

Development of Microglia in the Prenatal Rat Hippocampus

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ABSTRACT

The distribution and appearance of microglial cell precursors in the prenatal hippocampus were examined in embryonic day 14 (E14) to E21 rats by nucleoside diphosphatase histochemistry. For comparison, the differentiation of astroglial cells was analyzed from E17 by vimentin and glial fibrillary acidic protein immunohistochemistry.

Based on morphologic features, nucleoside diphosphatase-positive microglial cell precursors were classified as ameboid microglial cells and primitive ramified microglial cells. Ameboid microglia were present in the hippocampal primordium on E14. As the hippocampus developed, however, ameboid microglia gradually transformed into primitive ramified microglia, first recognized at E19. Microglial cell precursors, often related to nucleoside diphosphatase-labeled blood vessels, were particularly observed next to the pial surface on days E14 and E17 and in the highly vascularized area around the hippocampal fissure from E19. Within the brain parenchyma, the microglial cell precursors tended to be located within the differentiating cell and neuropil layers rather than in the germinative zones. The late developing dentate gyrus remained almost devoid of microglial cell precursors before birth. Vimentin-positive astroglial processes with radial orientation were observed throughout the hippocampal subregions from E17. In contrast, glial fibrillary acidic protein-positive, radial processes were barely discernible in the fimbria and the dentate gyrus before E19.

The results are discussed in relation to the possible interactive role of microglial cells in central nervous tissue development and histogenesis. Regarding the origin of hippocampal microglial cell precursors, the present observations support the view that these cells may well originate from different mesodermal sources depending on time and localization. *J. Comp. Neurol.* 377:70-84, 1997. © 1997 Wiley-Liss, Inc.

Indexing terms: nucleoside diphosphatase; glial fibrillary acidic protein; vimentin; fascia dentata; embryo

Since the first reports by del Río Hortega (1920a,b; 1932) of the invasion of the developing brain by mesodermal pial elements, the extracerebral or intracerebral origins of microglia have been discussed (Ling and Wong, 1993; Castellano and González, 1995). Most studies agree that the microglial cell precursors, the so-called ameboid microglial cells, derive from monocytes infiltrating the central nervous system (CNS) during development (Imamoto and Leblond, 1978; Ling et al., 1980; Murabe and Sano, 1982; Perry et al., 1985; Ashwell, 1991; Chugani et al., 1991). Alternative origins—in the meningeal connective tissue (Cammermeyer, 1970; Boya et al., 1991), from pericytes (Mori and Leblond, 1969; Baron and Gallego 1972), or even from neuroectodermic precursors (Rydberg, 1932; Vaughn and Peters 1968; Oehmichen, 1982; Kitamura et al., 1984;

Hao et al., 1991)—have not, however, been completely discarded. It has even been proposed that microglia in different cerebral areas might have more than one origin (see Ling, 1981; Jordan and Thomas, 1988; Theele and Streit, 1993).

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The functional role during development of ameboid microglia remains uncertain. Referring to their phagocytic capacity and their particular relationship with white matter, it has been suggested that they are involved in the elimination of exuberant neurons and axonal projections formed during neurogenesis (Innocenti et al., 1983a,b; Hume et al., 1983; Ashwell, 1990; 1991; Ferrer et al., 1990). Microglial derived neurotrophic factors and proteases (Mallat et al., 1989; Shimojo et al., 1991; Nakajima et al., 1992a,b; Nagata et al., 1993) as well as neurotoxic effects (Giulian et al., 1990; Théry et al., 1991) have been demonstrated *in vitro*. Other studies have indicated that the ameboid microglial cells may actively participate in angiogenesis (Giulian et al., 1988a,b) and gliogenesis (Giulian and Baker, 1985; Giulian et al., 1988a; Selmaj and Raine, 1988; Jenkins and Ikeda, 1992; Hamilton and Rome, 1994).

The aim of the present paper was to analyze thoroughly the morphology and distribution of the microglial cell precursors in the developing rat hippocampus. We chose the plasmalemma-bound enzyme nucleoside diphosphatase (NDPase) visualized by histochemistry (Murabe and Sano, 1982; Vorbrot and Wisniewski, 1982; Fujimoto et al., 1987, 1989; Schnitzer, 1989; Castellano et al., 1991a,b; Vela et al., 1995a,b) as a marker for microglia and microglial cell precursors. In the developing brain, the hippocampal region is remarkable by the entirely prenatal formation of hippocampal pyramidal cells and interneurons, contrasting the preferential postnatal formation of dentate granule cells combined with an early laminar, cellular, and connective differentiation (Zimmer, 1978; Zimmer and Haug, 1978; Bayer, 1980a,b; Altman and Bayer, 1990a,b,c). We anticipated that these features would facilitate a detailed study of the temporal and spatial relationship between the occurrence and differentiation of microglial cell precursors and the neurogenesis, gliogenesis, and angiogenesis in the different hippocampal subregions.

