying Neurons and Differentiation of Microglial Cells to Form a Regular Array in the Plexiform Layers

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ABSTRACT In the developing mouse retina degenerating neurons can be observed initially in the ganglion cell layer followed by a phase of cell death in the inner nuclear layer. Using an immunohistochemical method to localize the mouse macrophage specific antigen F4/80, we show that macrophages migrate from the vascular supply overlying the developing retina and phagocytose the degenerating neurons. The macrophages subsequently differentiate to become the microglia of the retina and form a regularly spaced distribution across the retina in the inner and outer plexiform layers. These experiments provide strong evidence for the mesodermal origin of central nervous system microglia.

There is considerable confusion as to the origins and functions of microglial cells in nervous tissue, although most workers favor the view that they arise from blood monocytes (1) and may be viewed as part of the mononuclear phagocyte system (2). Direct evidence supporting the operation of this pathway has been obtained from bone-marrow chimera and monocyte transfer experiments (3, 4). During the early development of nervous tissues there is considerable overproduction of neurons and selective programmed cell death occurs during subsequent maturation (5). Since macrophages possess the ability to recognize senescent erythrocytes (6) and neutrophils (7), we considered the possibility that monocytes/macrophages initially enter nervous tissue to dispose of dying neurons and subsequently differentiate to form microglia. The present paper is concerned with an investigation of this hypothesis in the developing mouse retina by immunohistochemical localization of the mouse macrophage specific antigen F4/80 (8).

MATERIALS AND METHODS

The animals (CBAT6/T6 and BALB/c; Pathology, Oxford, England) were killed with an overdose of ether. They were then perfused through the left ventricle with heparinized 0.1 M sodium cacodylate buffer, pH 7.2, followed by the fixative, 0.5% glutaraldehyde, in the same buffer. The eyes were then removed and the anterior chamber cut off. After a brief wash in buffer the eyes were dehydrated in a series of alcohols and embedded in polyethylene glycol 4000 distearate wax (Koch-Light Laboratories, England). During the dehydration some eyes were placed between two glass slides to flatten the eye and thus allow larger areas of retina to be cut in the horizontal plane. The eyes were cut in the vertical or horizontal plane at 5–10 μm. We examined retinae from animals at day 16 and 19 postconception, on the day of birth (referred to here as day 0), and also 5, 10, 20, and 40 d or more after birth, (referred to here as days 5, 10, 20, or adult). The method for immunocytochemical localization of antigen F4/80 is described elsewhere (8). The antigen was revealed by the avidin-biotin-complex (ABC) immunoperoxidase method of Hsu et al. (9), using reagents supplied by Vector Laboratories (via Sera-Laboratories, England). Endogenous peroxidase was eliminated by prior exposure to 0.3% H_2O_2 in methanol for 30 min. Control preparations without antibody F4/80 showed no reaction product. We counterstained tissues lightly with Mayer's haematoxylin. Sections were examined under dark- or bright-field illumination and photographed using an Ilford 303 blue filter to increase contrast of the brown reaction product.

RESULTS

In the developing mouse retina there are many degenerating neurons as reported previously in other rodent retinae (10–12). Dying cells can be identified readily because of their pyknotic nuclei, the condensed chromatin becomes more basophilic and stains intensely with haematoxylin (13). Cell death in the mouse retina commences initially in the ganglion cell layer between 14 and 16 days *in utero*, and the maximum number of pyknotic nuclei is observed in this layer on day 0. The phase of cell death occurs later in the inner nuclear layer and a peak pyknotic nucleus count is reached on day 5 after birth. Virtually no pyknotic nuclei are found in the outer nuclear layer at any time during development. These data are contained in Table I.

TABLE I

Pyknotic Nuclei and F4.80-labeled Macrophages in the Retinal Layers at Different Ages

Age (d)	Ganglion cell layer	Inner nuclear layer	Outer nuclear layer
16 day embryo	4 (3)	7 (5)	-
0	54 (34)	10 (1)	-
5	31 (24)	68 (16)	-
10	0	10 (2)	2
20	0	0	0

The pyknotic nuclei were counted in three sections through the center of two eyes from two CBAT6/T6 mice of each age. The numbers in parentheses indicate the number of pyknotic nuclei with positively stained processes surrounding them.

