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Microglia Development in the Quail Cerebellum

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ABSTRACT

We used the QH1 antibody to study changes in the morphological features and distribution of microglial cells throughout development in the quail cerebellum. Few microglial precursors were present in the cerebellar anlage before the ninth incubation day (E9), whereas many precursors apparently entered the cerebellum from the meninges in the basal region of the cerebellar peduncles between E9 and E16. From this point of entry into the nervous parenchyma, they spread through the cerebellar white matter, forming a 'stream' of labeled cells that could be seen until hatching (E16). The number of microglial cells in the cerebellar cortex increased during the last days of embryonic life and first posthatching week, whereas microglial density within the white matter decreased after hatching. As a consequence, the differences in microglial cell density observed in the cerebellar cortex and the white matter during embryonic life diminished after hatching, and microglia showed a nearly homogeneous pattern of distribution in adult cerebella. Ameboid and poorly ramified microglial cells were found in developing stages, whereas only mature microglia appeared in adult cerebella. Our observations suggest that microglial precursors enter the cerebellar anlage mainly by traversing the pial surface at the basal region of the peduncles, then migrate along the white matter, and finally move radially to the different cortical layers. Differentiation occurs after the microglial cells have reached their final position. In other brain regions the development of microglia follows similar stages, suggesting that these steps are general rules of microglial development in the central nervous system. J. Comp. Neurol. 389:390–401, 1997.

Indexing terms: QH1 antibody; CNS invasion; ameboid microglia; cell migration; differentiation

Knowledge about microglia did not advance significantly from the earliest descriptions by Rio-Hortega (reviewed in Rio-Hortega, 1932) until new tools to study these cells were developed. Of the techniques now used to label microglia, histochemical staining of nucleoside diphosphatase and thiamine pyrophosphatase activities have revealed the distribution and morphology of microglial cells in the developing and adult brain of rodents (Murabe and Sano, 1982; Vela et al., 1995), humans (Fujimoto et al., 1989), birds (Fujimoto et al., 1987), and lizards (Castellano, 1991). Different lectins also label microglial cells in some species (Streit and Kreutzberg, 1987; Hutchins et al., 1992; Acarin et al., 1994). In addition, antibodies recognizing microglial cells have been found in a number of species, including fishes (Dowding et al., 1991), amphibians (Goodbrand and Gaze, 1991), rodents (Perry et al., 1985; Imamura et al., 1990; Ling et al., 1990; Gehrmann and Kreutzberg, 1991; Perry and Gordon, 1991), and humans (Penfold et al., 1991).

The QH1 antibody, which recognizes endothelial and hemopoietic cells in the quail (Pardanaud et al., 1987), labels all stages of development of quail microglia (Cuadros et al., 1992). Taking advantage of this labeling, we studied the development of microglia in the quail optic tectum (Cuadros et al., 1994) and retina (Navascués et al., 1995). These studies revealed that microglia develop from precursors that invade the nervous tissue in a highly stereotypical manner. Although nonendothelial cells of hemangioblastic lineage appear in the avian nervous system at early developmental stages (Cuadros et al., 1993), many of the microglial precursors enter the nervous tissue at later

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stages through specific points in the pial surface. So, large numbers of microglial precursors concentrate at both sides of the pial surface in the ventrolateral region of the quail optic lobes during embryonic life (Cuadros et al., 1994) and could enter the quail retina from the pecten (Navascués et al., 1995).

Once they have entered the nervous parenchyma, microglial precursors migrate to their final location (Cuadros et al., 1994; Navascués et al., 1995), where they change from ameboid cells (also called brain macrophages) to ramified mature microglia (Ling and Wong, 1993). This transformation has been carefully analyzed in the corpus callosum and cortex of rats (Wu et al., 1992, 1993), and has also been described in the developing central nervous system (CNS) of humans (Fujimoto et al., 1989) and birds (Fujimoto et al., 1987; Cuadros et al., 1994). Microglial precursors become ramified microglia only after reaching their final position in the quail optic tectum (Cuadros et al., 1994) and retina (Navascués et al., 1995). Therefore, microglia seem to modify their morphological features as their surrounding environment changes, supporting the view that their phenotype depends on the place where the cells are located (Lawson et al., 1990; Perry and Gordon, 1991).

In this paper, we analyze the pattern of microglia development in the cerebellum, and compare it with that observed in the optic tectum and retina. Our observations reveal that during embryonic development a large proportion of microglial cells enter the cerebellum from the meninges of the basal region of the cerebellar peduncles and afterward distribute through the developing white matter and cortical layers.

MATERIALS AND METHODS

Animals and histology

Embryonic and hatched quails (*Coturnix coturnix japonica*) used in this study ranged from day 6 of incubation (E6) to E16, when quails normally hatch, and from the first postnatal day (P0) to adulthood (P45; Table 1). Embryos younger than E8 were decapitated and the heads immersed in Bouin's fixative for 1 to 3 days without further dissection. Older embryonic brains and those of hatched quails were dissected and fixed for 3 to 5 days in the same fixative. Quails older than P3 were anesthetized with ether and perfused through the heart before decapitation. Procedures for death of the animals and material retrieval were in accordance with specifications from the unit of Laboratory Animals of the University of Granada. The brains were embedded in paraffin, and serial sections 7- to 10-μm-thick, were obtained on a rotatory microtome. In addition, brains of E8, E12, E16, P3, and adult quails (Table 1) were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 4 to 6 hours, and sagittal sections 50- to 60-μm-thick were obtained by using a Vibratome.

TABLE 1. Number of Embryos Analyzed at Each Developmental Stage[1]

Age	ParaffinTS	Paraffin SS	Vibratome
E6	2	1	
E7	1	2	
E8	1	1	2
E9	2	2	
E10	2	1	
E11	1	1	
E12	2	1	2
E14	2	2	
E16	2	2	2
P1	1	2	
P2	1	2	2
P3	3		
P10	1	1	
P45	1	1	2

[1]E, embryonic day; P, posthatching day; TS, transverse sections; SS, sagittal sections.

Immunocytochemistry

Both paraffin and Vibratome sections were stained with QH1 antibody (Pardanaud et al., 1987). The antibody was obtained as culture supernatant from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences at Johns Hopkins University School of Medicine (Baltimore, MD) and the Department of Biology at the University of Iowa (Iowa City, IA), under contract number NO1-HD-2-3144 from the National Institute of Child Health and Human Development (NICHD).

Immunocytochemical processing of paraffin sections was performed as previously reported (Cuadros et al., 1992, 1993). After removing endogenous peroxidase activity by treating the sections with 0.3% hydrogen peroxide in PBS, the sections were incubated overnight in QH1 supernatant diluted 1:2, washed extensively in PBS, and successively incubated for 30 to 45 minutes in the secondary antibody (biotinylated goat anti-mouse IgG, Sigma, St. Louis, MO) and streptavidin-biotin-peroxidase complex (Extravidin, Sigma). Peroxidase activity was revealed with a PBS solution containing 0.025% diaminobenzidine and 0.0003% hydrogen peroxide. Sections were lightly counterstained with hematoxylin and mounted in DPX (BDH, Poole, UK).

For Vibratome sections, the immunocytochemical procedure was similar to that described above, with some modifications. Treatment with hydrogen peroxide was omitted, 1% Triton X-100 (Sigma) was added to the PBS used for washing, and incubation times were longer: the Vibratome sections were left for 30 to 40 hours in QH1 antibody, 10 to 12 hours in the secondary antibody and 3 to 4 hours in the streptavidin-biotin-peroxidase complex. Peroxidase activity was revealed in the same solution as in paraffin sections. The Vibratome sections were then dehydrated and mounted without counterstaining in DPX.

Analysis of cell density and distribution

The bodies of nonendothelial labeled cells were counted in ten 10-μm-thick parasagittal sections from one cerebellum per stage. The area of each section used was measured on drawings made with a camera lucida. After E9, the border of the developing cortex was clearly distinguishable, making it possible to determine the area occupied by the cortex and by the rest of the cerebellum (white matter and cerebellar nuclei). These measurements were used to estimate the density of labeled cells at each age. Additional measurements and counts in other specimens at E9, E16, and P2 gave analogous results. Sections selected for counting were always located close the midline, so that we could compare data from similar levels at different ages; in E6–E8 embryos, however, sections selected were more lateral as the cerebellar anlage is very thin near the midline and contains concentrations of labeled cells, which might have skewed the results. The QH1-labeled cells showed heterogeneous size and shape, frequently bore cell processes, and had general features that changed with age; these facts made the use of precise stereological methods difficult. In any case, although the approach used

here did not reveal the precise number of labeled cells, it provided general information about their number and distribution in the cerebellum, allowing us to compare them throughout development.