MATERIALS AND METHODS

Tissue collection and fixation

The experimental material consisted of rat embryos of both sexes from the Wistar strain, with ages ranging from embryonic day 14 (E14) to E21. A total of 23 embryos from

embryonic stage E14 (n = 3), E17 (n = 8), E19 (n = 8), and E21 (n = 4) were included. The embryos were delivered by cesarean section, with the mothers in deep sodium pentobarbital anaesthesia (50 mg/kg body weight).

After careful removal, the majority of fetal brains were fixed by immersion for 5–6 hours at 4°C in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) with 5% sucrose and then cut into 50-µm thick vibratome sections. The sections were collected in cacodylate buffer (0.1 M cacodylate buffer at pH 7.4 with 7.5% sucrose) for immediate processing for NDPase histochemistry (see below). Three brains per group (excluding E14) were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 4 hours at 4°C, cryoprotected with PBS containing 30% sucrose, and then frozen with CO₂ for later cryostat sectioning at 20 µm. These sections were mounted on gelatinated glass slides and stored in a freezer at -40°C, until they were stained immunohistochemically (see below) for glial fibrillary acidic protein (GFAP) and vimentin (VIM).

Histochemical demonstration of the NDPase activity

The demonstration of NDPase enzymatic activity for microglial visualization was performed as described earlier (Castellano et al., 1991a). Briefly, the vibratome sections were incubated at 38°C for 25 minutes in the medium described by Novikoff and Goldfisher (1961), containing 7 ml of distilled water, 80 mM Trizma maleate buffer (Sigma; T-3128, pH 7.4), 5.05 mM manganese chloride, 3.62 mM lead nitrate, and 2.16 mM sodium salt of inosine 5'-diphosphate (Sigma; catalog no. I 4375) as substrate. After incubation, the vibratome sections were rinsed (3 × 10 minutes) in cacodylate buffer, treated in 2% ammonium sulfide for 1 minute, rinsed in cacodylate buffer (2 × 10 minutes) and distilled water (2 × 1 minute), and then immersed in 1% silver nitrate and rinsed again in distilled water (2 × 1 minute). The stained sections were mounted on gelatine-coated glass slides, dehydrated in alcohol, cleared in xylene, and coverslipped with DPX synthetic resin. Some NDPase-stained sections from each age were lightly counterstained with 0.5% toluidine blue in 0.2 M Walpole acetate buffer (pH 4.5), others with the Feulgen nuclear reaction for DNA (Bancroft and Stewens, 1982).

As a control for the histochemical reaction, a few sections from each embryonic age were incubated in medium lacking the substrate inosine 5'-diphosphate.

Immunohistochemical demonstration of GFAP and VIM

Cells belonging to the astroglial cell lineage were visualized by immunohistochemical staining for GFAP and VIM using the avidin-biotin technique on cryostat sectioned embryonic brains.

After initial rinsing in 0.05 M Tris-buffered saline (TBS) at pH 7.4 and washing in TBS with 0.5% Triton X-100 (2 × 10 minutes), sections were incubated in 10% fetal calf serum (FCS) diluted in TBS for 30 minutes at room temperature. The incubation with the primary polyclonal rabbit antibody, raised against cow GFAP (Dako, Denmark) was performed overnight at 4°C in a dilution 1:1000 in TBS with 10% FCS. Following a rinse in TBS with 0.5% Triton X-100 (3 × 10 minutes), the sections were incubated at room temperature for 60 minutes with a biotinylated

Abbreviations

ac	alveolar channels
ah	Ammonic (Ammon's horn) neuroepithelium
AH	Ammon's horn
AHP	Ammon's horn, stratum pyramidale (pyramidal cell layer)
cp	choroid plexus
DG	dentate gyrus
DGe	external limb of dentate gyrus outer blade of stratum granulare
DGi	internal limb of dentate gyrus inner blade of stratum granulare
dgp	primary dentate neuroepithelium
dgm	dentate migration
DH	dentate hilus
fg	fimbrial gliopithelium
FI	fimbria
HiF	hippocampal fissure
izh	hippocampal intermediate zone
lv	lateral ventricle
ne	hippocampal neuroepithelium
su	subicular neuroepithelium
SU	subiculum