The F4/80 antigen is absent from mouse embryonic tissues including the brain and retina prior to vascularisation and appears first in the liver between 10 and 11 d after conception (Shia, G., D. Hume, and S. Gordon, unpublished results). In the retina of the 16-d embryo very few F4/80$^+$ cells are seen in the retinal layers, except amongst the ganglion cells. At this stage the F4/80$^+$ cells are relatively rounded with short processes. A considerable number of F4/80$^+$ cells, which have the appearance of blood monocytes, is found attached to the wall of the blood vessel adjacent to the ganglion cells (Fig. 1). The subsequent pattern of migration of F4/80$^+$ cells parallels the appearance of dying cells; in the neonate (day 0), the majority of the F4/80$^+$ cells are still in the ganglion cell layer (Fig. 2), but by day 5 large numbers of F4/80$^+$ cells are found in the inner nuclear layer and others delineate the boundary between the inner and outer nuclear layers prior to the formation of a visible distinct plexiform layer (Figs. 3 and 4). By day 10 F4/80$^+$ cells are not commonly seen within the neuronal cell layers and are restricted to the plexiform layers (Fig. 5).

During retinal maturation, the involvement of infiltrating F4/80$^+$ cells in the phagocytosis of dying neurons can be readily discerned. Between one-third and two-thirds of all pyknotic nuclei can be seen to be surrounded by F4/80$^+$ membrane processes (Table I); an example is seen in Fig. 6. As the wave of cell death declines the nature of the F4/80$^+$ cells changes and the extent and arborization of the membrane processes increases.

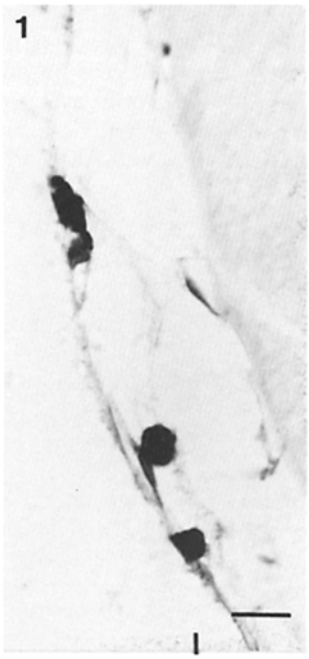
The F4/80$^+$ cells in the inner plexiform layer of the adult are bistratified and dendrites spanning the entire width of the plexiform layer have been observed (Fig. 7). The distribution and morphology of F4/80$^+$ cells in the plexiform layers of the adult retina can best be appreciated in thick (10 μm) horizontal sections. Fig. 8 shows clearly the regular spacing of F4/80$^+$ cells in the inner plexiform layer. Because of the stratification of the cells in the inner plexiform layer, the full extent of their dendritic processes is not seen. The F4/80$^+$ cells spread in a much narrower plane in the outer plexiform layer and an entire cell can be viewed in a single 10-μm section (Fig. 9). As in the inner plexiform layer, each F4/80$^+$ cell appears to have a "zone of influence," with little overlapping of membrane processes between adjacent cells.

DISCUSSION

The F4/80 antigen is a mouse macrophage differentiation marker (14, 15). Immunoprecipitation after lactoperoxidase and biosynthetic labeling has shown that antigen F4/80 is part of a 160-kdalton plasma membrane protein produced by all mature mouse macrophages in culture. We have found using the immunohistochemical method described here that F4/80 labels all well-defined resident macrophage populations of the mouse including liver, lung, spleen, and bone-marrow (8; unpublished results) (Hume, D. A., A. P. Robinson, G. G. MacPherson, and S. Gordon, manuscript submitted for publication) and does not cross react with any other blood or tissue cell type in the embryo or adult. The F4/80$^+$ cells observed in the adult mouse retina are similar in morphology and distribution to retinal microglia observed in Golgi preparations of mammalian retinae (16). These cells do not resemble astrocytes or Müller cells, the other glial cells of the retina. Indeed the distribution of the F4/80$^+$ cells is quite distinct from that found for the astroglia and Müller cells (17, 18) and we have found no evidence for cross reaction of F4/80 with these cells. Antigen F4/80 also selectively stains microglia elsewhere in the central nervous system (unpublished observations).

