GLIA 46:261–273 (2004)

Radial Migration of Developing Microglial Cells in Quail Retina: A Confocal Microscopy Study

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KEY WORDS Müller cells; microglial ramification; tenascin; s-laminin

ABSTRACT Microglial cells spread within the nervous system by tangential and radial migration. The cellular mechanism of tangential migration of microglia has been described in the quail retina but the mechanism of their radial migration has not been studied. In this work, we clarify some aspects of this mechanism by analyzing morphological features of microglial cells at different steps of their radial migration in the quail retina. Microglial cells migrate in the vitreal half of the retina by successive jumps from the vitreal border to progressively more scleral levels located at the vitreal border, intermediate regions, and scleral border of the inner plexiform layer (IPL). The cellular mechanism used for each jump consists of the emission of a leading thin radial process that ramifies at a more scleral level before retraction of the rear of the cell. Hence, radial migration and ramification of microglial cells are simultaneous events. Once at the scleral border of the IPL, microglial cells migrate through the inner nuclear layer to the outer plexiform layer by another mechanism: they retract cell processes, become round, and squeeze through neuronal bodies. Microglial cells use radial processes of s-laminin-expressing Müller cells as substratum for radial migration. Levels where microglial cells stop and ramify at each jump are always interfaces between retinal strata with strong tenascin immunostaining and strata showing weak or no tenascin immunoreactivity. When microglial cell radial migration ends, tenascin immunostaining is no longer present in the retina. These findings suggest that tenascin plays a role in the stopping and ramification of radially migrating microglial cells.

INTRODUCTION

Cellular mechanisms and molecular factors that control the motility of microglial cells have been studied in vitro in brain slices (Brockhaus et al., 1996; Schiefer et al., 1999; Stence et al., 2001) and microglial cell cultures (Booth and Thomas, 1991; Ward et al., 1991; Abd-el-Basset and Fedoroff, 1995; Nolte et al., 1996, 1997; Badie et al., 1999; Faff and Nolte, 2000; Honda et al., 2001; Rezaie et al., 2002). However, the migratory behavior of microglial cells is altered in cultures. Therefore, in situ studies of the central nervous system (CNS) are necessary to understand the cellular mechanisms of microglial migration. Studies of this type in the developing CNS revealed that microglial cells use tangential and radial migration to go from their entry points into the CNS to their final destinations (Navascués et al., 2000, 2002).

The quail embryo retina offers a particularly useful model to study microglial cell migration because it is a well-demarcated region of the CNS with no blood vessels, ruling out the possibility of microglial precursors reaching their final destinations directly from the blood. In

Grant sponsor: the Spanish Ministry of Science and Technology; Grant number: PM97-0178 and BMC2001-3274.

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Received 3 October 2003; Accepted 18 November 2003

DOI 10.1002/glia.20007

Published online 10 February 2004 in Wiley InterScience (www.interscience.wiley.com).

addition, whole-mounted retinas facilitate the analysis of complete unsectioned cells, even of those with complex morphological features, such as ramified microglial cells. The adult quail retina contains a dense network of ramified microglia (Navascués et al., 1994), which derive from amoeboid microglial cells that enter the developing retina between 7 and 16 days of incubation (E7–E16). They enter from the optic nerve head and the base of the pecten and then migrate tangentially on its vitreal surface in contact with Müller cell endfeet in a central-to-peripheral direction (Navascués et al., 1995; Marín-Teva et al., 1998). From E9 to the first half of the first posthatching week, microglial cells migrating on the vitreal surface of the retina can move radially in a vitreal-to-scleral direction to gain access to the inner plexiform layer (IPL) and the outer plexiform layer (OPL), where they become ramified microglia (Navascués et al., 1995; Marín-Teva et al., 1999c). Observations by various studies appear to support the view that tangential (Diaz-Araya et al., 1995a, 1995b; Provis et al., 1996) and radial (Hume et al., 1983; Bodeutsch and Thanos, 2000) migration of amoeboid microglia also occurs in the retina of mammals.

Some aspects of the mechanism of tangential migration of microglia in the quail retina have been clarified (Marín-Teva et al., 1998). Tangentially migrating amoeboid microglial cells are elongated in shape and emit broad lamellipodia that adhere to Müller cell endfeet. The lamellipodia seem to explore the vitreal environment of the retina and guide the migration of the microglia. The cellular mechanism of this movement involves polarized extension of lamellipodia at the leading edge of the cell, strong cell-to-substratum attachment, forward translocation of the cell body, and retraction of the rear of the cell. Moreover, amoeboid microglial cells alternate periods of active movement with pauses for mitosis (Marín-Teva et al., 1999a).

The chronotopographical pattern of the radial migration of microglial cells has been described in the quail retina (Marín-Teva et al., 1999c), but the cellular mechanisms involved in this migration remain unknown. In the present work, morphological features of microglial cells were studied during different phases of their radial migration using confocal laser scanning microscopy in whole-mounted quail embryo retinas. This analysis revealed that the cellular mechanisms involved in the radial and tangential migration of microglial cells are very different, with microglial cells ramifying while radially migrating. We also found that microglial cells use radial processes of Müller cells as the substratum for their radial migration. Finally, the chronotopographical pattern of tenascin expression in the retina suggested that this extracellular matrix molecule may play a role in the stopping and ramification of microglial cells at distinct levels of the retinal thickness.

MATERIALS AND METHODS

The study mainly used whole-mounted retinas and retinal sections from quail (*Coturnix coturnix japonica*) embryos at E9–E15, when radial migration of microglial cells is occurring (Navascués et al., 1995). Retinas from quails at 3–10 days posthatching (P3–P10) and from adult quails were also used to complete the chronotopographical pattern of tenascin distribution. The quails were killed in accordance with the euthanasia methods approved by the Laboratory Animal Unit of the University of Granada.

Preparation of Whole-Mounted Retinas and Retinal Sections

Retinas were isolated by rapid eye dissection in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), removing the cornea, lens, ciliary body, sclera, and vitreous body. Whole-mounted retinas were obtained by making radial incisions in isolated retinas that permitted their flattening on slides. They were then additionally fixed in 4% paraformaldehyde in 0.1 M PBS for 1–3 days.

To obtain retinal sections, isolated retinas were fixed in 4% paraformaldehyde in 0.1 M PBS for 2 h, washed in 0.1 M PBS, and incubated in 30% sucrose in PBS for 24 h. They were then soaked in OCT cryocompound (Sakura Finetek Europe, Zoeterwoude, The Netherlands), frozen, and stored at −40°C before sectioning on a Leica CM1850 cryostat. Retinal transverse sections 20 μm in thickness were captured onto Superfrost slides (Menzel-Glaser, Braunschweig, Germany) and immunocytochemically treated as described below.

Immunocytochemistry

Microglial cells in the quail retina were identified by immunocytochemical labeling using the monoclonal antibody QH1 [Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa City, IA], which recognizes all phases of development of quail microglial cells (Cuadros et al., 1992). Müller cells were identified by immunostaining with the monoclonal antibody H5 (DSHB), which recognizes vimentin, a component of the Müller cell cytoskeleton (Belmonte et al., 2000). Monoclonal antibodies C4 and M1B4 (both from DSHB) were used to label s-laminin and tenascin, respectively; s-laminin is an adhesion-related glycoprotein expressed in the retina by Müller cells (Libby et al., 1997; Marín-Teva et al., 1999b), and tenascin is an extracellular matrix protein expressed in the plexiform layers of the embryonic retina (Perez and Halfter, 1993; Belmonte et al., 2000).