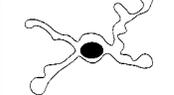
Type of cell	Shape	Cell processes	Measuring	NDPase activity	Time course of appearance	Cell morphology
Ameboid microglia type 1	Roundish and sometimes lobular	None	15-40 μm	Very high	From day E14	
Ameboid microglia type 2	Round	None, occasional filopodia	15-20 μm	High	From day E19	
Ameboid microglia type 3	Pleomorphic	Filopodia and/or pseudopodia	20-50 μm	Moderate	From day E14	
Primitive ramified microglia	Elongated	Scantly developed processes showing a beaded shape	50-110 μm	Low	From day E19	

Fig. 1. Classification of the morphologic types of NDPase-labeled microglial cell precursors in the prenatal rat hippocampus.

anti-rabbit antibody (Amersham, United Kingdom) diluted 1:300 in TBS with 10% FCS. After rinsing in TBS with 0.5% Triton X-100 (3×10 minutes), the samples were incubated at room temperature for 60 minutes in a 1:400 dilution of avidin-labeled peroxidase (Dako, Denmark) in TBS with 10% FCS. Finally, sections were rinsed in TBS with 0.5% Triton X-100 (1×10 minutes) and TBS (2×10 minutes) before visualization of the peroxidase using 3-3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen (50 mg DAB in 100 ml 0.05M TBS, pH 7.4, with 0.033 ml H_2O_2 added just before use).

VIM immunohistochemistry was performed as described above, using a monoclonal mouse antibody raised against human VIM (Dako, Denmark) as the primary reagent (dilution 1:500) and a biotinylated anti-mouse antibody (Amersham, United Kingdom) as the secondary reagent (dilution 1:200). After immunocytochemistry, the sections stained for GFAP or VIM were dehydrated in alcohol, cleared in xylene, and coverslipped with DPX synthetic resin.

For control, other sections were processed with omission of the primary antibodies.

RESULTS

Throughout the E14–E21 fetal period, NDPase histochemistry revealed a highly polymorphic population of black-brownish cells within the hippocampus. The stained cells were identified as microglial cell precursors in accordance with previous studies using NDPase histochemistry as a marker for microglia in the normal developing and adult CNS (Murabe and Sano, 1982; Vorbrodt and Wisniewski, 1982; Fujimoto et al., 1987, 1989; Schnitzer, 1989; Castellano et al., 1991a,b; Vela et al., 1995a,b). The terminology used in the description of the different morphologic types of microglial precursor cells is based on the one proposed by Murabe and Sano (1982). The term ameboid microglia (AM) thus designates the heterogeneous population of polymorphic NDPase reactive cells observed from the earliest ages, whereas the term primitive ramified microglia (PRM) designates the population of poorly ramified NDPase-stained cells appearing later.

In the following text we describe the morphologic features of the different NDPase-stained cell types, followed

by their occurrence at different ages, and finally the distribution of the different cell types at septal, midposterior, and temporal levels of the developing hippocampus. For each group we also describe the association between the microglial cell precursors and the blood vessels (endothelial cells also stain by NDPase histochemistry) and the occurrence of differentiating astroglial cells as demonstrated by VIM and GFAP immunohistochemistry. For the developing hippocampus we used the terminology of Altman and Bayer (1990a,b,c, 1995).

Morphology of the NDPase-labeled microglial cell precursors

Ameboid microglial cells. Ameboid microglial cells (AM) displayed considerable diversity of shape and size, but a consistent high NDPase activity. The following AM types were defined.

AM type 1 cells (Figs. 1, 2) were present on E14 but in considerably larger numbers at E17. They had a roundish shape, a diameter ranging from 15 to 40 μm , a ruffled surface, and lacked well-defined cell processes. This cell type also displayed a vacuolated cytoplasm in certain hippocampal subregions and occasionally took on the morphology of blackberry-shaped cells. The cells were often located next to the pial surface and displayed high NDPase activity. The presence of occasional pairs of cells suggested ongoing proliferative activity.

AM type 2 cells (Figs. 1, 2) were present at days E19 and E21 as round cells, ranging in diameter between 15 and 20 μm . They were devoid of cell processes except for occasional thin filopodia-like processes. They were often found next to blood vessels and sometimes intimately related to these. The NDPase activity of type 2 cells was higher than that of type 1.