It has recently been demonstrated that the microglia of the central nervous system can be stained using a histochemical method to localize the enzyme thiamine pyrophosphatase (19). Cells stained by this method were shown to have the ultrastructural characteristics of microglia and the authors argued for a monocytic origin for the microglia (19). We have stained whole-mounts of adult mice retinae for thiamine pyrophosphatase and find that the positively stained cells are similar in morphology and distribution to our F4/80$^+$ cells (unpublished observations). However the thiamine pyrophosphatase is found not only on the microglia but also on the capillaries and other retinal elements and is thus a less selective method for the demonstration of microglia than the F4/80 antigen.

FIGURES 1–4 Immunohistochemical localisation of F4/80 antigen in developing mouse retina. The antigenic sites are indicated by dark diaminobenzidine precipitate under bright-field illumination. Under dark field the precipitate reflects light and appears bright. Fig. 1: F4/80$^+$ cells (dark immunoprecipitate) adhere to the wall of a vessel underlying the ganglion cell layer in a 16-d embryonic retina. Bar, 20 μm. × 400. Fig. 2: Bright-field (*a*) and dark-field (*b*) views of a vertical section through the retina of a newborn BALB/c mouse. Arrows indicate F4/80$^+$ cells in the vascular supply and within the ganglion cell layer. *gcl*, Ganglion cell layer. *ipl*, Inner plexiform layer. *inl*, inner nuclear layer. Bar, 20 μm. × 300. Fig. 3: Bright field (*a*) and dark field (*b*) of an oblique section