For immunostaining with QH1, fixed whole-mounted retinas were washed free-floating in 0.1 M PBS and incubated in 0.1 M PBS with 1% Triton X-100 for 1 h with agitation. They were then incubated in normal goat serum (NGS) diluted 1:30 in 1% bovine serum albumin in 0.1 M PBS (BSA-PBS) for 2 h and incubated in QH1 supernatant diluted 1:4 in BSA-PBS for 1–3 days at 4°C with agitation. Subsequently, they were

washed in 0.1 M PBS, incubated in fluorescein isothiocyanate (FITC)-conjugated antimouse IgG (Sigma, St. Louis, MO) diluted 1:50 in BSA-PBS for 4–6 h at room temperature with agitation, and washed again in 0.1 M PBS. Finally, they were spread on slides with the vitreous side facing up and coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

The following double-labeled immunofluorescence experiments were carried out on retinal sections: H5/QH1, C4/QH1, and M1B4/QH1. Cell nuclei in double-labeled sections were revealed with Hoechst 33342 (Sigma) to identify the different retinal layers. Specifically, retinal sections were washed in 0.1 M PBS for 30 min, incubated in 0.1 M PBS with 0.3% Triton X-100 for 30 min, and treated with NGS diluted 1:30 in BSA-PBS for 1 h. They were then incubated in supernatant of the first primary antibody diluted in BSA-PBS (dilution 1:10 for H5 and M1B4, 1:2 for C4) for 24 h at 4°C, washed in 0.1 M PBS, and incubated in FITC-conjugated antimouse IgG diluted 1:60 in BSA-PBS for 3 h. Retinal sections were then washed in 0.1 M PBS, incubated in NGS diluted 1:30 in BSA-PBS for 2 h, incubated in QH1 supernatant diluted 1:4 in BSA-PBS for 24 h at 4°C, washed again in 0.1 M PBS, incubated in tetramethylrhodamine isothiocyanate (TRITC)-conjugated antimouse IgG (Sigma) diluted 1:50 in BSA-PBS for 2 h, and washed in 0.1 M PBS. Finally, sections were treated with Hoechst 33342 diluted in 0.1 M PBS (20 μg/ml) for 5 min, washed, and coverslipped with Vectashield. Results in double-labeled immunofluorescence sections were proven to be identical to those observed when antibodies were separately applied. Negative controls omitting the primary antibody were performed.

Confocal Microscopy

Information on the mechanism of radial migration of microglia was obtained by analyzing the morphological features of microglial cells at different steps of their migration. Radially migrating microglial cells were studied on QH1 immunostained whole-mounted retinas by using a Leitz DMRB microscope equipped with a Leica TCS-SP scanning laser confocal imaging system.

Each whole-mounted retina was previously observed by nonconfocal fluorescence microscopy to select microscopic fields for analysis with confocal microscopy. Confocal microscopic observations were made under a 63×/1.32 oil PLAN APO objective. In each microscopic field, radially migrating microglial cells were observed from two angles, parallel (XY views) and perpendicular (XZ views) to the retinal surface, facilitating their morphological analysis. For this purpose, stacks of optical sections in planes parallel to the retinal surface (XY optical sections) were collected at 0.5–1.5 μm intervals throughout the retinal thickness. Six to eight scans were carried out for each optical section in order to improve the signal-to-noise ratio of the confocal image. Optical section images were stored as TIF files of 1,024 × 1,024 pixel eight-bit gray scale. XY optical sections from stacks in each microscopic field were classified in different series according to their location in different retinal layers. Leica Confocal Software (Leica, Heidelberg, Germany) was used to superimpose optical sections from each series onto two-dimensional (2D) projection images (XY 2D projection images) of the profiles of all microglial cells contained in each retinal layer (Fig. 1A–C). XY 2D projection images were obtained in the following layers: the nerve fiber layer (NFL) together with the ganglion cell layer (GCL; XY NFL + GCL; Fig. 1A), the vitreal part of the IPL (XY vIPL; Fig. 1B), the intermediate zone of the IPL (XY iIPL; Fig. 1C), the scleral part of the IPL, the inner nuclear layer (INL), and the OPL. At developmental stages in which radially migrating microglial cells had not yet reached some of these layers, their projection images were omitted because they did not contain microglial cell profiles.

Microglial cells of particular interest in microscopic fields in which XY 2D projection images had previously been obtained were then selected to generate stacks of virtual optical sections in a plane perpendicular to the retinal surface (XZ optical sections) at intervals of 0.5–1 μm. 2D projection images (XZ 2D projection images) were then obtained from XZ optical section stacks (Fig. 1D–F). They showed microglial cell profiles through the retinal thickness, highlighting the levels at which the soma and ramified processes of these cells were located.

Confocal microscopic observations on double-immunolabeled retinal transverse sections were made by obtaining 2D projection images from stacks of optical sections through variable portions of the section thickness. FITC and TRITC fluorescence from immunostained retinal components was detected at 500–540 nm using Argon laser (488 nm) illumination and at 590–630 nm using helium-neon laser (543 nm) illumination, respectively. The sequential scan option was used to minimize the cross-talk between fluorochromes. Hoechst 33342-stained cell nuclei were imaged by nonconfocal microscopy using a Leica DC200 digital camera (1,280 × 1,024 pixel resolution) adapted to the Leitz DMRB microscope described above. Observations were performed with an ultraviolet filter set (illumination path BP340–380, observation path LP425). Photoshop 5.5 (Adobe Systems) was used to merge the Hoechst-labeled nonconfocal image with FITC- and TRITC-labeled confocal images of each microscopic field.

RESULTS

Ramification Levels of Radially Migrating Microglial Cells

Amoeboid microglial cells migrated tangentially on endfeet of Müller cells in the vitreal part of the quail