AM type 3 cells (Figs. 1, 3) had a polymorphic shape and were observed at all fetal ages studied. Typically, their cell bodies had an irregular outline, with a ruffled surface and size, which along the long axis ranged between 20 and 50 μm . Fine filopodia and/or pseudopodia were often formed. Occasionally, the cells had a few thin and short processes. This cell type with typical high NDPase activity was observed at all distances from blood vessels.

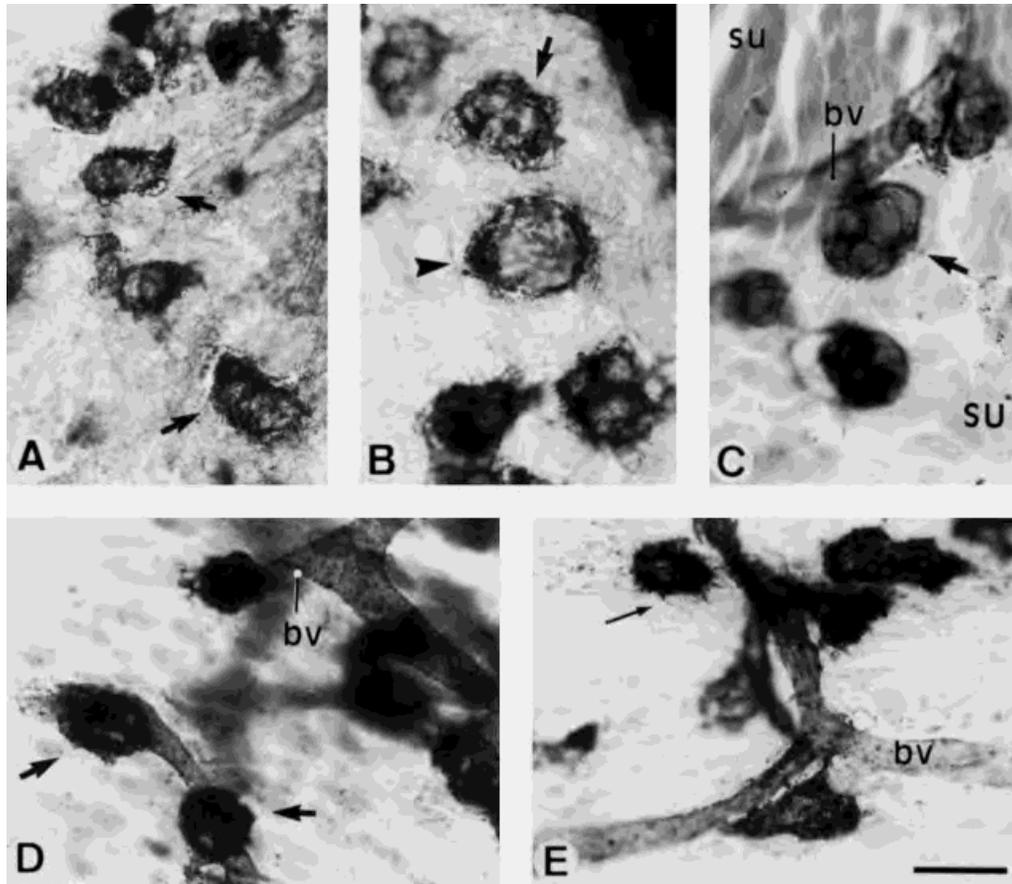


Fig. 2. **A:** Ameboid microglia type 1 cells (arrows), located in the differentiating field of the hippocampus on embryonic day 17, are often found next to the surrounding pia. **B:** Developing fornix on embryonic day 17 (E17). Ameboid microglia type 1 are also observed as rounded large cells (arrowhead) showing a vacuolated cytoplasm (arrow) in developing hippocampal white matter areas. **C:** Ameboid microglia type 1 in the border of the subicular neuroepithelium (su) and the differentiating field of the subiculum (SU) on E19. Blackberry-shaped

forms of this cell type (arrow) are occasionally found in relation to the blood vessels (bv) in the subicular area on E19, but are largely found on E21. **D:** Ameboid microglia type 2 in the gray matter at the septal level of the hippocampus. Type 2 cells (arrows) are widely situated nearby blood vessels (bv) and mainly appear from E19. **E:** Ameboid microglia (type 2) in the hippocampal commissure. Type 2 cells lack cell processes but sometimes bear filopodia-like processes (arrow). Scale bar = 18 μ m.