across the retina of a 5-d-old CBA mouse. Note the large rounded F4/80$^+$ cells in the ganglion cell layer compared with the finer processes of F4/80$^+$ cells in the inner plexiform layer (small arrows). The outer plexiform layer (large arrow in *a*) is just beginning to form. A detail of the outer plexiform layer in a five day retina is shown in Fig. 4. *onl*, outer nuclear layer. Bar, 20 μm. × 110. Fig. 4: Bright-field (*a*) and dark-field (*b*) views of an oblique section through the outer plexiform layer of the retina of a 5-d-old CBA mouse. Note the F4/80$^+$ positive cells in the inner nuclear layer associated with pyknotic nuclei (small arrows). Three F4/80$^+$ cells are found in the outer plexiform layer (large arrows) but no F4/80$^+$ cells can be seen in the outer nuclear layer (upper half of field). Bar, 20 μm. × 450.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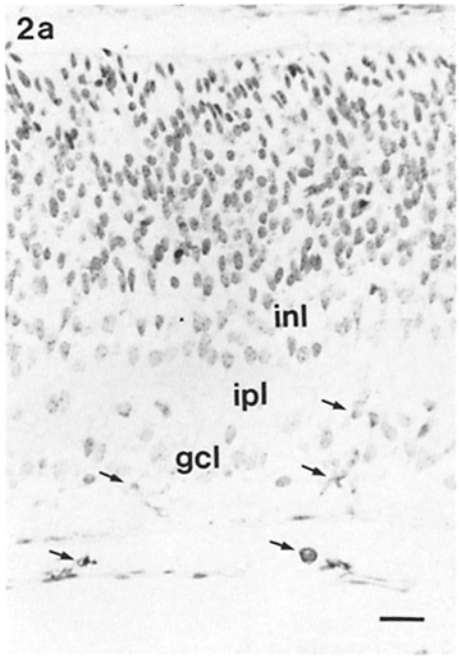
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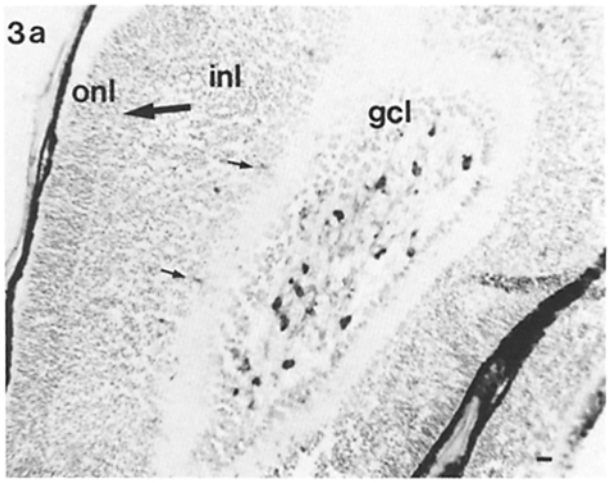
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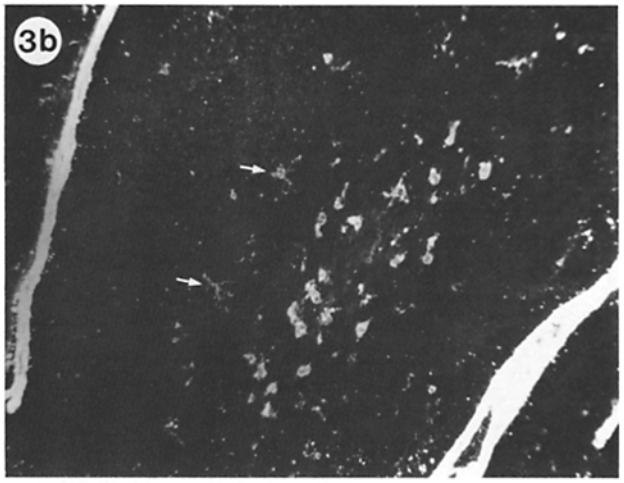
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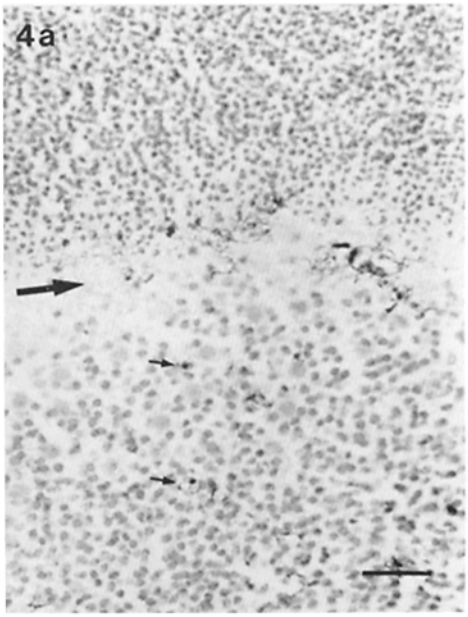
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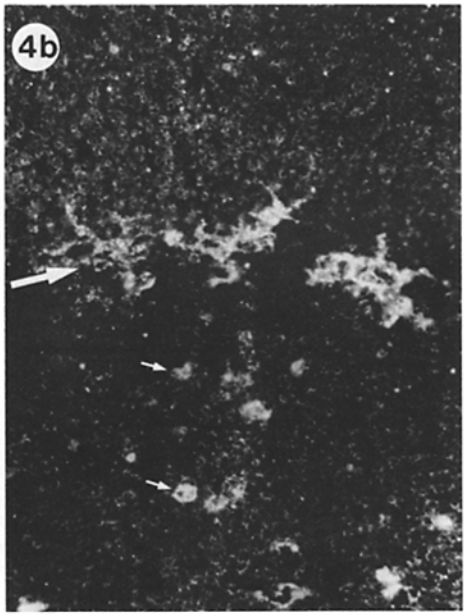
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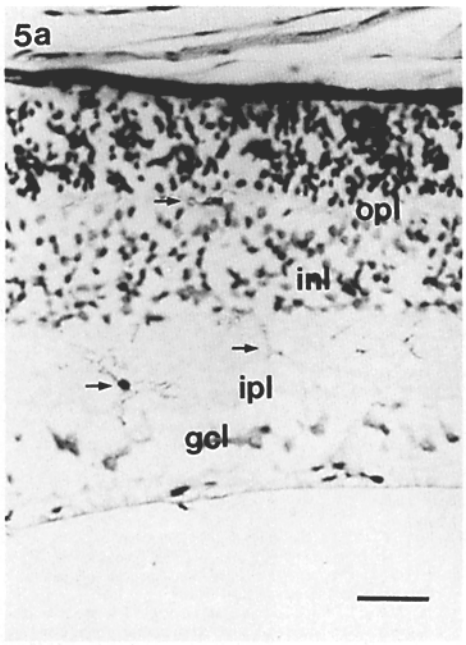
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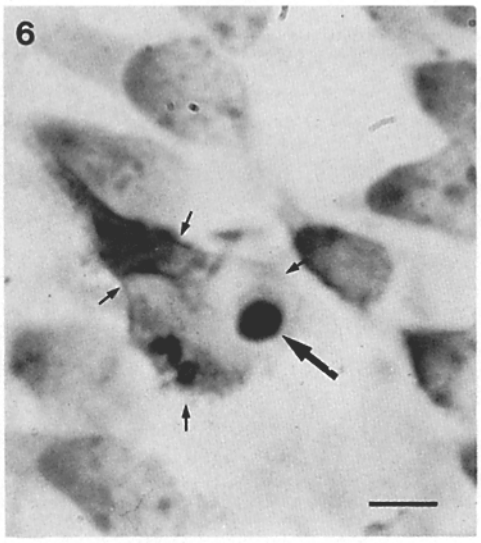
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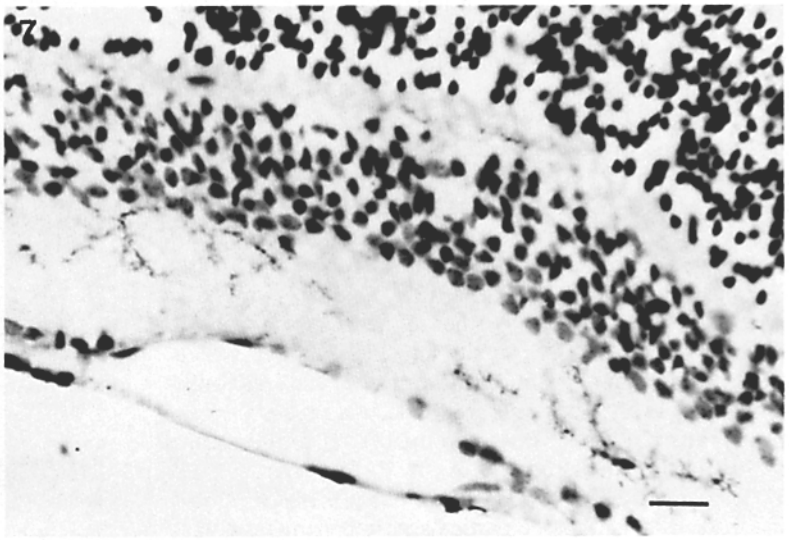