Drawings of representative sections were made by using a camera lucida with a ×6.3 objective. In these sections the precise locations of macrophages and microglial cells were noted and subsequently checked with a ×16 objective to eliminate cross-sections of blood vessels incorrectly marked as macrophages or microglial cells at lower magnification.

RESULTS

Previous work in our laboratory (Cuadros et al., 1992, 1994) showed that the QH1 antibody recognizes in the developing and mature quail brain all the microglial cell types described in mammals (Fujimoto et al., 1989; Milligan et al., 1991; Perry and Gordon, 1991; Ling and Wong, 1993). Although blood vessels show QH1 immunoreactivity in both developing and adult brains, we will use the terms 'QH1+ cells' and 'labeled cells' to refer only to nonendothelial immunoreactive cells, i.e., presumptive microglial cells.

QH1+ cell density during cerebellar development

We estimated the density of QH1+ cells in the midsagittal region of the developing and mature cerebella as an indication of the number of microglial cells in each developmental stage. Figure 1A,B show the variations in labeled cell density observed during development. Little variation in labeled cell density was observed between E6 and E9; because the cerebellar anlage increased in size during this period (Fig. 1C) new QH1+ cells would appear within it at a rate sufficient to maintain a constant density. The density of labeled cells increased at E10 and E11 and decreased at later embryonic stages. Although increasing density necessarily implies that new QH1+ cells appear in the cerebellar anlage, decreasing density does not imply that the absolute number of labeled cells diminishes. In fact, new labeled cells may continue to appear within the cerebellar anlage, but the rate of addition of such cells would be lower than the rate of growth of the cerebellum, and, therefore their density decreases. The density of labeled cells varied little after hatching, suggesting that new cells once again entered the cerebellum at a rate that maintained a constant cell density despite the growth of the cerebellum.

Presumptive cortical regions and regions that will become white matter and cerebellar nuclei could be clearly distinguished from E10 onward, making it possible to determine labeled cell densities in each of these zones. Figure 1B shows that the density of QH1-labeled cells in the white matter and cerebellar nuclei increased until hatching and decreased during the first posthatching days. In cortical layers QH1+ cell density was lower during embryonic and early posthatching development, and it did not show variations, in contrast with the white matter and cerebellar nuclei. In the adult, QH1+ cell densities were similar in both regions, confirming that the labeled cells were distributed almost homogeneously throughout the cerebellum.

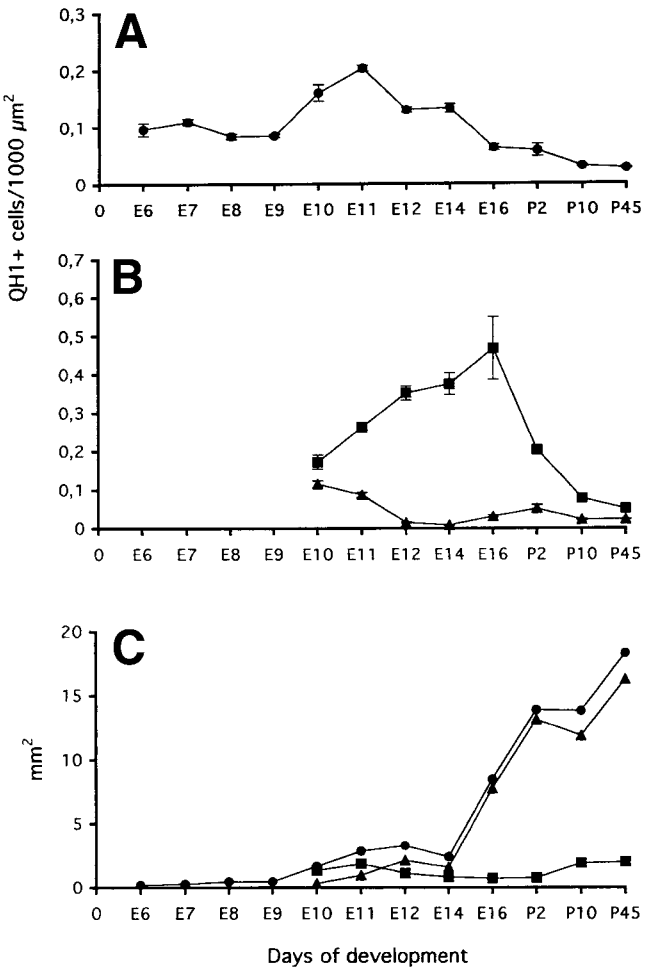

Fig. 1. Plots of the density of QH1-labeled nonendothelial cells in the developing cerebellum from E6 to adulthood. **A:** Labeled cell density in the entire cerebellum. **B:** Densities recorded in the white matter and cerebellar nuclei (squares) and cortex (triangles). Note that this plot begins at E10, when the anlage of the cortical layers can be clearly determined. Each point in A and B is the mean value obtained in a single specimen, and bars represent standard errors. **C:** Surface area of the sections used to estimate the QH1-labeled cells density at each stage. The size of the entire cerebellar area (circles), that of the white matter and cerebellar nuclei (squares), and that occupied by the cortical layers (triangles) are represented.

QH1+ cell distribution during cerebellar development

Previous work showed that QH1+ nonendothelial cells were present in presumptive regions of the cerebellum in E2–E3 quail embryos (Cuadros et al., 1993), well before the youngest developmental stage considered here (E6). At E6 the cerebellar anlage was constituted by a thick neuroepithelial layer lining the ventricle, and a nonhomogeneous mantle layer in which fiber-rich and cell-rich regions could be distinguished. Round or ameboid cells appeared in both the neuroepithelial and the mantle layer (Figs. 2A, 3). Many QH1+ cells were seen along the very thin dorsal midline (Fig. 3A).

The pattern of distribution of labeled cells in E7 and E8 embryos was very similar to that in E6. At E9, QH1-labeled cells located around the ventricles were more

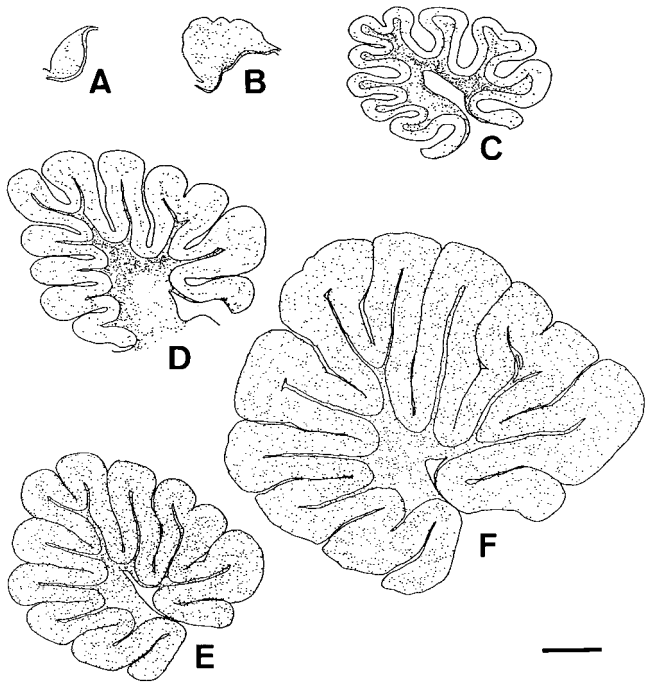

Fig. 2. Camera lucida drawings from parasagittal paraffin sections of the cerebellum, in which QH1-labeled nonendothelial cells are shown. **A:** E6; **B:** E9; **C:** E12; **D:** E16; **E:** P2; **F:** P45. In all the drawings, anterior is on the left and dorsal on the top. Scale bar = 850 μm.

numerous than in previous stages, and fewer labeled cells were present in other regions (Figs. 2B, 4). Many of these cells were dendritic in shape, with processes that frequently ended in flat expansions (Fig. 4).

The definitive laminar organization of the cerebellum emerged during subsequent development, when the internal granule layer and the Purkinje cell layer became discernible (Fig. 5A). At E10–E12 numerous labeled cells were seen both in and around the neuroepithelial layer, and in the developing white matter (Figs. 2C, 5B). These cells were usually round with large cytoplasmic vacuoles and occasionally cell debris. Labeled cells that appeared in the presumptive cerebellar nuclei and cortex were fewer in number and more ramified than those in the white matter (Fig. 5C). Labeled cells in the molecular layer were frequently elongated and oriented parallel to the boundaries between the molecular layer and the external granule layer (Fig. 5D). Some of the labeled cells in the molecular layer had processes that entered the external granule layer, but no soma of labeled nonendothelial cells were found within this layer.