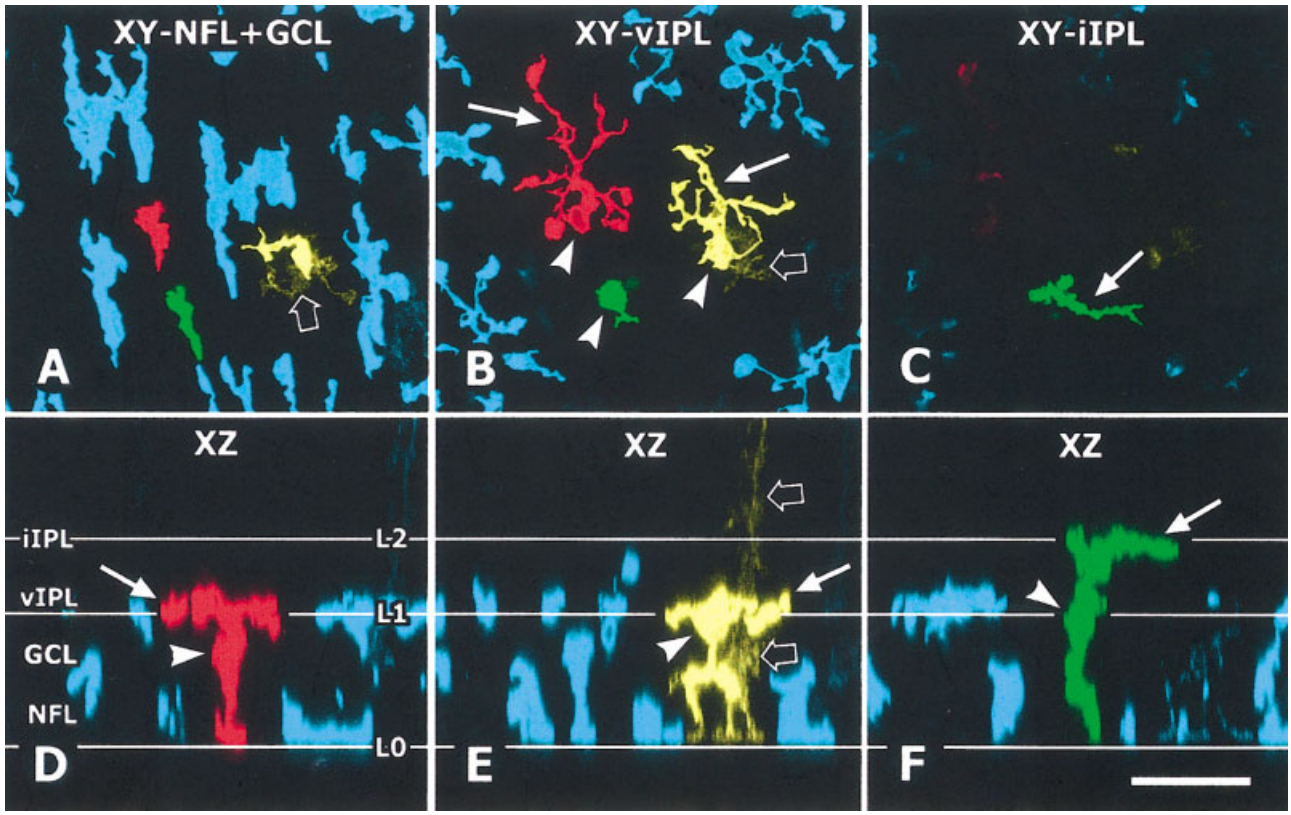

Fig. 1. 2D projection images of QH1-immunolabeled microglial cells. **A–C:** XY 2D projection images generated from XY optical sections contained in the nerve fiber layer and ganglion cell layer (XY NFL + GCL; A), in the vitreal part of the inner plexiform layer (XY vIPL; B), and in the intermediate part of the inner plexiform layer (XY iIPL; C). **D–F:** XZ 2D projection images generated from stacks of XZ optical sections containing the red (D), yellow (E), and green (F) microglial cells. The white line at the bottom of each picture represents the vitreal border of the retina (L0), where microglial cells do not ramify. The white lines marked with L1 and L2 represent microglial cell ramification levels at the vitreal border of the inner plexiform layer (L1) and at an intermediate zone of the inner plexiform layer (L2). The vitreal ends of the red, yellow, and green microglial cells are located at L0, whereas their ramified scleral portions (thin arrows in D–F) are located at L1 (red and yellow cells in D and E, respectively) and at L2 (green cell in F). Arrowheads in B and D–F indicate the bodies of these three cells. The yellow microglial cell is closely associated to vitreal processes of a Müller cell showing weak QH1 immunolabeling (thick open arrows in A, B, and E). Red and yellow microglial cells are migrating from the nerve fiber layer (A, D, and E) to the vitreal border of the inner plexiform layer (B, D, and E), where they ramify (thin arrows in B, D, and E). The green microglial cell is migrating from the nerve fiber layer (A, and F) to the intermediate part of the inner plexiform layer (C, and F), where it is beginning to ramify (thin arrow in C, and F). Scale bar: 25 μm.

embryonic retina (Marín-Teva et al., 1998). In the present study, this vitreal part was termed level 0 (L0), in which migrating microglial cells were amoeboid in shape and did not ramify. At E9, a few microglial cells changed the direction of their migration, beginning to move radially in a vitreal-to-scleral direction (Navascués et al., 1995; Marín-Teva et al., 1999c). From E9 on, more and more microglial cells migrated radially from L0 to four distinct levels in the retinal thickness, where they ramified horizontally, i.e., parallel to the retinal surface. These levels (Fig. 2) were located at the vitreal border (level 1, L1), intermediate regions (level 2, L2), and scleral border (level 3, L3) of the IPL and the vitreal border of the OPL (level 4, L4).

Radial Migration From L0 to L3

At E9, some amoeboid microglial cells migrating tangentially at L0 showed lamellipodia closely adhered to the vitreal border of the retina, and a long thin process perpendicular to the retinal surface (Fig. 3A) that ended in an expanded region (Fig. 3A and C). This process reached L1 by crossing through the NFL and GCL (Fig. 3A–C). The nucleus of other microglial cells with lamellipodia at L0 was observed to be within a radially oriented process that ended at L1, where it bifurcated (Fig. 3D–F). Other microglial cells showed a soma containing the nucleus at L1 connected with lamellipodia at L0 by a radially oriented process (Figs. 1A, B, D, and E and 3G–I). This last group of cells showed an incipient horizontal ramification at L1, where their soma bore several cell branches oriented parallel to the retinal surface. In addition, ramified microglial cells with no lamellipodia were entirely localized at L1 (Fig. 3J–L).

The above observations suggested that amoeboid microglial cells migrating tangentially at L0 began to migrate radially by emitting a radially oriented leading process that reached L1. Then, the cell nucleus ap-

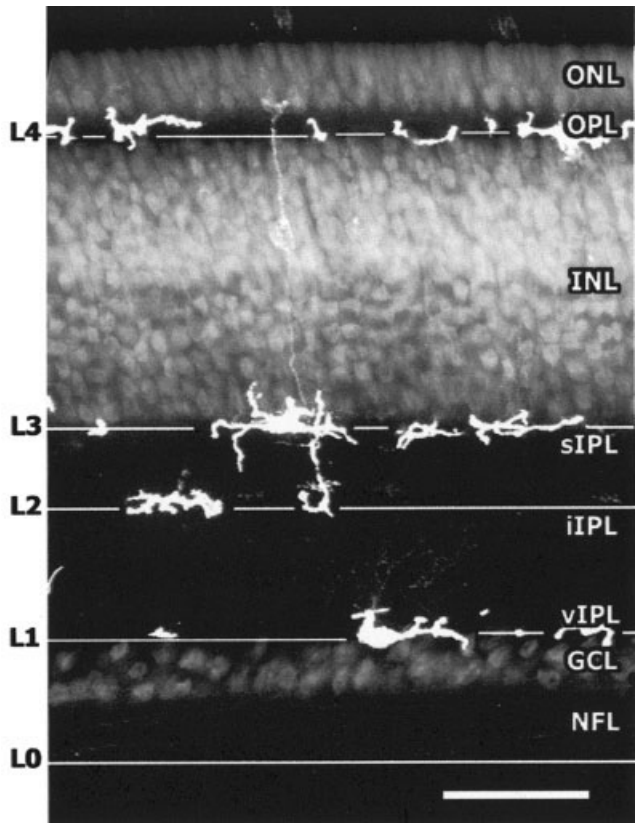

Fig. 2. Ramification levels of radially migrating microglial cells shown in a transverse section of a P4 quail retina immunostained with QH1 to reveal microglial cells (white) and treated with Hoechst 33342 to stain cell nuclei (different shades of gray), distinguishing the different retinal layers. The white line marked with L0 represents the vitreal border of the retina. Ramified microglial cells are seen in four levels of the retinal thickness. These levels, marked with L1, L2, L3, and L4, are indicated with white lines at the vitreal border (vIPL), intermediate zone (iIPL), and scleral border (sIPL) of the inner plexiform layer, and the vitreal border of the OPL, respectively. ONL, outer nuclear layer. Scale bar: 37 μm.

peared to translocate from L0 to L1 by moving through the leading process. The expanded region at the end of this leading process ramified horizontally at L1 simultaneously to the translocation of the nucleus. Then, microglial cells retracted the lamellipodia and radially oriented process, losing contact with the vitreal surface of the retina and becoming entirely located at L1. The first microglial cells reaching L1 were seen at E9–E10. Further microglial cells migrated radially from L0 to L1 at subsequent developmental stages until the first posthatching days.

A similar mechanism to that described for migration from L0 to L1 appeared to be used by ramified microglial cells to jump from L1 to L2 (Fig. 4A–J) and from L2 to L3 (not illustrated). In fact, ramified microglial cells at L1 (Fig. 4A and C) emitted a radial process ending at L2 (Fig. 4A and D), i.e., in an intermediate zone of the IPL, where it ramified (Fig. 4F and I). Then, cell branches at L1 progressively retracted (Fig. 4F and H), and the nucleus translocated through the radial process to become situated at L2. Finally, the radial process retracted and the microglial cell became completely located at L2 (mc3 in Fig. 4N).

Morphological features of some ramified microglial cells at L2 suggested they began to migrate from L2 to L3 before completing their migration from L1 to L2. The horizontal branches of these cells at L2 were radially connected with both the soma at L1 and an incipiently ramified process at L3 (Fig. 4P–T). Other microglial cells appeared to migrate directly from L1 to L3 without previous ramification at L2. The soma of the latter cells, which bore some horizontal processes, was located at L1 and emitted a radial process that traversed the entire thickness of the IPL, ending at L3 (Fig. 4K–O, mc5). Presumably, the end of the radial process subsequently ramified. Microglial cells migrating from L1 to either L2 or L3 were seen from E12 until the first posthatching days.

Radial Migration From L3 to L4

Microglial cells apparently migrating through the INL from L3 to L4 were occasionally observed at E13 and were more numerous from E14 on. The mechanism by which microglial cells migrated from L3 to L4 apparently differed from that used to move from L1 to L2 and L3. Thus, most ramified microglial cells migrating from L3 to L4 first emitted a protuberance that contained the nucleus and entered the vitreal portion of the INL (Fig. 5A, D, and E). Then, their cell processes at L3 retracted, and the cells became round (Fig. 5G and K) with short pseudopods (Fig. 6C and F), squeezing through neuronal bodies in the INL to reach the OPL. Once in this layer, the cells emitted horizontal cell processes to adopt a ramified morphology (Fig. 5M and R).