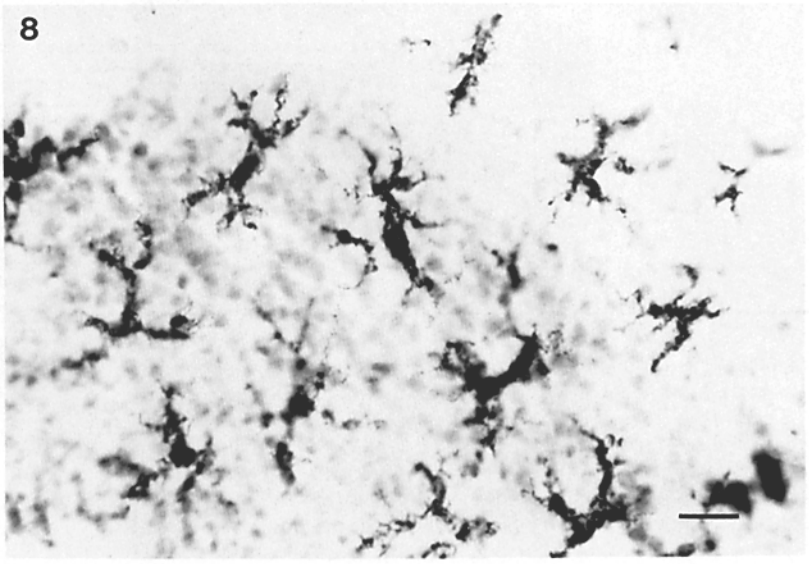
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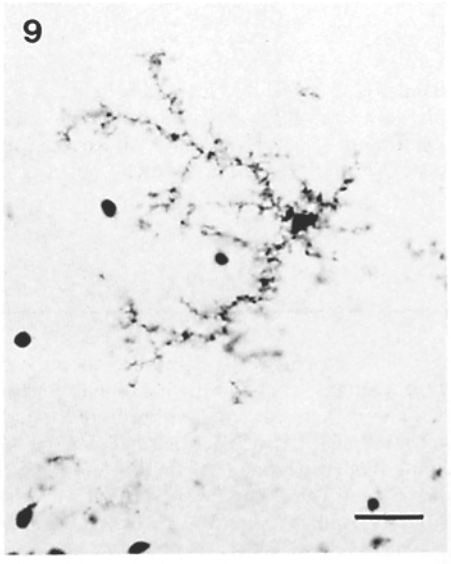
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During the development of the retina progressive stages in the differentiation of F4/80$^+$ monocytes into F4/80$^+$ microglial cells can be distinguished (see Figs. 1–5). In the early stages of this developmental sequence the F4/80$^+$ cells resemble so-called reactive or amoeboid microglia (1). The transformation of ameboid microglia into fully extended "resting" microglia has been described by others studying developing brain (20–24) and occurs also when monocytes infiltrate the brain in response to inflammatory stimuli in the adult (1, 4).