At later stages of embryonic development, labeled cells were still more numerous within the cerebellar white matter than in cortical layers (Fig. 2D). The proportion of ameboid cells in the white matter decreased between E12 and E16, and a larger proportion of the cells were elongated or ramified. Elongated cells were oriented parallel to the fibers running toward the apex of cerebellar folia (Fig. 6A). QH1+ cells within the cerebellar nuclei had more ramified morphology than those in the surrounding white matter (Fig. 6B). In addition, QH1-labeled cells were more numerous within cortical layers, where they showed ramified shapes that increased in complexity with age (Fig. 6C). Many labeled cells appeared within the internal granule layer, were less numerous in the molecular layer, and were not detected within the external granule layer.

In hatched quails, ramified QH1 labeled cells became more frequent in the cortical layers, whereas labeled cells in the white matter were stained more weakly. Because of the increase in labeled cells in the cortex, clear differences in QH1+ cell density were no longer observable in the cerebellum after hatching, and microglial cells were distributed homogeneously in the different regions of the mature cerebellum (Figs. 1B, 2E,F). Few, if any, labeled ameboid cells were found in the cerebellum 1 week after hatching (Fig. 7), and all labeled cells observed in the adult cerebella were ramified (Fig. 8). Microglial cells in the adult cerebellum showed differences in staining intensity and morphology. In the molecular layer, microglial cells were more strongly labeled and had thicker processes than in other layers (Fig. 8A). In the white matter QH1 labeling was weak, and most cell processes were oriented parallel to the fiber bundles (Fig. 8C,D).

Migration of labeled cells in the cerebellum

No clear routes of entry were detected in E6–E8 embryos. From E9 on, numerous labeled cells were seen in the basal portion of the cerebellar peduncles, from which a continuous stream of labeled cells originated and penetrated the white matter (Fig. 9A). Labeled cells in the basal peduncles appeared on both sides of the pial surface, i.e., in the meninges and in the subpial nervous parenchyma (Fig. 9B). The number of cells within the stream increased during embryonic life, reaching more and more distant regions within the white matter, so that by E12, cells of the stream had reached portions of white matter in the core of cerebellar folia. Before E12 many of the labeled cells constituting the stream were round or ameboid (Fig. 9C), but subsequently the proportion of elongated cells increased; elongated cells were frequently oriented parallel to axon bundles in the white matter (Fig. 9D). The stream was still present at E16, but no traces of it were recognizable in cerebella from hatched quails. Therefore, new labeled cells apparently enter the cerebellum by traversing the pial surface in the basal region of the cerebellar peduncles and moving through the white matter. The external granule layer, the most external layer of the developing cerebellum, was always completely devoid of nonendothelial labeled cells.

Many round, ameboid, and ramified labeled cells made contact with blood vessels either at their soma or via cell processes (Fig. 9E). Sometimes clusters of small round QH1+ cells were observed around a vessel (Fig. 9F). Labeled cells were also frequently found close to the ventricular surface, sometimes within the width of the neuroepithelial layer. We also saw labeled cells within the ventricular lumen, where some of them were apparently in the act of traversing the ventricular layer (Fig. 9G,H).

DISCUSSION

The specificity of the labeling of microglia with the QH1 antibody has been discussed previously (Cuadros et al., 1992, 1994; Navascués et al., 1994, 1996). Like in the retina, where QH1 labeled some Müller cells (Navascués et al., 1994), this antibody also marked some Bergmann glia of the cerebellar cortex (Fig. 7C). This labeling was generally weaker than that of microglial cells, affected

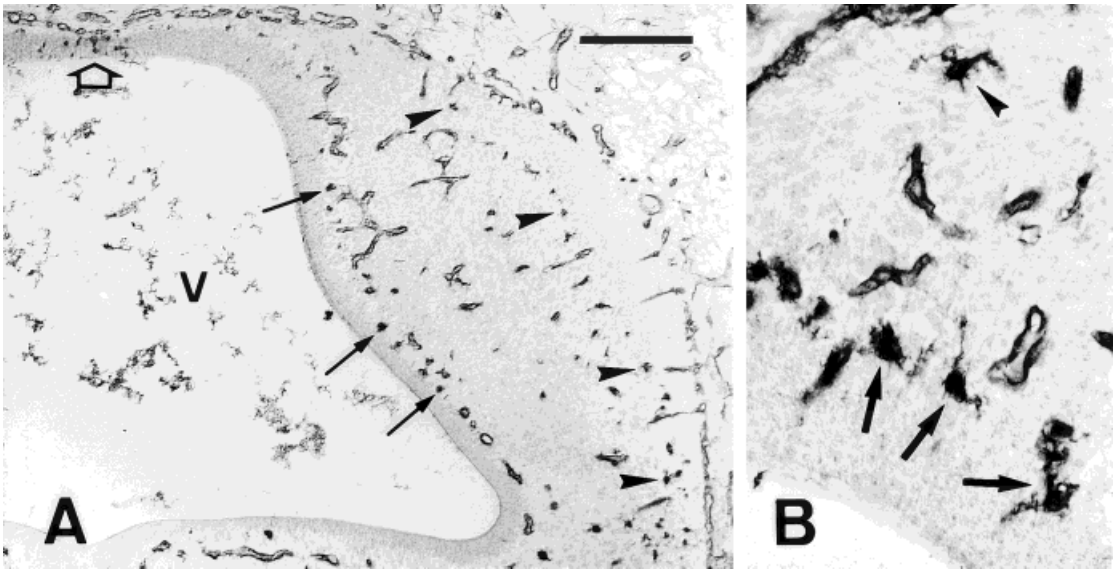

Fig. 3. QH1-labeled cells in E6 cerebella. **A:** Transverse section showing labeled cells near the ventricular layer (arrows) and scattered throughout the mantle layer (arrowheads). Note the thin roof of the cerebellar anlage (open arrow), where some labeled cells appear. V, ventricle. Paraffin section. **B:** Labeled cells near the ventricular layer (arrows) and in the mantle layer (arrowhead). Blood vessels are also labeled by QH1. Transverse paraffin section. Scale bar = 150 μm in A, 50 μm in B.

only a small proportion of Bergmann cells, which had morphological features clearly different from those of microglial cells, and occurred only during the last part of development. Therefore, it did not interfere with the identification of microglial cells, and we conclude that the QH1 antibody remains a useful tool to analyze this cell type during cerebellar development.

That quail microglial cells are labeled by an antibody recognizing hematopoietic cells suggests that they belong to the blood cell line. However, this is not a definitive proof, because some nonhematogenous cells also show QH1 immunoreactivity in the retina (Navascués et al., 1995) and cerebellum (this article).

Our observations provide new insights about the ways microglial precursors enter the developing cerebellum, the distribution of microglial cells during development, and the morphological differentiation of microglial cells. Each one of these topics will be discussed.

Entry of microglial cells into the CNS

If microglial precursors originate outside the neuroepithelium, they must invade the nervous parenchyma. Three main routes of invasion of the CNS by microglial precursors have been proposed: (i) from the meninges, by traversing the pial surface, (ii) from the ventricles, by traversing the ventricular (or ependymal) layer, and (iii) from the blood circulation, by traversing the endothelial wall. Microglial precursors probably use all three routes (Jordan and Thomas, 1988; Cuadros et al., 1994; Navascués et al., 1996); the role of each may vary in different regions of the central nervous system, and in different periods of development. Some labeled mitotic cells were observed within the cerebellum during embryonic stages, but they were scarce, so that proliferation did not appear to be the main mechanism accounting for the increase in number during development.

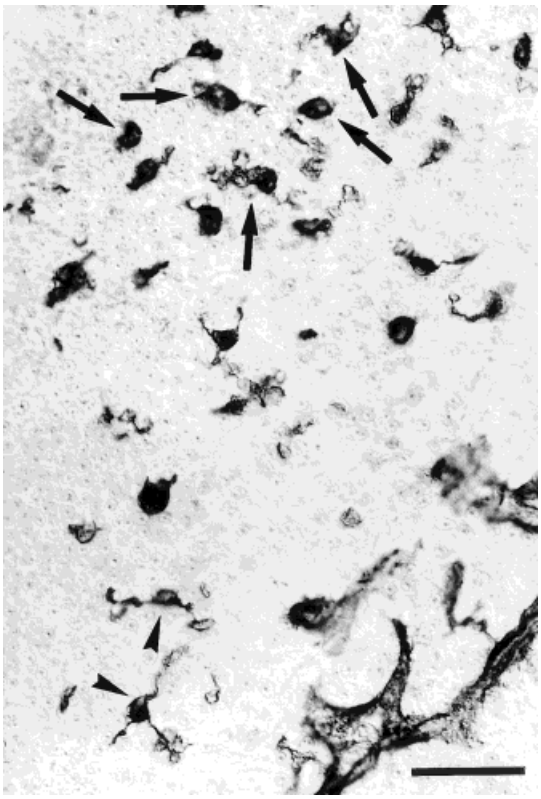

Fig. 4. QH1+ cells in a E9 cerebellum. The ventricular layer, cut tangentially in this parasagittal section, shows ameboid cells (arrows) and cells with dendritic morphology (arrowheads). Paraffin section. Scale bar = 35 μm.