Primitive ramified microglia cells (PRM) (Figs. 1, 4). This cell type was first observed at E19. All these cells were sparsely ramified with an elongated cell body with a maximal diameter ranging between 50 and 110 μ m. The processes of PRM were initially (at E19) slightly developed, but got both longer and thinner at later stages, with a single branching point and small swellings of variable diameter giving them a beaded shape. At E19, PRM most frequently were found close to blood vessels; at later stages, they were distributed further into the neuropil. The NDPase activity was less intense than that of the ameboid microglia.

Temporal and spatial occurrence of NDPase-labeled microglial cell precursors

E14 (Fig. 5). The hippocampal primordium at E14 consisted of the Ammonic neuroepithelium and the developing subiculum. In the Ammonic neuroepithelium, AM type 1 and 3 were found subjacent to the surrounding pia, in close relation to the NDPase-labeled blood vessels arising from the vascular plexus in the pia, and in the periventricular region. In the subiculum, AM types 1 and 3

were rarely seen and, if so, next to the pia. Finally, roundish, NDPase-stained cells, ranging from 15 to 20 μ m in diameter, were observed scattered among the blood vessels of the pia immediately external to the hippocampal primordium and in the border zone of the hippocampal primordium and the pial membrane.

E17 (Fig. 6). At this stage, the distribution of microglial cell precursors was almost identical in both the midposterior and temporal levels of the hippocampus. AM types 1 and 3 were concentrated within the differentiating parts of the hippocampus, which had already formed. Normally, the AM were located subjacent to the pia or in association with the vascular network. Occasionally, single AM type 3 were seen in the proliferative zone of the hippocampal neuroepithelium. At this age, the primary dentate neuroepithelium and the fimbrial gliopithelium are already distinguishable. In the region where the fimbria forms, almost no blood vessels were seen and the few AM types 1 and 3 observed were mostly located near the pial limiting membrane and rarely in the fimbrial gliopithelium. In the subiculum, blackberry-shaped AM type 1 were seen occasionally. Finally, roundish NDPase-

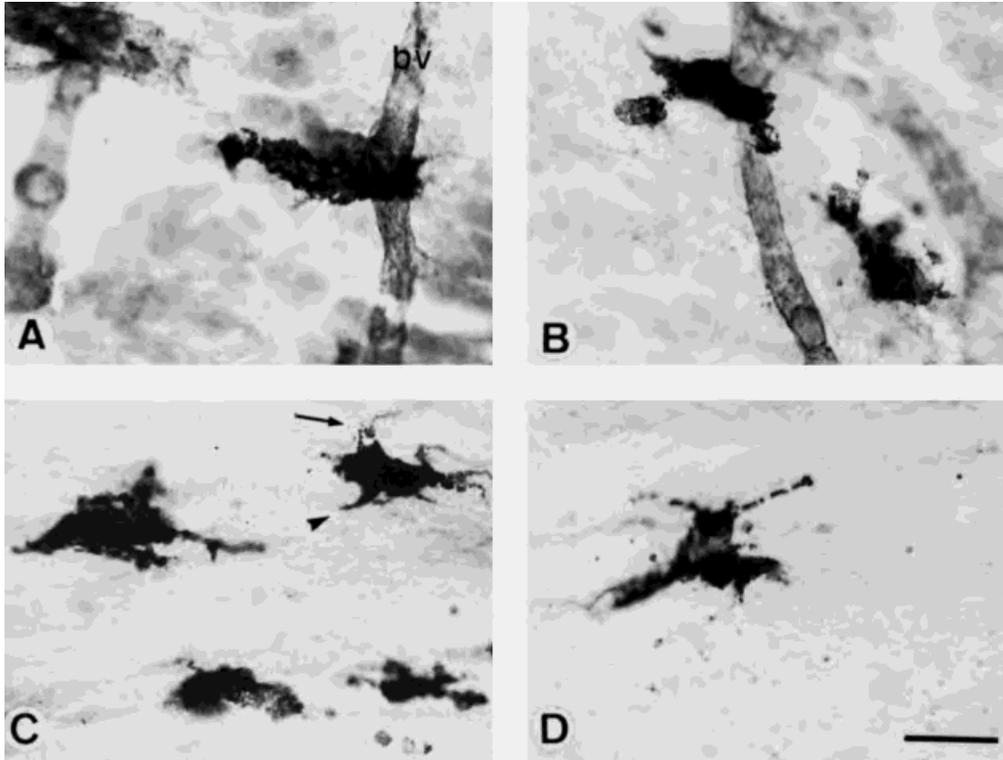


Fig. 3. **A:** Differentiating field of E17 hippocampus. Ameboid microglia type 3 often demonstrate a close relationship to blood vessels (bv) in the early stages. **B:** Ameboid microglia type 3 in the developing gray matter of the hippocampus on E19. **C:** Fimbria at E19. Ameboid microglia type 3 are pleomorphic cells often bearing filopodia (arrow)

and/or pseudopodia (arrowhead). **D:** Ameboid microglia type 3 in the ventral hippocampal commissure on E21. Type 3 are arranged in parallel to the fibers in the developing white matter areas. Scale bar = 18 μ m.

labeled cells were again observed within the pial membrane overlaying the hippocampus.