Occasionally, some ramified microglial cells at L3 bore a radial process that traversed the INL and ended in the OPL (Fig. 5S and V–X). These observations suggested that some microglial cells did not become round during their migration from L3 to L4, but rather migrated by a similar mechanism to that described above for migration between L0 and L3.

Migrating Through Two Ramification Levels in One Step

Most radially migrating cells moved either from L0 to L1, from L1 to L2, from L2 to L3, or from L3 to L4, i.e., they migrated from one level to the next. However, some microglial cells appeared to migrate from L0 to L2 in one step. These cells were radially oriented and extended from L0 to L2, where they began to ramify horizontally (green cell in Fig. 1A–C and F). Other microglial cells appeared to migrate in one step from L1 to L3 (mc5 in Fig. 4K and M–O). However, no images were observed that suggested cell migration from L1 or L2 to L4 in one step.

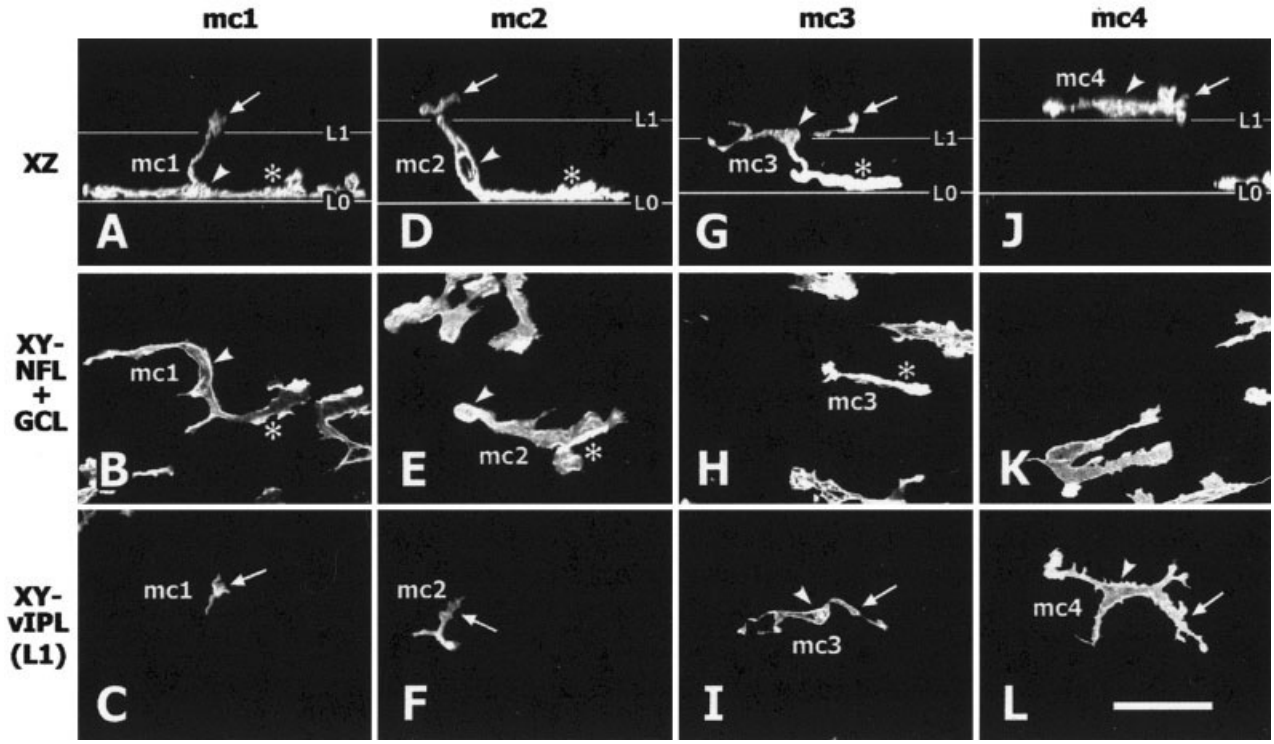

Fig. 3. Four microglial cells (mc1–mc4) at different steps of their radial migration from the vitreal border of the retina (L0) to the vitreal border of the inner plexiform layer (L1) in E10 whole-mounted retinas immunostained with QH1. Each column of micrographs (A–C, D–F, G–I, and J–L) shows an XZ 2D projection image of a radially migrating microglial cell (XZ; A, D, G, and J) and XY 2D projection images of the same cell obtained separately at the nerve fiber layer and ganglion cell layer (XY NFL + GCL; B, E, H, and K) and at the vitreal part of the inner plexiform layer (XY vIPL; C, F, I, and L). In A, D, G, and J, the white lines marked with L0 represent the vitreal border of the retina, and those marked with L1 represent the ramification level at the vitreal border of the inner plexiform layer. **A–C:** Microglial cell (mc1) with the soma (arrowheads in A and B) and lamellipodia (asterisks in A and B) in close contact with the vitreal border of the retina. A thin cell process from the soma crosses the nerve fiber layer and the ganglion cell layer and ends in an expanded region (arrows in A and C) at L1. **D–F:** Microglial cell (mc2) showing lamellipodia (asterisks in D and E) in close contact with the vitreal border of the retina and a soma (arrowheads in D and E) that is radially traversing the nerve fiber layer. The short process at the scleral pole of the soma is beginning to ramify (arrows in D and F) at L1. **G–I:** Microglial cell (mc3) showing an incipient ramification (arrows in G and I) at L1. Its soma (arrowheads in G and I) is connected with a lamellipodium (asterisks in G and H) that contacts the vitreal border of the retina. **J–L:** Microglial cell (mc4) with its soma (arrowheads in J and L) and cell branches (arrows in J and L) located at L1. Scale bar: 30 μm.

Adhesion of Radially Migrating Microglial Cells to Radial Processes of Müller Cells

Immunolabeling for vimentin with H5 monoclonal antibody revealed a dense network of vimentin-positive radial processes of Müller cells in the quail embryonic retina (Fig. 6A). Double immunolabeling with H5 and QH1 antibodies showed that microglial cells migrating radially through the retinal thickness closely adhered to vimentin-positive Müller radial processes. Adhesions between these two cell types were evident in the NFL (Fig. 6B), IPL (not shown), and INL (Fig. 6C). The same microglial cell could adhere to different Müller radial processes, suggesting that microglial cells jumped from one Müller radial process to another during their radial migration.

In the developing quail retina, strong s-laminin immunostaining of Müller cell bodies and radial processes was observed in the INL and NFL, but it was less apparent in the IPL and OPL (Fig. 6D–F). Radially migrating microglial cells were seen closely adhered to s-laminin-positive Müller cells (Fig. 6E and F).

Tenascin Distribution in Developing Plexiform Layers and Relationship With Horizontal Ramification of Radially Migrating Microglial Cells

Immunolabeling with M1B4 antibody revealed that tenascin was present in the retina from early developmental stages until the end of the first posthatching week (Fig. 7). The vitrealmost part of the retina was tenascin-positive at E7 (not shown). At later developmental stages (E10, E13, and E15), the differentiated plexiform layers were strongly tenascin-positive (Fig. 7A–D), whereas the NFL and GCL were tenascin-negative. The INL was also tenascin-negative, although it occasionally showed a weak reticulate immunolabeling (Fig. 7A and B).