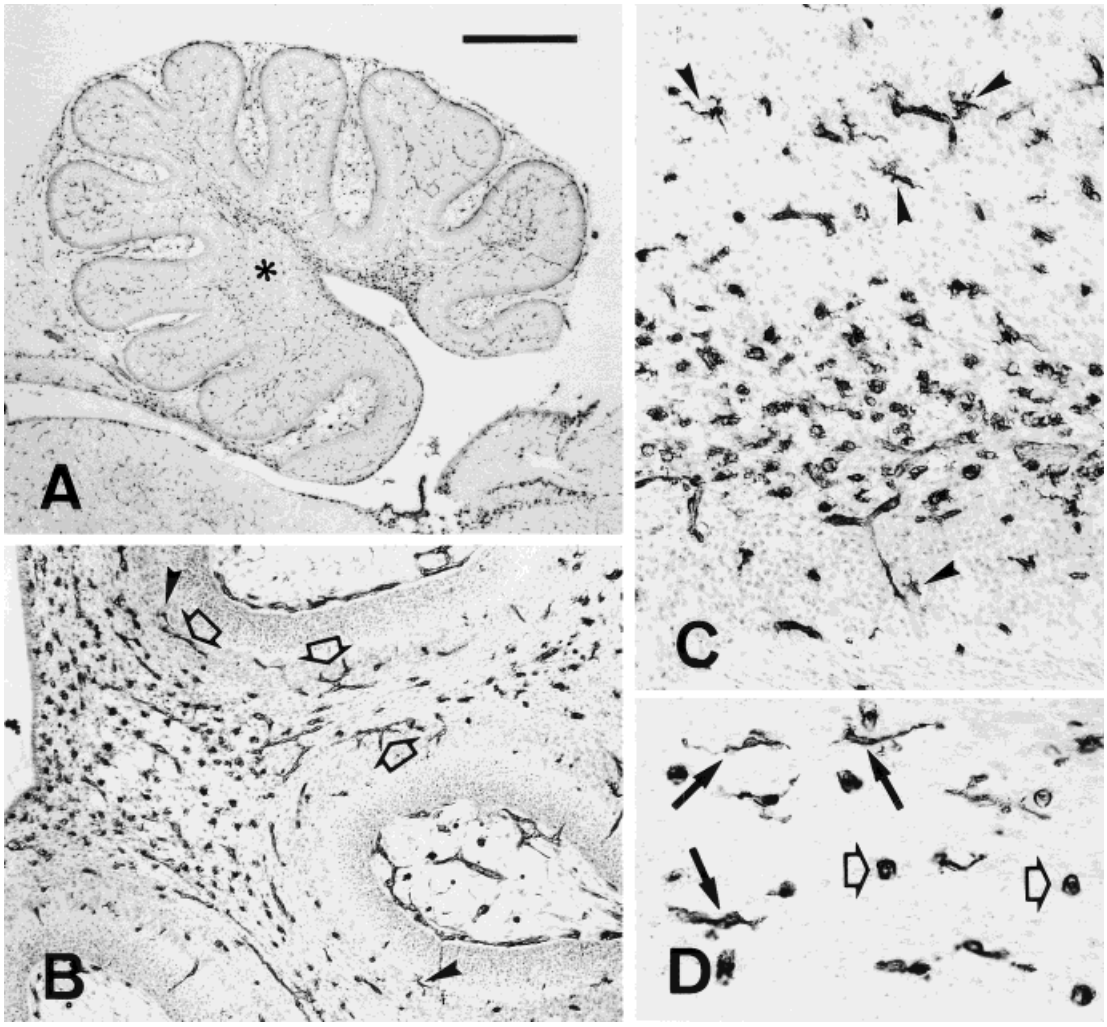

Fig. 5. QH1+ cells in E10–E12 cerebella. **A:** Parasagittal section of an E11 cerebellum. Concentrations of labeled cells are present in the white matter, constituting the stream. Few of such cells are present in other regions. Some individual labeled cells cannot be distinguished from blood vessel profiles at this magnification. Asterisk, presumptive cerebellar nuclei. Paraffin section. **B:** Higher magnification of a parasagittal section of an E12 cerebellum. Round and ameboid cells gather in the developing white matter near the ventricle (on the left), while smaller numbers of such cells appear in the white matter of the developing folia. Ramified cells located in the cortex are marked by arrowheads. Open arrows, blood vessels. Paraffin section. **C:** Transverse section of an E11 cerebellum. Many ameboid cells are present in the white matter, constituting the stream. Some ramified cells (arrowheads) can be seen in the presumptive cortex (bottom of the figure) and cerebellar nuclei (top of the figure). Paraffin section. **D:** Transverse section of an E10 cerebellum. This section cuts tangentially the border between the external granule layer (in the background) and the molecular layer, and reveals the morphological features of cells located at this border. Elongated labeled cells (arrows) are apparently in the act of migrating parallel to the border of the external granule layer. Some processes of these cells penetrate the external granule layer, but their body is always located outside this layer. Blood vessels are indicated by open arrows. Scale bar = 590 μm in A, 175 μm in B, 80 μm in C, 50 μm in D.

Our observations strongly suggest that a large proportion of microglial precursors enter the cerebellar anlage by traversing the pial surface at the basal region of the cerebellar peduncles, where concentrations of labeled cells appear during embryonic stages. Similar concentrations of microglial cells have been described during postnatal development in the mouse and rat (Ashwell, 1990; Boya et al., 1991; Wolswijk, 1995). We cannot rule out that some of these cells reach this region by migrating beneath the pial surface of the brainstem, following the axon bundles of the lemniscus spinalis (identified according to Youngren and Phillips, 1978) and enter the cerebellum at these points. It is not clear whether Rio-Hortega (1932) alluded to the same region when he wrote that 'the penetration of microglia in the cerebellum takes place chiefly through the angles formed by the medullary veils.' In any case, the regions of the cerebellar peduncles crowded by large numbers of microglial cells probably correspond to the 'sources of microglia' described by Rio-Hortega (1932) in the developing brain.

In addition to the massive invasion of microglia precursors at certain points, individual microglial cells may enter the nervous parenchyma by traversing the pial surface in other regions (Boya et al., 1979; Cuadros et al., 1994; unpublished observations). However, this diffuse entry of precursors through the pial surface does not occur in the quail (this study) or rat (Boya et al., 1991) cerebellum. During development, the most external layer of the cerebel-

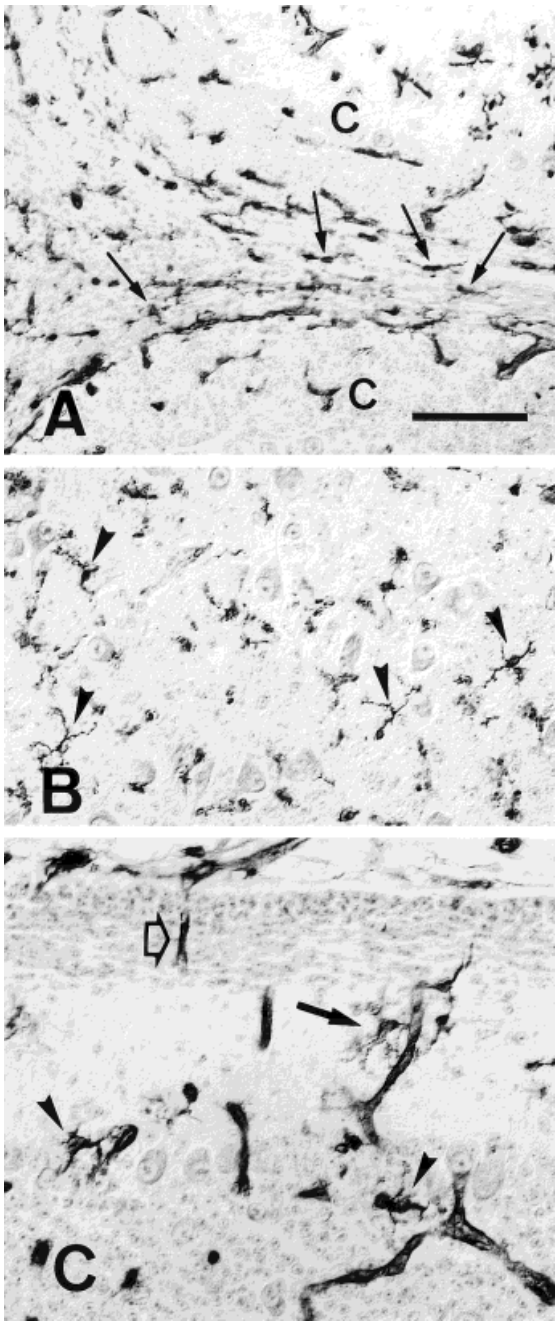

Fig. 6. QH1-labeled cells in E14–E16 cerebella. **A:** Elongated microglial cells (arrows) in the white matter of an E16 cerebellum. C, cerebellar cortex. Parasagittal paraffin section. **B:** Ramified microglial cells (arrowheads) in the presumptive cerebellar nuclei of an E14 cerebellum. Note the presence of large neurons in this area. Transverse paraffin section. **C:** Ramified cells in the cortex of an E16 cerebellum. One of these cells (arrow) is associated to a blood vessel that traverse the molecular layer. Other cells (arrowheads) lie near the Purkinje cell layer. Note that the external granule layer lacks of labeled nonendothelial cells. The open arrow marks a blood vessel in the external granule layer. Transverse paraffin section. Scale bar = 70 μm in A, 60 μm in B, 50 μm in C.