At the septal levels of the developing hippocampus, AM types 1 and 3 were present in the differentiating cell and neuropil layers, whereas no AM were found in the neuroepithelium where only blood vessels stained for NDPase. Clusters of AM type 1 of globoid shape and vacuolated cytoplasm were found in the developing fornix. Double labeling with NDPase histochemistry and the Feulgen nuclear reaction showed pyknotic nuclei engulfed by these cells. Also at this level, roundish NDPase-labeled cells were observed in the surrounding pia.

VIM immunoreactive radially oriented glial processes were distributed throughout the hippocampus. GFAP staining was not observed at this age.

E19–E21 (Figs. 7, 8). The midposterior and temporal levels of the hippocampus displayed grossly identical microglial distribution patterns at both ages. On E19, the population of microglial cell precursors within the Ammon's horn consisted of both AM and PRM. At E21, the latter became the most predominant. AM types 2 and 3, PRM and cell types in transition from AM to PRM were located in the developing stratum radiatum and in relation to the blood vessels of the hippocampal fissure and pia. AM type 1 were rarely found in the hippocampal fissure and, if so, predominantly on E19 in the vicinity of the pial membrane. The PRM were located differently and were found particularly on E21 along the apical pole of stratum pyramidale. This layer is quite distinct at this age, curving

in the direction of the dentate gyrus. Next to the reduced Ammonic neuroepithelium and accumulating at the edges of the hippocampal intermediate zone, were (at both ages) cell types in transition from AM type 3 to PRM, just as some PRM were observed at E21 next to the vascular network in this area. In the hippocampal intermediate zone, which underwent a significant size reduction from E19 to E21, we observed a gradual differentiation of microglial precursor cell types. At E19, there were predominantly cells of AM type 3 and cell types in transition from AM to PRM, whereas cells in transition to PRM and PRM predominated at E21. These cells were related to the blood vessels and, being specific for this area, were flanking the alveolar channels, which already were present at E19 but more developed at E21. Within CA1, PRM were found apical to the developing pyramidal cell layer. Occasionally, AM types 2 and 3 were observed in the pyramidal cell layer at midposterior levels, but not at temporal levels of the Ammon's horn on E19. These cells were often located next to the vascular network.

At the temporal levels of the dentate gyrus, AM type 3 and cell types in transition from AM to PRM were located in the developing hilus. The cells were generally located next to the vessels. Similar cells were only rarely observed at the midposterior levels. At E19, microglial cell precursors were located on both sides of the dentate migration path linking the secondary dentate matrix with the forming dentate gyrus. Primitive ramified microglia were located on the Ammon's horn side, whereas AM type 2 and