In the IPL of embryonic retinas from E11 to hatching, the distribution of tenascin appeared stratified, with several strongly tenascin-immunoreactive sublayers separated by strata with weak tenascin immunolabeling (Fig. 7A–D). During the first posthatching week,

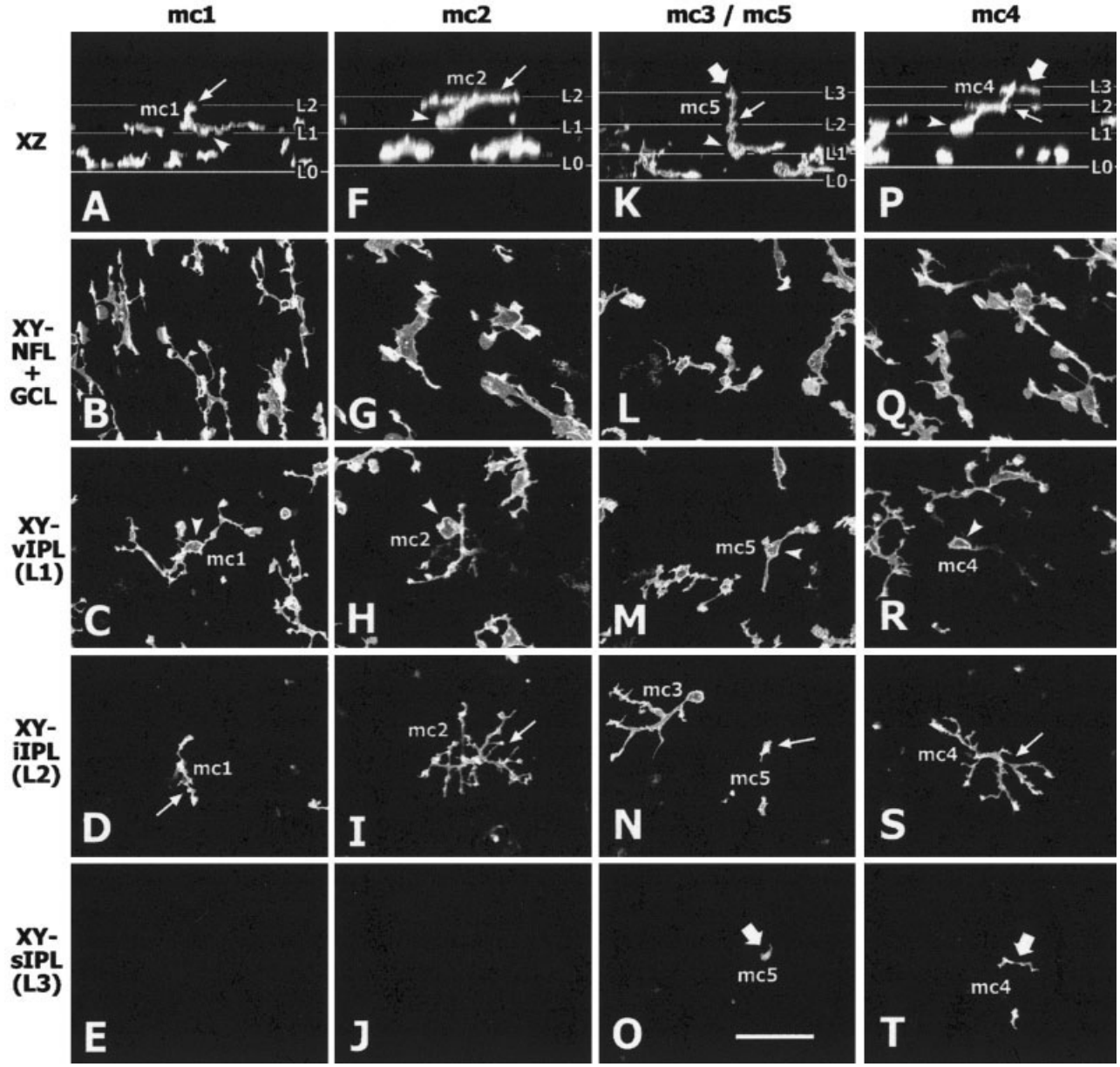

Fig. 4. Five microglial cells (mc1–mc5) at different steps of their radial migration within the inner plexiform layer, from its vitreal border (L1) to intermediate zones (L2) and the scleral border of this layer (L3), in E13 whole-mounted retinas immunostained with QH1. Each column of micrographs (A–E, F–J, K–O, and P–T) shows an XZ 2D projection image of a radially migrating microglial cell (XZ; A, F, K, and P) and XY 2D projection images of the same cell obtained separately at the vitreal (XY vIPL; C, H, M, and R), intermediate (XY iIPL; D, I, N, and S), and scleral (XY sIPL; E, J, O, and T) parts of the inner plexiform layer. No profiles of cells mc1–mc5 are observed in the 2D projection images obtained at the nerve fiber layer and ganglion cell layer (XY NFL + GCL; B, G, L, and Q). In A, F, K, and P, the white lines marked with L0 represent the vitreal border of the retina and those marked with L1, L2, and L3 represent the ramification levels at the vitreal border, intermediate zones, and scleral border of the inner plexiform layer, respectively. **A–E:** Microglial cell (mc1) with its soma (arrowheads in A and C) and most of its cell branches located at L1. A cell process from the soma reaches L2, where it is beginning to ramify (thin arrows in A and D). No portions of this cell have reached L3 (E). **F–J:** Microglial cell (mc2) showing a soma (arrowheads in F and H) and a few cell branches located at L1. A radial process ramifies profusely (thin arrows in F and I) at L2 but does not reach L3 (J). **K–O:** Microglial cell (mc3 in N) with its soma and cell branches entirely located at L2. No portions of this cell are observed at L1 (M) or L3 (O). Another microglial cell (mc5) has its soma (arrowheads in K and M) at L1 and shows a radial process traversing the inner plexiform layer with small protuberances at L2 (thin arrows at K and N) and L3 (thick arrows at K and O). **P–T:** Microglial cell (mc4) with its soma (arrowheads in P and R) located at L1, which emits a radial process traversing the inner plexiform layer. This process ramifies profusely at L2 (thin arrows in P and S) and extends to L3, where it is beginning to emit cell branches (thick arrows in P and T). Scale bar: 30 μm.

the weak tenascin-immunoreactive sublayers increased their thickness (Fig. 7E) and progressively lost their weak immunoreactivity. Finally, the entire thickness of the IPL became tenascin-negative after the first posthatching week and remained so in the retina of adult quails (Fig. 7F).