lar anlage is the external granule layer, which is consistently devoid of nonendothelial QH1 labeled cells. This layer may be a barrier to microglial invasion, precluding the diffuse migration observed in other regions of the developing CNS.

Ashwell (1990) proposed that microglial precursors entered the cerebellum from the ventricle. This possibility is supported by the observation of clusters of QH1+ cells close the ventricular layer, and the presence of labeled cells in the ventricle and within the ventricular layer. Clusters of QH1+ cells were sometimes seen around a vessel, suggesting that these cells had entered the nervous parenchyma from the blood. However, our results shed no light on this possible route of migration, as the QH1 antibody also labels the endothelial cells lining the vessel.

In brief, microglial precursors invade the developing quail cerebellum through the pial and ventricular surfaces mainly during embryonic life. In hatched quails the concentrations of labeled cells in the basal region of the peduncles and close the ventricle have disappeared. Thus, massive migration of microglial precursors apparently ends around the time of hatching. Nevertheless, new microglial cells are probably added after this time, as microglial density did not decrease during growth of the cerebellum until adulthood.

Distribution of microglial cells during development

As in the cerebellum of other species (Ashwell, 1990; Boya et al., 1991; Chugani et al., 1991; Milligan et al., 1991; Wolswijk, 1995), mostly ameboid microglial cells appear within the cerebellar white matter during quail development. Clusters of ameboid cells have been described in the white matter of several areas of the developing brain in a number of species (Murabe and Sano, 1982; Lent et al., 1985; Perry et al., 1985; Ashwell, 1991; Chugani et al., 1991; Milligan et al., 1991; Cuadros et al., 1994). The presence of such clusters of ameboid cells has been related to the removal of transient or exuberant axons (Ling, 1981; Innocenti et al., 1983; Milligan et al., 1991), but in the avian optic tectum they have been interpreted as groups of microglial precursors spreading within this region of the central nervous system (Cuadros et al., 1994). In the cerebellum, ameboid microglial cells are arranged in a continuous stream that originates in the basal region of the cerebellar peduncles and extends through the white matter. This stream may be a 'highway' in which axon bundles provide an oriented substrate for tangential migration of microglial precursors. This migration probably ends around the time of hatching, because the stream is no longer recognizable in hatched quails. After reaching particular points within the stream, individual microglia precursors appear to migrate radially along different 'secondary roads' to invade the cortical layers. Radial migration occurs during embryonic life and the first posthatching days, and eventually causes that microglial cells had similar density in cortical layers and white matter in the cerebellum of adult quails. This sequence of events explain the differences in cell density observed during development.

In both tangential and radial migration of microglial precursors, structures oriented in the direction of migration are available to precursors, and may guide their migration (Navascués et al., 1996). The white matter contains large axon bundles that enter the cerebellar

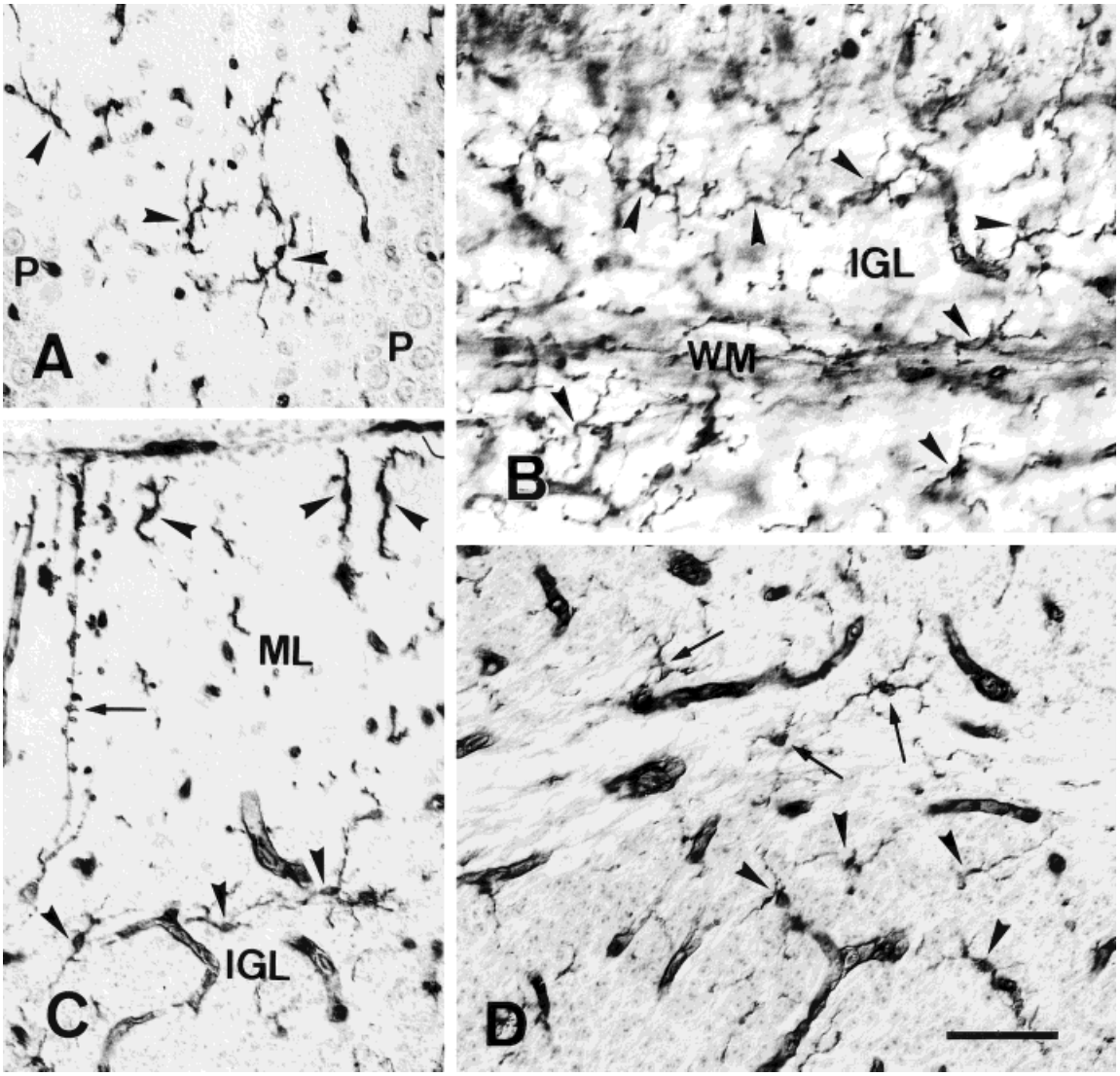

Fig. 7. QH1-labeled cells in P2–P10 cerebella. **A:** The molecular layer has been cut tangentially in this transverse section of a P2 cerebellum. Some ramified labeled cells (arrowheads) send out branches that lie parallel to the border of this layer. P, Purkinje cells. Paraffin section. **B:** Vibratome section of a P2 cerebellum. Ramified microglia (arrowheads) are seen in both the internal granule layer (IGL) and the white matter (WM). **C:** Cerebellar cortex of a P10 cerebellum. Microglial cells (arrowheads) are seen in the internal granule layer (IGL) and molecular layer (ML). The arrow points out a QH1-labeled Bergmann cell. Transverse paraffin section. **D:** Ramified microglia in the white matter (arrows) and internal granule layer (arrowheads) in a parasagittal section of a P10 cerebellum. Paraffin section. Scale bar = 50 μm in A, 35 μm in B, 45 μm in C, 40 μm in D.

anlage at the cerebellar peduncles and then spread to the apex of each folia. These bundles may guide the migration of precursors within the stream. Radially oriented elements such as radial glia and axons coursing toward the molecular layer may play a similar role in the radial migration of microglia precursors within the cerebellum. Axons and glia have been implicated in guiding cell migration within the developing cerebellum (Hatten, 1990; Mason et al., 1990).