Our results support the hypothesis that the initial infiltration of mononuclear phagocytes into the retina is associated with neuronal death and that the invading cells are involved in phagocytosis and degradation of the resulting cellular debris. It is interesting to note that we found no F4/80$^+$ cells in the outer plexiform layer at any age and we observed that pyknotic nuclei are only rarely found in this layer. The scavenger function of the macrophages does not provide a conclusive explanation for their subsequent migration and differentiation. We found that the F4/80$^+$ cells tend initially to reside at the boundaries between the cellular layers prior to the formation of visible plexiform layers and that they are later observed in regular ordered arrays in both inner and outer plexiform layers. One possibility is that the macrophages themselves promote neurite extension to form the plexiform layers. Plasminogen activator, a secretory product of inflammatory macrophages (25), is apparently involved in neuronal cell migration (26).

The precise positioning of microglial cells could possibly result from their interaction with dying neurons. It has been shown in the cat retina that ganglion cells, horizontal cells, and photoreceptor cells are all distributed in regular ordered arrays (27). It follows that the death of excess neurons may also occur in a pattern dictated by the array of surviving cells. However, it is equally possible that infiltrating macrophages form specific associations with other retinal cells that lead to their migration to a particular site and their differentiation into microglia. There is a precedent for this in the epidermis where F4/80$^+$ Langerhans cells are found in a regular hexagonal array interdigitating between groups of 9–10 proliferating epidermal basal cells (Hume, D. A., A. P. Robinson, G. G. MacPherson, and S. Gordon, manuscript submitted for publication). It will therefore be particularly interesting to compare the distribution of F4/80$^+$ microglia with that of other retinal cell types.

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FIGURES 5–9 Immunohistochemical localization of F4/80 antigen in adult mouse retina. Fig. 5: Bright-field (*a*) and dark-field (*b*) views of a vertical section through a retina from a 10-d-old CBA mouse. The F4/80$^+$ cells and their processes (arrows) are largely restricted to the inner and outer plexiform layers. Bar, 20 μm. × 475. Fig. 6: A pyknotic nucleus (large arrow) being phagocytosed by an F4/80$^+$ cell. The small arrows indicate the boundary of the F4/80$^+$ plasma membrane. Bar, 5 μm. × 1,800. Fig. 7: A vertical section through the retina of an adult. Note the well developed processes of the F4/80$^+$ cells spanning the full width of the inner plexiform layer. Bar, 20 μm. × 375. Fig. 8: Horizontal section through the inner plexiform layer to show the regular distribution of F4/80$^+$ cells. Bar 20 μm. × 375. Fig. 9: A horizontal section showing an F4/80$^+$ cell in the outer plexiform layer of an adult retina. Bar, 20 μm. × 450.