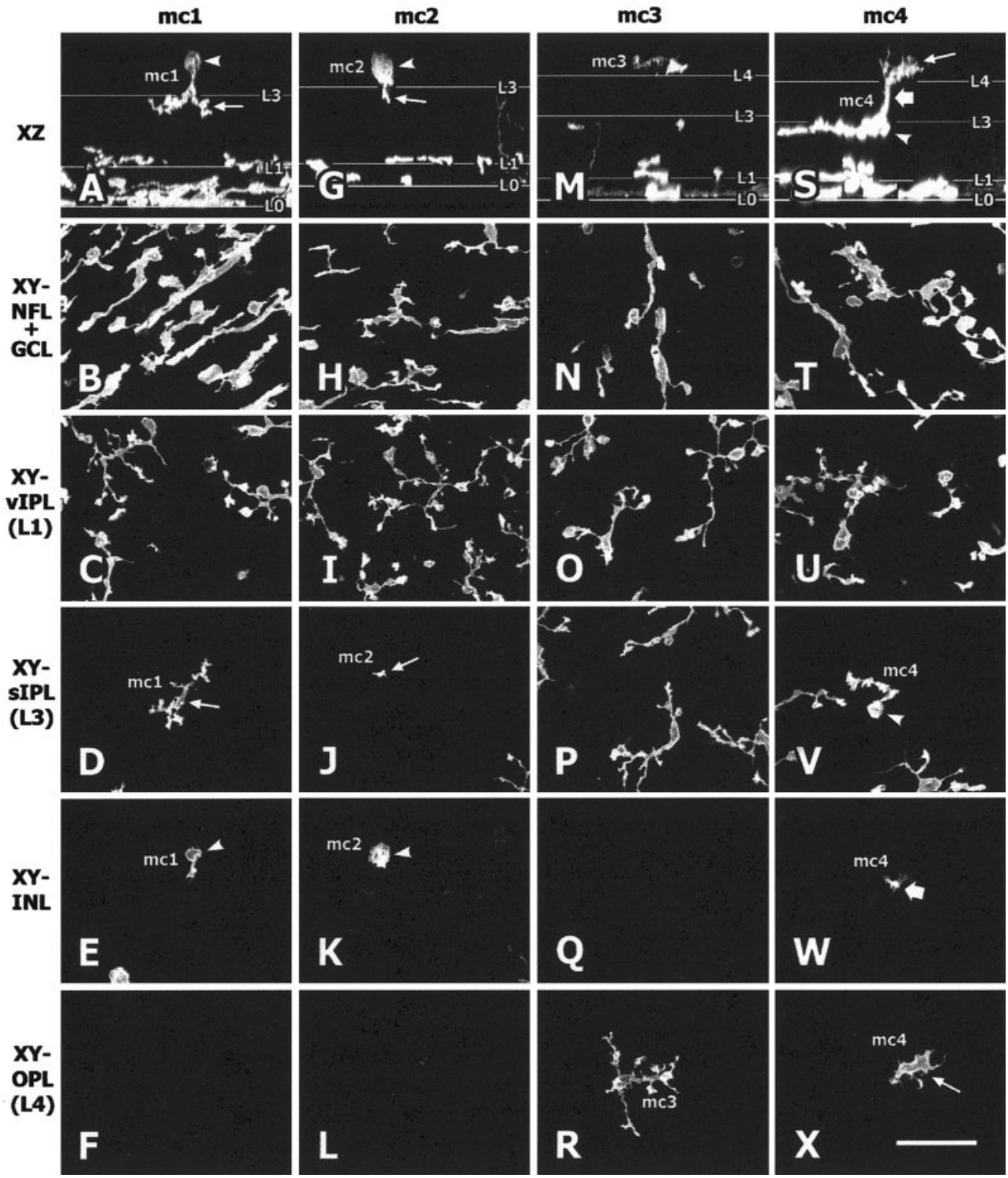

Fig. 5. Four microglial cells (mc1–mc4) are seen at different steps of their radial migration from the scleral border of the inner plexiform layer (L3) to the outer plexiform layer (L4) in E13 (A–F) and E15 (G–X) whole-mounted retinas immunostained with QH1. Each column of micrographs (A–F, G–L, M–R, and S–X) shows an XZ 2D projection image of a radially migrating microglial cell (XZ; A, G, M, and S) and XY 2D projection images of the same cell obtained separately at the scleral border of the inner plexiform layer (XY sIPL; D, J, P, and V), inner nuclear layer (XY INL; E, K, Q, and W), and outer plexiform layer (XY OPL; F, L, R, and X). No profiles of cells mc1–mc4 are observed in the 2D projection images obtained at the nerve fiber layer and ganglion cell layer (XY NFL + GCL; B, H, N, and T) or the vitreal part of the inner plexiform layer (XY vIPL; C, I, O, and U). In A, G, M, and S, the white lines marked with L0 represent the vitreal border of the retina, and those marked with L1, L3, and L4 represent the ramification levels at the vitreal and scleral borders of the inner plexiform layer and the vitreal border of the outer plexiform layer, respectively. L2 is not represented because no microglial cells are observed at this level. **A–F:** Microglial cell (mc1) ramified at L3 (thin arrows in A and D) showing a process that contains the nucleus (arrowheads in A and E) protruding toward the inner nuclear layer. **G–L:** Round microglial cell (mc2, arrowheads in G and K) within the inner nuclear layer. The cell shows a short process (thin arrows in G and J) at L3. **M–R:** Ramified microglial cell (mc3 in M and R) entirely located at L4. **S–X:** Microglial cell (mc4) with its soma (arrowheads in S and V) and a few cell branches located at L3. A radial process from the soma (thick arrows in S and W) traverses the inner nuclear layer and ends at L4, where it is beginning to ramify (thin arrows in S and X). Scale bar: 30 μm.

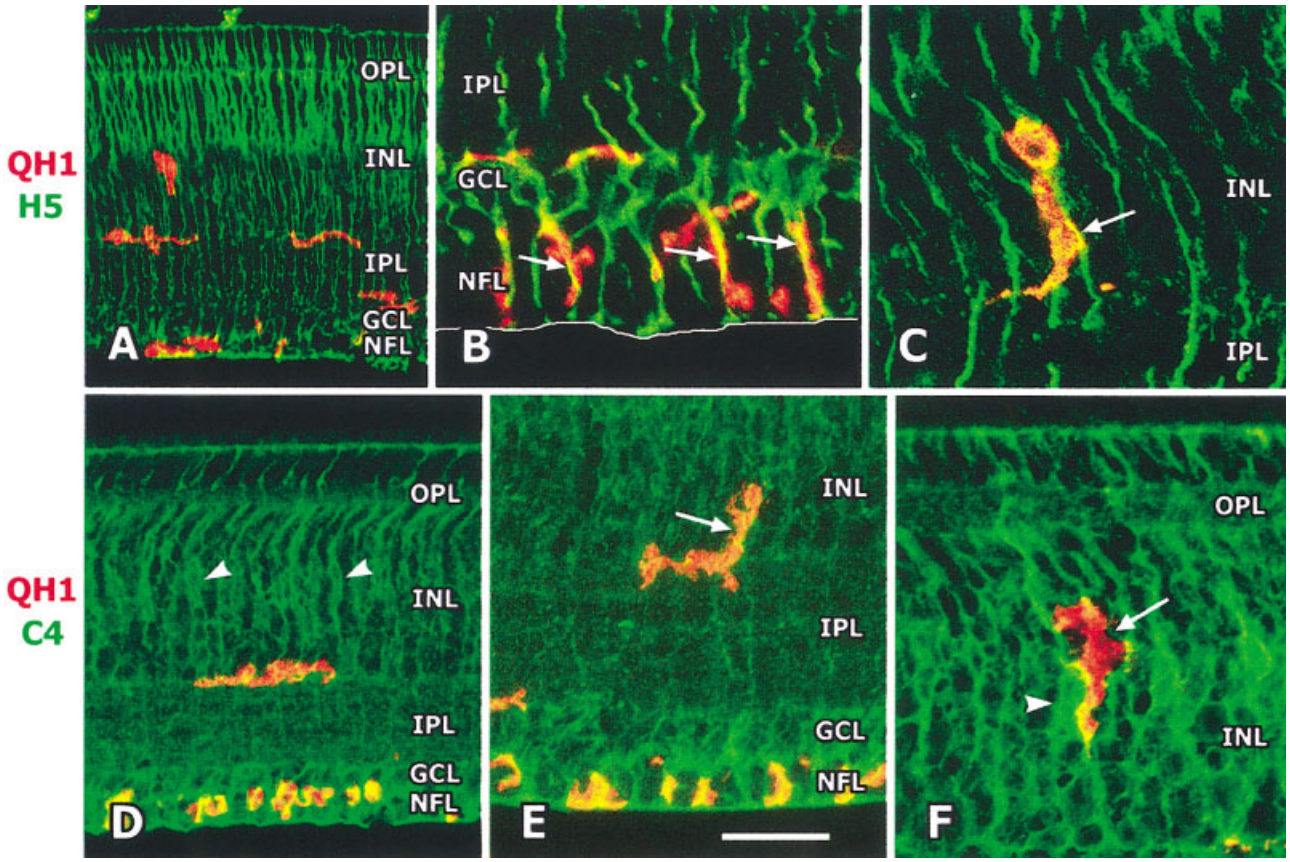

Fig. 6. Confocal microscopic pictures of transverse sections of retinas from E13 (**A**–**E**) and E15 (**F**) quail embryos treated with double immunostaining with the antibodies QH1, revealing microglial cells (red), and either H5 (A–C) or C4 (D–F), revealing vimentin (green in A–C) or s-laminin (green in D–F) in Müller cells, respectively. Abbreviations indicate locations of the different retinal layers. A–C: A scaffolding of Müller cell radial processes is seen throughout the retinal thickness (green in A). Microglial cells (red in B and C) closely contact Müller cell radial processes (green in B and C) at the nerve fiber layer (B) and inner nuclear layer (C). B and C are 2D projection images from only two optical sections selected from a stack of optical sections obtained at 0.6 μm intervals throughout the thickness of a retinal transverse section. Yellow profiles (arrows in B and C) show superimpositions of Müller cell radial processes and microglial cells corresponding to close contacts between them. The white line at the bottom of B represents the vitreal border of the retina. D–F: Strong s-laminin expression (green) in bodies (arrowheads in D and F) and radial processes of Müller cells at the inner nuclear, ganglion cell, and nerve fiber layers. Müller cell processes at the inner and outer plexiform layers show a much weaker labeling. Microglial cells (arrows in E and F) migrating through the inner nuclear layer are closely adhered to either s-laminin-positive radial processes (E) or bodies (arrowhead in F) of Müller cells. Scale bar: 46 μm for A; 22 μm for B; 20 μm for C, E, and F; and 36 μm for D.