In addition to contact-guidance factors, diffusible factors may also affect microglial precursor migration. Studies in vitro have shown that microglial cells respond to chemotactic stimuli (Yao et al., 1990; Gilad and Gilad, 1995), but thus far no factors operating in vivo have been identified. Some of the factors may be produced by dying cells. Ashwell (1990) reported that concentrations of microglia in the developing cerebellum largely match the distribution of pyknotic figures during postnatal development in the mouse, except in the external granule layer, and Wolswijk (1995) found large numbers of ameboid cells in regions with high levels of cell death in the rat (Krueger et al., 1995). So, factors released during death may therefore attract microglial precursors. However, the correlation between the distribution of cell death and the presence of microglial precursors is not exact; as already indicated, no microglial cells appear within the external granule layer, despite that cell death is frequent in this layer. Moreover, the distribution of microglial precursors does not match that of cell death in the developing optic tectum (Cuadros et al., 1994) or retina (unpublished results). Therefore,

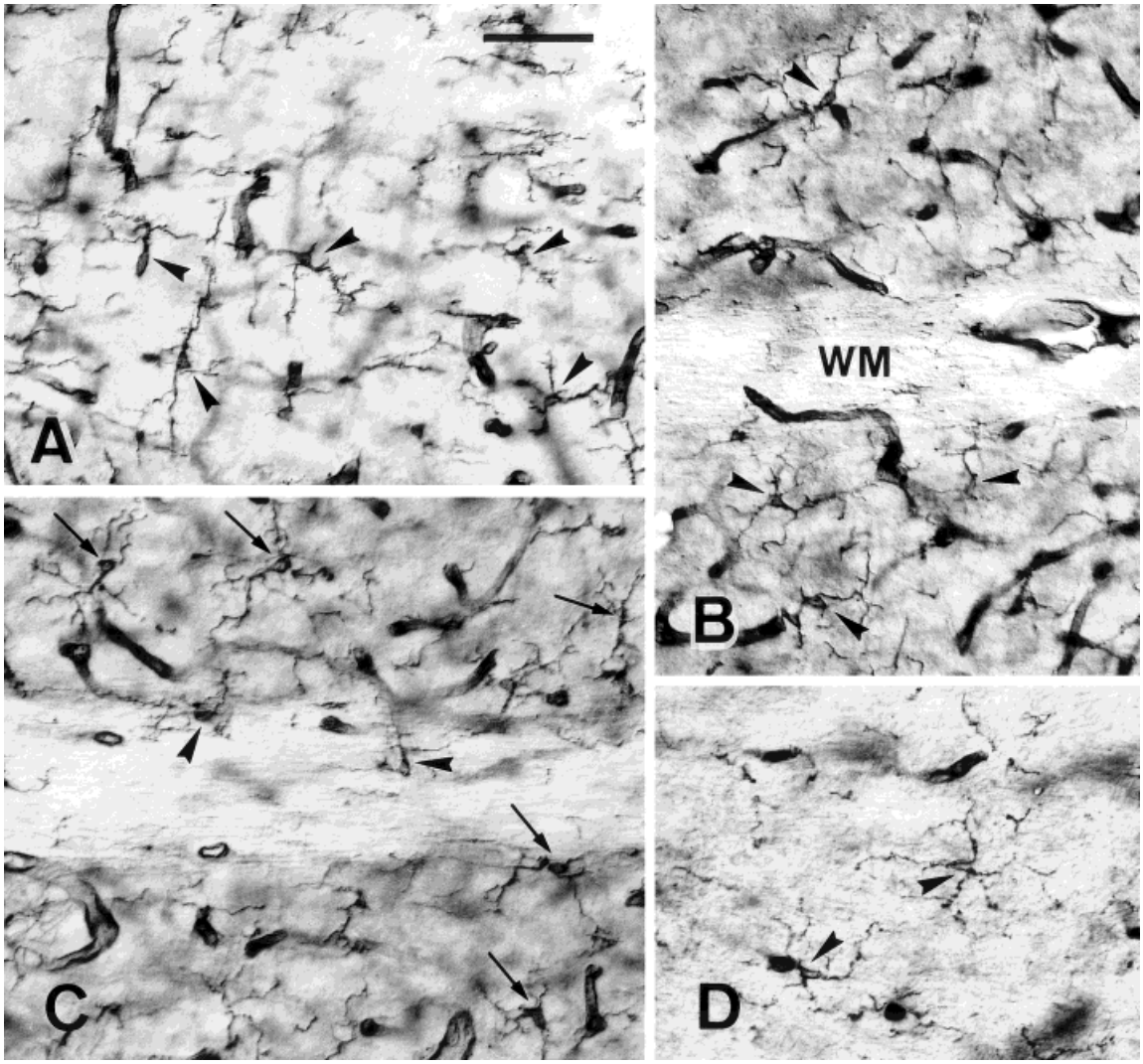

Fig. 8. QH1-labeled cells in Vibratome sections of adult cerebella. **A:** Ramified microglia (arrowheads) in the molecular layer. Many microglial cell processes oriented perpendicular or parallel to the cerebellar surface. **B:** Labeled mature microglia (arrowheads) in the internal granule layer. WM, white matter. **C:** A similar field in the cerebellum of another animal. In addition to the microglia in the internal granule layer (arrows), two microglial cells are seen in the white matter (arrowheads). **D:** Ramified microglia (arrowheads) in the white matter near the cerebellar nuclei. Scale bar = 45 μm in A,C, 50 μm in B, 40 μm in D.

other stimuli, in addition to those produced during cell and axonal degeneration, must be involved in the migration of microglial precursors.

Although our quantitative data do not allow to set definitive conclusions, it is clear that the density of microglial precursors during development is greater in the cerebellar anlage than in the optic tectum (compare Fig. 1 with Fig. 3 in Cuadros et al., 1994). Analysis in the developing cerebellum and tectum were done with similar procedures, and the discrepancies observed cannot be attributed to methodological factors. Microglial cells may be more numerous in the cerebellum during development because they play a role in organogenetic processes in this part of the central nervous system, and which are more complex than in the optic tectum. In any case, the density of microglial cells in the adult cerebellum is similar to that observed in the adult optic tectum of the quail (Cuadros et al., 1994) and is comparable to the density in the adult mouse cerebellum reported by Lawson et al. (1990) but is much lower than the surface density values inferred from the data reported in Vela et al. (1995).

Differentiation of microglial cells

Ramified microglia are thought to originate from ameboid cells (Ling and Wong, 1993) through intermediate forms with progressively more complex ramification patterns. This concept has received support from observations in the developing quail optic tectum (Cuadros et al., 1994), retina (Navascués et al., 1995), and cerebellum (this article). Microglial cell differentiation does not take place simultaneously in different parts of the quail cerebellum; more elaborate ramification patterns were observed at each developmental stage in the cortical layers than in the white matter. Thus, it seems that microglial cells differentiate after having reached their definitive location outside the highway (the white matter) on which they migrate,