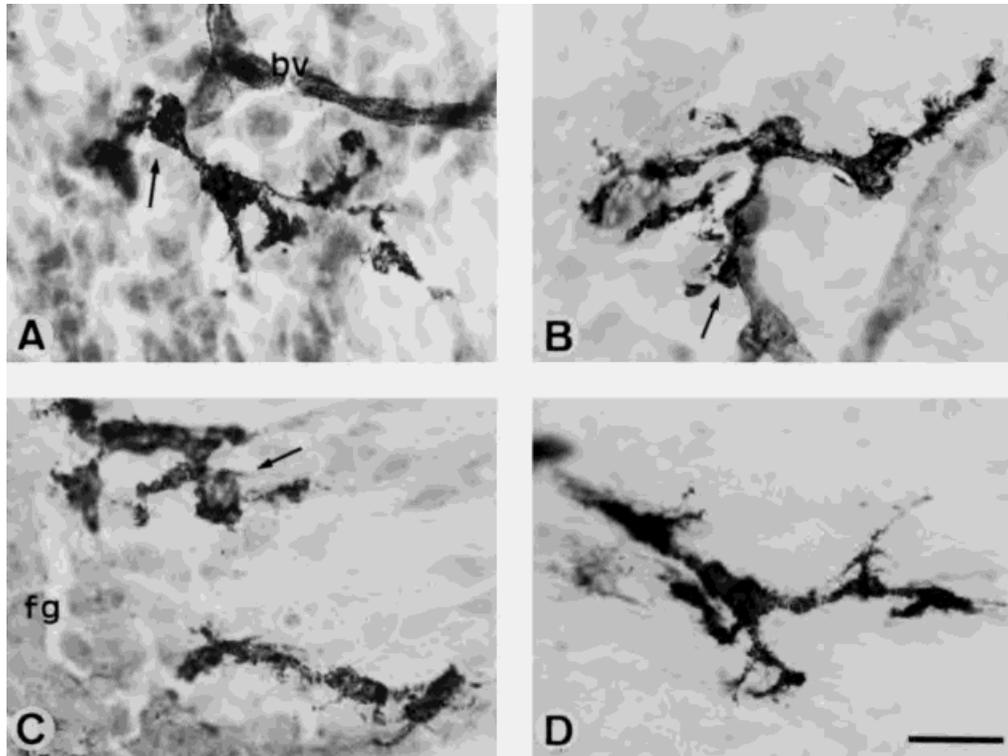


Fig. 4. **A:** Primitive ramified microglial cells in the vicinities of blood vessels (bv) at the hippocampal intermediate zone on E19. Characteristically, primitive ramified microglia (PRM) show a poorly ramified shape with cell processes containing small swellings (arrow). **B:** Hippocampal intermediate zone on E21. With age, PRM display a more mature shape although still displaying the characteristic swellings (arrow) along their cell processes. **C:** Primitive ramified microglia

in the fimbria bordering the fimbrial glioepithelium (fg) on E19. PRM in the developing white matter also contain swellings (arrow) in the cell processes, but in contrast to PRM in gray matter, they show a less branched shape. **D:** Hippocampal commissure on E21. Similar to amoeboid microglia, PRM cells arrange parallel to the axons. Scale bar = 18 μ m.

particularly type 3 cells were located on the fimbrial side. At E21, the inner blade of the dentate granule cell layer and its subjacent dentate hilus are morphologically distinct and the outer blade of the dentate granule cell layer is distinguishable. At this age, a few PRM were also seen just deep to the outer blade of the stratum granulare and in the dentate hilus, next to blood vessels. At both ages, AM type 3 and cell types in transition from AM to PRM were also observed in close relation to blood vessels at the border of the primary dentate neuroepithelium. Neither AM nor PRM were observed in the developing granule cell layer at any of these ages.

In the fimbria, at midposterior and temporal levels, AM type 2 and preferentially type 3 were arranged in parallel with the fibers rather than being associated with the very few NDPase-labeled blood vessels present in the fimbria at this age. AM types 2 and 3 were observed in the putative fimbrial glioepithelium and next to the pia. When related to the putative fimbrial glioepithelium, the AM were located next to the blood vessels at least in the ventral part, whereas they appeared to be related to the glioepithelium in the dorsal part. Primitive ramified microglia were occasionally present and then mainly in the ventral part. Amoeboid microglia were sometimes also found in the developing cortical white matter in relation to the midposterior part of the subicular area at E19.

At the midposterior levels of the subiculum, scattered AM types 2 and 3 were distributed along the basal part of

the pyramidal cell layer, just as blackberry-shaped AM type 1 occasionally were encountered next to the blood vessels in stratum moleculare. These cells had transformed to PRM on E21. Microglia were infrequently found at the temporal level of the subiculum at these ages.

At the septal levels on E19, a remarkable presence of AM types 2 and 3 often related to the blood vessels were particularly seen in the stratum radiatum of Ammon's horn. AM type 3 cells were also observed next to the induseum griseum at this age. At these levels at E21, clusters of AM type 1 with a vacuolated cytoplasm and AM type 3 were found, especially in association with the vascular network next to the stratum pyramidale of subiculum, bordering the developing white matter of the cerebral cortex. Counterstaining of NDPase-stained sections with the Feulgen reaction showed pyknotic nuclei engulfed by these AM cell types. At both ages, AM type 3 cells were seen in the developing dentate hilus.