The ramification levels of microglial cells (L1 to L4) matched interfaces between strongly tenascin-positive retinal strata and strata with weak or no tenascin immunoreactivity. Thus, horizontally ramified microglial cells at L1 were located at the interface between the tenascin-negative GCL and the strongly tenascin-positive IPL (Fig. 7A). Microglial cell horizontal processes at L2 were located in intermediate areas of the IPL, always coinciding with weakly tenascin-positive strata sandwiched between strongly tenascin-positive strata (Fig. 7B). Horizontally ramified microglia at the scleral border of the IPL (L3) were located between the strongly tenascin-positive IPL and the tenascin-negative INL (Fig. 7C). Likewise, horizontal microglial processes at L4 were located at the interface between the tenascin-negative INL and the strongly tenascin-positive OPL (Fig. 7D).

Taken together, these observations suggested that radially migrating microglial cells were induced to ramify when they reached an interface between retinal strata with different degrees of tenascin immunostaining, with the cells ramifying along the plane of the interface.

DISCUSSION

We concluded from our results that microglial cells migrate radially in the quail retina by successive jumps from the vitreal part to progressively more scleral levels of the retinal thickness, at which they stop and ramify horizontally; these cells use Müller radial processes as the substratum for radial migration; morphological features of radially migrating microglial cells are different from those of microglial cells in the process of tangential migration, indicating that the mechanisms of radial and tangential migration differ; and the chronotopographical pattern of tenascin expression suggests a role for this extracellular matrix glycopro-

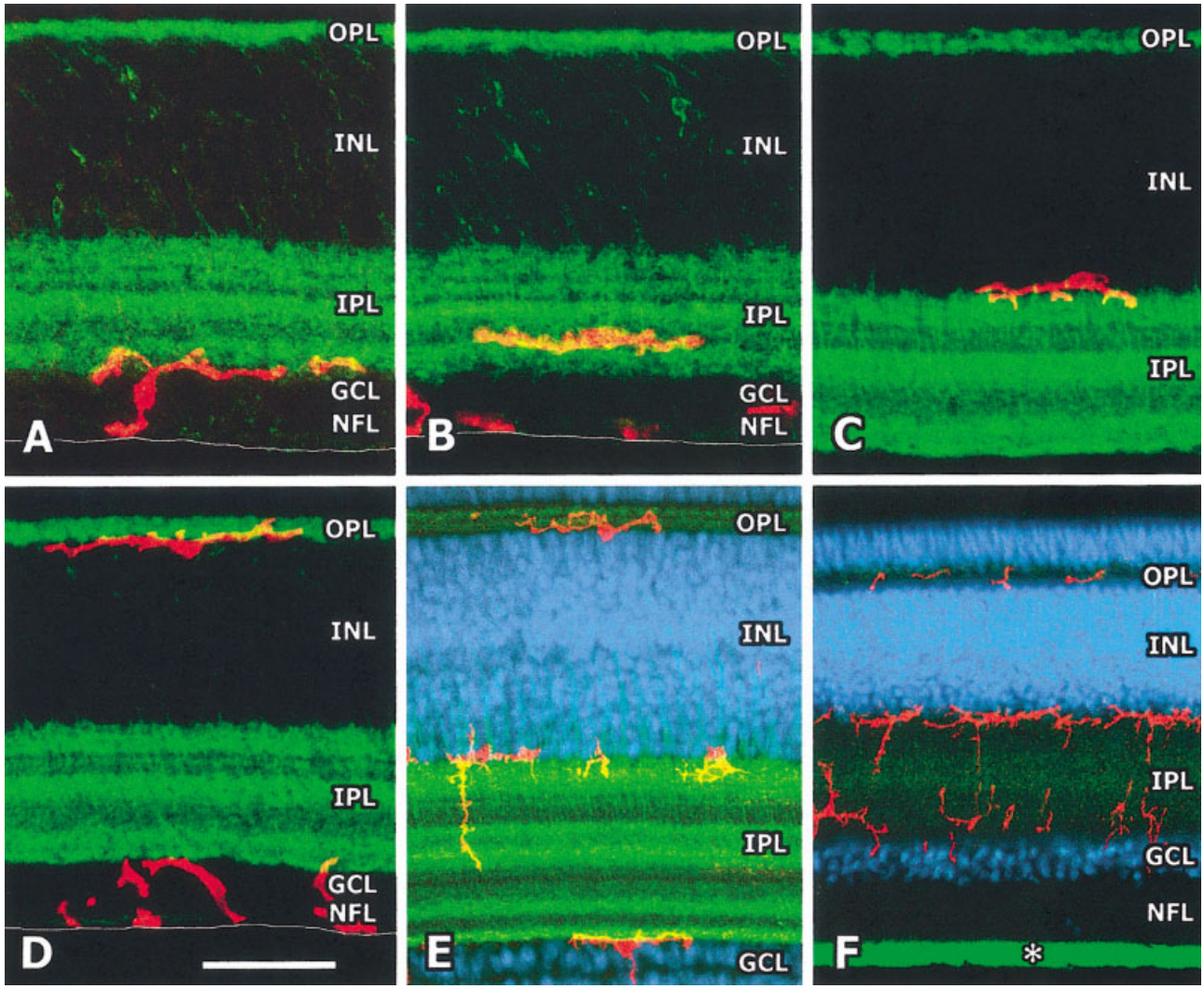

Fig. 7. Confocal microscopy of transverse sections of E13 (**A** and **B**), E15 (**C** and **D**), P3 (**E**), and mature (**F**) quail retinas with double immunostaining with QH1, revealing microglial cells (red), and M1B4, revealing tenascin (green). Cell nuclei (blue) are shown in E and F by Hoechst staining. Abbreviations indicate locations of the different retinal layers. At embryonic developmental stages (A–D), the plexiform layers are strongly tenascin-positive, whereas the nerve fiber and the ganglion cell layers are tenascin-negative. The inner nuclear layer is mostly tenascin-negative, although a weak pericellular immunolabeling is occasionally observed (A and B). Strongly and weakly tenascin-positive sublayers alternate within the inner plexiform layer (A–D). A microglial cell apparently migrating from the vitreal border of the retina (white line at the bottom of A, B, and D) to the vitreal border of the inner plexiform layer shows horizontal branches located at the interface between tenascin-negative and tenascin-positive territories (A). Branches of ramified microglial cells at the scleral border of the inner plexiform layer (C) and the vitreal border of the outer plexiform layer (D) also coincide with interfaces between tenascin-positive and tenascin-negative territories. The horizontal branches of ramified microglial cells in an intermediate area of the inner plexiform layer (B) are located at the interface between two strata of different tenascin-immunoreactivity. At posthatching developmental stages (E), weakly tenascin-positive sublayers within the inner plexiform layer are thicker than those at embryonic stages. In the mature retina (F), tenascin immunoreactivity has been lost in the inner and outer plexiform layers. The strongly tenascin-positive band (asterisk in F) corresponds to vitreous remains adhered to the retinal surface. Scale bar: 35 μm for A and B; 40 μm for C and D; 48 μm for E; and 65 μm for F.

tein in the mechanism of radial migration, signaling levels of the retinal thickness at which microglial cells stop and ramify. These findings are discussed separately below.

Radial Migration and Ramification of Microglial Cells

Our observations on fixed whole-mounted retinas of developing quails are like snapshots of microglial cells depicted during their process of radial migration. The morphological features of these fixed cells suggest that they use a similar mechanism to move from L0 to L1, from L1 to L2, and from L2 to L3. Microglial cells in a retinal level first emit a process (leading process) directed toward the next ramification level; then, the nucleus enters and travels along the leading process, reaching its end at the next level. Simultaneously, the end of the leading process ramifies. Subsequently, the rear process, which connects the cell to the ramification

level from which it moves, retracts. The outcome of these events is that the microglial cell is located and ramified at a more scleral level.