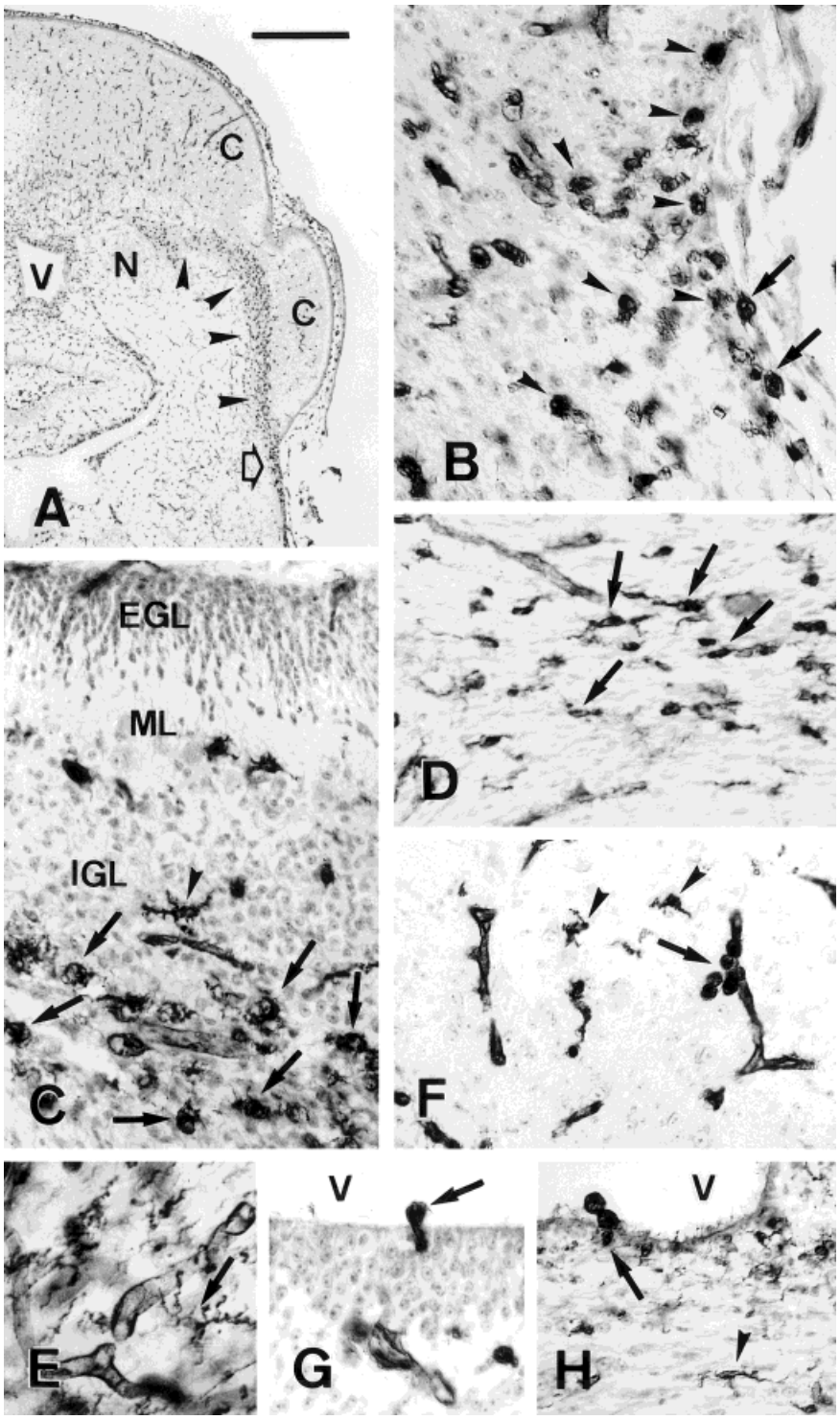

Fig. 9. Entry and migration of labeled cells in the cerebellar anlage. **A:** Transverse section of an E11 cerebellum. The stream of labeled cells begins under the pial surface (open arrow) and continues through the white matter (arrowheads). Note that the stream does not penetrate the developing cortical layers (C) or the presumptive cerebellar nuclei (N). V, ventricle. Paraffin section. **B:** Transverse section of an E14 cerebellum. Pial surface of the cerebellar peduncle, showing some labeled cells (arrows) adhering to the meningeal side of the pial surface. Other labeled cells (arrowheads) are seen within the nervous parenchyma. Paraffin section. **C:** Detail of the stream in a transverse section of an E10 cerebellum. The cells in the stream are ameboid (arrows), whereas a poorly ramified cell (arrowhead) is visible in the developing internal granule layer (IGL). EGL, external granule layer; ML, molecular layer. Paraffin section. **D:** White matter of an E12 cerebellum. Most of the labeled cells (arrows) in the stream are elongated. Sagittal paraffin section. **E:** Ramified cell (arrow) contacting a blood vessel in the internal granule layer of an adult cerebellum. Vibratome section. **F:** Cluster of round labeled cells (arrow) around a vessel in the developing cerebellar nuclei of an E14 embryo. Two ameboid cells (arrowheads) are also seen. Transverse paraffin section. **G:** A labeled cell (arrow) in the ventricle (V) showing part of its cytoplasm in the ventricular layer of the developing cerebellum. Parasagittal section of an E6 cerebellum. **H:** Two labeled cells in the cerebellar ventricle (V) of an E14 embryo. One of these cells sends out a process (arrow) that traverses the ventricular surface and reaches the nervous parenchyma. The arrowhead points out a poorly ramified labeled cell. Transverse paraffin section. Scale bar = 520 μm in A, 45 μm in B,H, 40 μm in C,E,G, 50 μm in D,F.

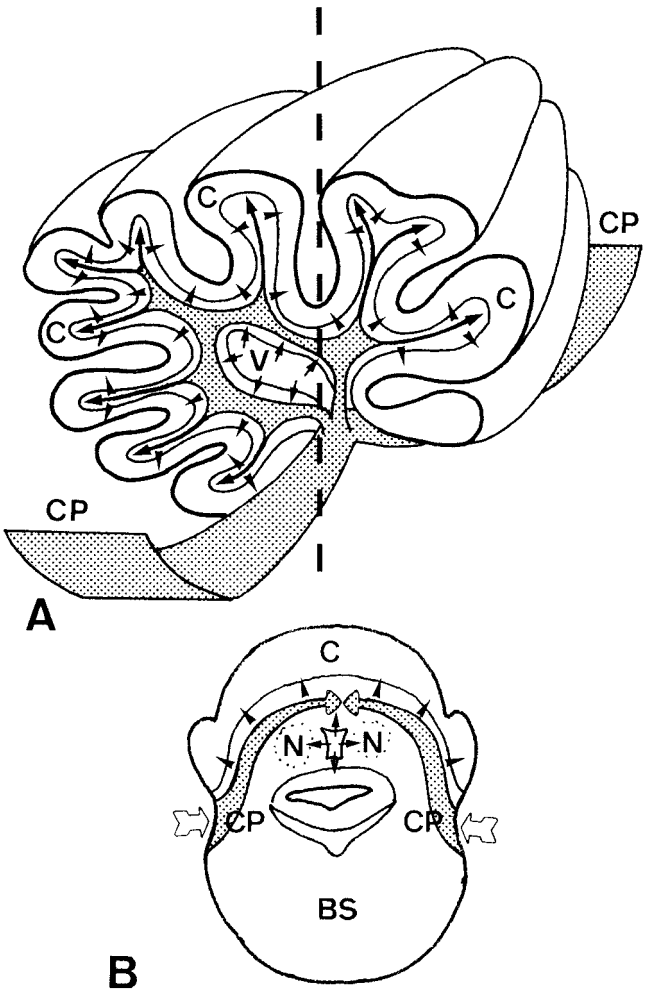

Fig. 10. Schematic drawings showing the proposed migration of microglial precursors in the cerebellum. **A:** Three-dimensional drawing of an E11–E12 cerebellum sectioned along a parasagittal plane. **B:** Transverse section at the level marked by the vertical dotted line in A. Many of the microglial precursors enter the cerebellar anlage from the meninges at the base of the cerebellar peduncles (CP) at the points marked by open arrows in B. The microglial precursors migrate through the white matter constituting the stream described in the text (dotted region in A and B). Individual microglial precursors migrate radially from this region (arrowheads in A and B) to invade the cortex (C in A and B). In addition, other microglial precursors may enter by traversing the ventricular layer (small arrows in A and B) or endothelial wall (not shown). BS, brain stem; N, cerebellar nuclei; V, ventricle.

and that the differentiation program of the microglia varies depending on the environmental factors present in each region. These factors account for the differences in morphological features of microglia during development (Cuadros et al., 1992, 1994; Wu et al., 1993) and in different regions of the adult brain (Lawson et al., 1990; Vela et al., 1995).

CONCLUSIONS

Although definitive conclusions cannot be drawn without experimental work, our observations suggest a picture of microglia development summarized in Figure 10. The main route of entry of microglial precursors in the cerebellar anlage is across the pial surface at the basal region of the cerebellar peduncles. This invasion apparently takes place from E8–E9 to E16. Microglial precursors may also invade the nervous parenchyma of the cerebellum by other routes, i.e., traversing the wall of blood vessels and/or the ventricular layer, but these routes are used by much smaller numbers of cells than those that cross the pial surface near the cerebellar peduncles. Microglial cells from the peduncles migrate tangentially along the cerebellar white matter between E9 and hatching, and constitute a stream of labeled cells. Individual microglial cells leave the stream at some points and migrate radially to their final locations within the developing cortex. Radial migration into the cerebellar cortex probably begins shortly after tangential migration within the stream has started, and lasts until the second posthatching week. Finally, cells differentiate once they have reached their final position in the cerebellar nuclei, the cortical layers or the white matter.

The development of microglia in the quail cerebellum proceeds in several steps: invasion of the nervous parenchyma at specific points, long-distance tangential migration along an oriented substrate, radial migration, and differentiation. This sequence is the same as that found during microglial development in the optic tectum (Cuadros et al., 1994) and retina (Navascués et al., 1995); we therefore suggest that it reflects a set of general rules in microglial development in the quail brain.

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LITERATURE CITED

Acarin, L., J.M. Vela, B. González, and B. Castellano (1994) Demonstration of poly-*N*-acetyl lactosamine residues in ameboid and ramified microglia cells in rat brain by tomato lectin binding. J. Histochem. Cytochem. *42:*1033–1041.

Ashwell, K. (1990) Microglia and cell death in the developing mouse cerebellum. Dev. Brain Res. *55:*219–230.

Ashwell, K. (1991) The distribution of microglia and cell death in the fetal rat forebrain. Dev. Brain Res. *58:*1–12.

Boya, J., J. Calvo, and A. Prado (1979) The origin of microglial cells. J. Anat. *129:*177–186.

Boya, J., J.L. Calvo, A.L. Carbonell, and A. Borregón (1991) A lectin histochemistry study on the development of rat microglial cells. J. Anat. *175:*229–236.

Castellano, B., B. González, I. Dalmau, and J.M. Vela (1991) Identification and distribution of microglial cells in the cerebral cortex of the lizard: A histochemical study. J. Comp. Neurol. *311:*434–444.

Chugani, D.C., N.L. Kedersha, and L.H. Rome (1991) Vault immunofluorescence in the brain: New insights regarding the origin of microglia. J. Neurosci. *11:*256–287.

Cuadros, M.A., A. Moujahid, G. Martín-Partido, and J. Navascués (1992) Microglia in the mature and developing quail brain as revealed by a monoclonal antibody recognizing hemopoietic cells. Neurosci. Lett. *148:*11–14.

Cuadros, M.A., C. Martin, P. Coltey, A. Almendros, and J. Navascués (1993) First appearance, distribution, and origin of macrophages in the early development of the avian central nervous system. J. Comp. Neurol. *330:*113–129.

Cuadros, M.A., A. Moujahid, A. Quesada, and J. Navascués (1994) Development of microglia in the quail optic tectum. J. Comp. Neurol. *348:*207–224.

Dowding, A.J., A. Maggs, and J. Scholes (1991) Diversity amongst the microglia in growing and regenerating fish CNS: Immunohistochemical characterization using FL.1, an anti-macrophage monoclonal antibody. Glia *4:*345–364.

Fujimoto, E., A. Miki, and H. Mizoguti (1987) Histochemical studies of the differentiation of microglial cells in the cerebral hemispheres of chick embryos and chicks. Histochemistry *87:*209–216.

Fujimoto, E., A. Miki, and H. Mizoguti (1989) Histochemical study of the differentiation of microglial cells in the developing human cerebral hemispheres. J. Anat. *166:*253–264.

Gehrmann, J., and G.W. Kreutzberg (1991) Characterisation of two new monoclonal antibodies directed against rat microglia. J. Comp. Neurol. *313:*409–430.

Gilad, G.M., and V.H. Gilad (1995) Chemotaxis and accumulation of nerve growth factor by microglia and macrophages. J. Neurosci. Res. *41:*594–602.

Goodbrand, I.A., and R.M. Gaze (1991) Microglia in tadpoles of *Xenopus laevis*: Normal distribution and the response to optic nerve injury. Anat. Embryol. *184:*71–82.

Hatten, M.E. (1990) Riding the glial monorail: A common mechanism for glial-guided neuronal migration in different regions of the developing mammalian brain. Trends Neurosci. *13:*179–184.

Hutchins, K.D., D.W. Dickson, W.K. Rashbaum, and W.D. Lyman (1992) Localization of morphologically distinct microglial populations in the developing human fetal brain: Implications for ontogeny. Dev. Brain Res. *55:*95–102.

Imamura, K., M. Ito, A. Suzumura, J. Asai, and A. Takahashi (1990) Generation and characterization of monoclonal antibodies against rat microglia and ontogenic distribution of positive cells. Lab. Invest. *63:*853–861.

Innocenti, G.M., S. Clarke, and H. Koppel (1983) Transitory macrophages in the white matter of the developing visual cortex: II. Development and relations with axonal pathways. Dev. Brain Res. *11:*55–66.

Jordan, F.L., and W.E. Thomas (1988) Brain macrophages: Questions of origin and interrelationship. Brain Res. Rev. *13:*165–178.

Krueger, B.K., J.F. Burne, and M.C. Raff (1995) Evidence for large-scale astrocyte death in the developing cerebellum. J. Neurosci. *15:*3366–3374.

Lawson, L.J., V.H. Perry, P. Dri, and S. Gordon (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience *39:*151–170.

Lent, R., R. Linden, and L. Cavalcante (1985) Transient populations of presumptive macrophages in the brain of the developing hamster, as indicated by endocytosis of blood-borne horseradish peroxidase. Neuroscience *15:*1203–1215.

Ling, E.A. (1981) The origin and nature of microglia. In S. Fedoroff and L. Hertz (eds): Advances in Cell Neurobiology, Vol 2. London: Academic Press, pp. 33–82.

Ling, E.A., C. Kaur, T.Y. Yick, and W.C. Wong (1990) Immunocytochemical localization of CR3 complement receptors with OX-42 in ameboid microglia in postnatal rats. Anat. Embryol. *182:*481–486.

Ling, E.A., and W.C. Wong (1993) The origin and nature of ramified and ameboid microglia: A historical review and current concepts. Glia *7:*9–18.

Mason, C.A., S. Christakos, and S. Catalano (1990) Early climbing fiber interactions with Purkinje cells in the postnatal mouse cerebellum. J. Comp. Neurol. *297:*77–90.

Milligan, C.E., T.J. Cunningham, and P. Levitt (1991) Differential immunochemical markers reveal the normal distribution of brain macrophages and microglia in the developing rat brain. J. Comp. Neurol. *314:*125–135.

Murabe, Y., and Y. Sano (1982) Morphological studies on neuroglia. VI. Postnatal development of microglial cells. Cell Tissue Res. *225:*469–485.

Navascués, J., A. Moujahid, A. Quesada, and M.A. Cuadros (1994) Microglia in the avian retina: Immunocytochemical demonstration in the adult quail. J. Comp. Neurol. *350:*171–186.

Navascués, J., A. Moujahid, A. Almendros, J.L. Marín-Teva, and M.A. Cuadros (1995) Origin of microglia in the quail retina: Central-to-peripheral and vitreal-to-scleral migration of microglial precursors during development. J. Comp. Neurol. *354:*209–228.

Navascués, J., M.A. Cuadros, and A. Almendros (1996) Development of microglia: Evidence from studies in the avian central nervous system. In E.A. Ling, C.K. Tan, and C.B.C. Tan (eds): Topical Issues in Microglial Research. Singapore: Singapore Neuroscience Association, pp. 43–64.

Pardanaud, L., C. Altmann, P. Kitos, F. Dieterlen-Lièvre, and C.A. Buck (1987) Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. Development *100:*339–349.

Penfold, P.L., M.C. Madigan, and J.M. Provis (1991) Antibodies to human leucocyte antigens indicate subpopulations of microglia in human retina. Vis. Neurosci. *7:*383–388.

Perry, V.H., D.A. Hume, and S. Gordon (1985) Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. Neuroscience *15:*313–326.

Perry, V.H., and S. Gordon (1991) Macrophages and the nervous system. Int. Rev. Cytol. *125:*203–244.

Rio-Hortega, P. (1932) Microglia. In W. Penfield (ed): Cytology and Cellular Pathology of the Nervous System, Vol 2. New York: Paul B. Hoeber, pp. 482–534.

Streit, W.J., and G.W. Kreutzberg (1987) Lectin binding by resting and reactive microglia. J. Neurocytol. *16:*249–260.

Vela, J.M., I. Dalmau, B. González, and B. Castellano (1995) Morphology and distribution of microglial cells in the young and adult mouse cerebellum. J. Comp. Neurol. *361:*602–616.

Wolswijk, G. (1995) Strongly G_{D3}+ cells in the developing and adult rat cerebellum belong to the microglial lineage rather than to the oligodendrocyte lineage. Glia *13:*13–26.

Wu, C.H., C.Y. Wen, J.Y. Shieh, and E.A. Ling (1992) A quantitative and morphometric study of the transformation of amoeboid microglia into ramified microglia in the developing corpus callosum in rats. J. Anat. *181:*423–430.

Wu, C.H., C.Y. Wen, J.Y. Shieh, and E.A. Ling (1993) A quantitative study of the differentiation of microglial cells in the developing cerebral cortex in rats. J. Anat. *182:*403–413.

Yao, J., L. Harvath, D.L. Gilbert, and C.A. Colton (1990) Chemotaxis by a CNS macrophage, the microglia. J. Neurosci. Res. *27:*36–42.

Youngren, O.M., and R.E. Phillips (1978) A stereotaxic atlas of the brain of the three-day-old domestic chick. J. Comp. Neurol. *181:*567–600.