Most microglial cells migrating through the INL from L3 to L4 show morphological features suggesting that a different mechanism of cell migration is involved. Ramified microglial cells at L3 round when they enter the INL and do not emit a leading process but have short and thick pseudopods to squeeze through neuronal bodies in the INL. This cell migration mechanism appears to be similar to that reported in activated microglial cells migrating within brain slices in vitro (Brockhaus et al., 1996; Schiefer et al., 1999; Stence et al., 2001). The causes of the differences in the mechanisms of radial migration through different retinal layers are unknown, although different cell microenvironments in the plexiform and nuclear layers may exert an influence.

We do not know the factors responsible for microglial cell ramification at distinct retinal levels. Many studies have highlighted contact with astrocytes as a major factor triggering microglia ramification (Liu et al., 1994; Tanaka and Maeda, 1996; Kloss et al., 1997; Rosenstiel et al., 2001; Rezaie et al., 2002). In the retina, contact of radially migrating microglial cells with Müller cells may play a role in this process. Nevertheless, microglial cells migrating tangentially in the vitrealmost part of the retina do not ramify, despite their close contact with Müller cell endfeet (Marín-Teva et al., 1998). This apparent contradiction can be readily explained by the functional polarization of Müller cells, with one domain located at the vitrealmost part of the retina (endfeet) and another extending over the rest of the cell. These domains express distinct molecules (Nagelhus et al., 1998) and exert different influences on retinal cells (Bauch et al., 1998; Steinbach and Schlosshauer, 2000). Therefore, it is possible that the vitreal domain does not stimulate microglial ramification, whereas the nonvitreal domain does. In addition, the presence in different retinal layers of extracellular matrix molecules such as tenascin may also be involved in establishing the levels of microglia ramification.

Our observations show that radial migration of microglial cells in the retina occurs by successive jumps from one ramification level to a more scleral level until they reach their final location in the retinal thickness. This intermittent cell migration is partially similar to the so-called locomotion mode of radial migration used by some neurons in the cerebral cortex, which shows periods of rapid movement interspersed with pauses (Nadarajah et al., 2001; Morest and Silver, 2003), and to the recently described multipolar neuronal migration (Tabata and Nakajima, 2003).

Besides showing that radial migration and ramification of microglia are simultaneous processes, our results reveal that microglial cells ramifying at a certain level of the retinal thickness are able to migrate again to a more scleral level. These findings agree with those of in vitro studies that highlighted the capability of completely ramified microglia to migrate (Booth and Thomas, 1991; Ward et al., 1991; Rezaie et al., 2002). On the other hand, the rate of movement of ramified microglia has been reported to be relatively low and to increase as the number of cell branches decreases (Rezaie et al., 2002). This is consistent with our observation that the ramification of radially migrating microglial cells is less profuse than that of microglial cells in the mature retina [compare Figs. 4 and 5 in this article with Fig. 1 in Navascués et al. (1994)].

Radially Migrating Microglial Cells Adhere to Müller Cell Radial Processes

Our study shows that radially migrating microglial cells closely adhere to radial processes of Müller cells throughout the retinal thickness. This supports the view that Müller cells are the substratum for radial migration of microglial cells in the avian retina, as previously suggested (Cuadros and Navascués, 1998; Navascués et al., 2000, 2002). This is not surprising, because Müller cells are radially oriented retinal components. The blood vessel network also appears to act as a substratum for microglia radial migration in other parts of the CNS (Navascués et al., 1996). However, this migratory pathway is not present in the avascular quail retina.

The radial distribution of Müller cell processes appears to be a major mechanical factor that facilitates radial migration of microglial cells. Müller cells also express adhesion molecules, essential for cell movement. Adhesion molecules, such as L1/NgCAM (Sharma and Johnson, 2000), the 5A11 antigen (Fadool and Linser, 1993), and the integrin β1-subunit (Hering et al., 2000) are expressed in Müller cells of the developing avian retina. Our results demonstrate that Müller cells also express s-laminin during embryonic development of the quail retina, as previously described in the developing and adult retina of other vertebrate species (Libby et al., 1997). This molecule appears to participate in the adhesion of different neuronal types to the substratum (Hunter et al., 1991; Porter and Sanes, 1995a, 1995b). Therefore, it is not unreasonable to assume that it could play a role in adhesion mechanisms between Müller cells and radially migrating microglial cells, although no evidence of this is provided by the present study.

Mechanism of Radial Migration of Microglial Cells Differs From That of Tangential Migration

Studies in our laboratory (Navascués et al., 1995; Marín-Teva et al., 1998) analyzed morphological features of amoeboid microglial cells migrating tangentially in the vitrealmost part of the quail embryo retina. Comparison of these morphological features with those of radially migrating microglial cells in the present study shows marked differences, suggesting

that the mechanisms of tangential and radial migration of microglial cells are very different.

The mechanism of microglial cell tangential migration in the quail retina includes polarized emission of lamellipodia at the leading part of the cell (Marín-Teva et al., 1998). However, no lamellipodia are seen during radial migration of microglial cells. This could be a consequence of the different substrata used by retinal microglial cells for tangential and radial migration, with tangential migration occurring on a carpet of Müller cell endfeet (Marín-Teva et al., 1998) and radial migration using thin radial processes of Müller cells. Another major difference between these migration mechanisms is that microglial cells ramify simultaneously with radial migration, whereas tangentially migrating cells do not ramify. Further studies are required to improve our understanding of these mechanisms.

Possible Involvement of Tenascin in Stopping and Ramification of Microglial Cells at Distinct Levels of Retinal Thickness

Our results show that tenascin is transiently detected in the IPL and OPL of the quail retina during developmental stages at which microglia radial migration occurs. This pattern of expression is identical to that of tenascin-C in the chick embryo retina (Perez and Halfter, 1993; Vaughan et al., 1994; Bartsch et al., 1995; D'Alessandri et al., 1995; Belmonte et al., 2000). Tenascin expression in the plexiform layers of the quail retina persists through the first posthatching week but disappears at the beginning of the second week, coinciding with the ending of the radial migration and ramification of retinal microglia. This chronotopographical pattern of tenascin distribution suggests that this molecule may have a role in microglial radial migration.

Abrupt interfaces between strongly tenascin-positive and tenascin-negative territories are present in the quail embryo retina. These interfaces match retinal levels in which microglial cells ramify horizontally after radial migration (Fig. 7), suggesting that radially migrating microglial cells stop their movement when they reach an interface between two territories with different expressions of tenascin. In support of this hypothesis, tenascin has been shown to be antiadhesive for various cell types, including microglial cells (Angelov et al., 1998; Pesheva and Probstmeier, 2000; Pesheva et al., 2001). The proposition that tenascin may also induce the ramification of microglial cells at these interfaces is supported by a report that tenascin induces changes in the cytoskeleton of cultured fibroblasts, favoring the emission of thin actin-rich processes (Wenk et al., 2000).

The barrier properties of tenascin would explain the stopping of radially migrating microglial cells at L1 (GCL-IPL interface). However, microglial cells that ramify at L1 can then resume their radial migration to reach L2 (interface between sublayers within the IPL) and L3 (IPL-INL interface) by traversing the tenascin-rich territory of the IPL. This would be explained by the presence in the tenascin molecule of domains with opposite functions, adhesive and antiadhesive (Husmann et al., 1995; Norenberg et al., 1995; Faissner, 1997; Ghert et al., 2001), and by the ability of this molecule to bind to different receptors (Schnapp et al., 1995; Mackie, 1997; Zacharias et al., 1999; Pesheva and Probstmeier, 2000). Therefore, tenascin is able to exert both inhibitory and stimulatory effects on cell migration (Faissner, 1997). Nevertheless, further experimental research is needed to test the hypothesis that tenascin is involved in the stopping and ramification of radially migrating microglial cells.

ACKNOWLEDGMENTS

The monoclonal antibodies QH1, developed by F. Dieterlen-Lièvre, and H5, C4, and M1B4, developed by J.R. Sanes, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD (National Institute of Child Health and Human Development) and maintained by the Department of Biological Sciences at University of Iowa in Iowa City, Iowa. The authors thank Richard Davies for improving the English style of the article.

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