Most rostrally at the septal level, the fimbria displayed the same microglial distribution pattern as observed at the midposterior level. In the hippocampal commissure at E19 and in the vicinities of the glioepithelium, AM types 2 and 3 were arranged in parallel with the fiber tracts, whereas transitional forms to PRM and a few PRM were observed in the other areas of the hippocampal commissure. At E21, PRM were distributed in the entire hippocampal commissure. Amoeboid microglia formed clusters of cells next to the

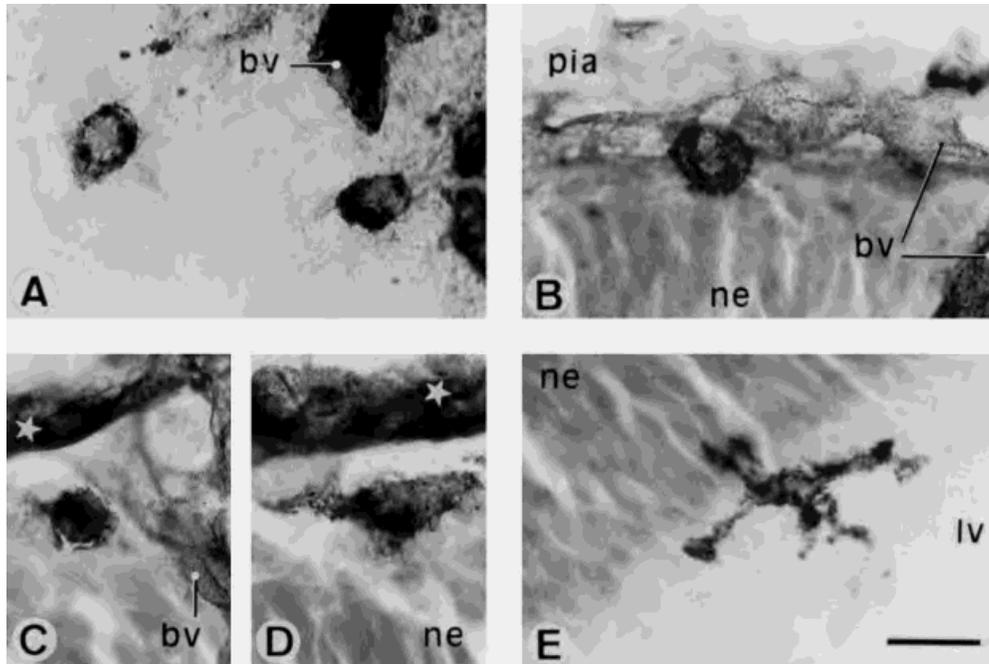


Fig. 5. Hippocampal primordium of a 14-day-old embryo. **A:** Round NDPase-labeled cells located between the blood vessels (bv) in the mesenchyme outside the hippocampal primordium. **B:** NDPase histochemistry demonstrates a cell next to the pia close to the hippocampal neuroepithelium (ne). **C,D:** At this age, amoeboid microglia often

localize in the vicinity of the pia external (white star) to the hippocampal neuroepithelium (ne). **E:** Apart from roundish cells, ramified NDPase-labeled cells are even seen at the periventricular region of the neuroepithelium (ne) nearby the lateral ventricle (lv). Scale bar = 18 μ m.

dorsal area of the periventricular zone. Roundish NDPase-labeled cells were observed within the pia at both ages.

VIM-immunoreactive radial glial processes were observed in all hippocampal subregions at E19 and E21, but there was less intense staining in stratum pyramidale of the Ammon's horn and subiculum and in the outer blade of the granule cell layer and the adjacent dentate hilus on E21. At this age, radial glial cell processes in the developing cell and neuropil layers of the subiculum, Ammon's horn, the dentate migration zone, and the fimbria showed a more elaborate parallel fiber pattern and a higher GFAP immunoreactivity than observed on E19. Within the dentate gyrus, the GFAP-positive processes were predominantly seen in the outermost part of the developing molecular layer, in the dentate hilus, and next to the fimbria and the fimbrio-dentate junction.

DISCUSSION

NDPase-reactive microglial cell precursors in the prenatal E14–E21 hippocampus display significant time-related and site-related changes in morphology with a significant increase in density from E19. The NDPase-labeled microglial precursors could be grouped into three subpopulations of amoeboid microglial cells (AM) and into primitive ramified microglia (PRM). It was possible to follow the migration of AM into the brain parenchyma and their differentiation from the rounded immature forms into the primitive forms of PRM. The principal location of the microglial precursor cells was within the differentiating cell and neuropil layers, rather than the germinative zones that are the Ammonic neuroepithelium, primary dentate gyrus, secondary dentate matrix and tertiary dentate matrix, and

subicular neuroepithelium. In accordance with the late development of the dentate gyrus, the occurrence and differentiation of microglial cells were delayed in this structure compared with other subregions of the hippocampus and the hippocampal white matter (hippocampal commissure, fimbria, and fornix). At all stages there was a close relationship between the NDPase-stained cells and the NDPase-stained vascular network. The onset of the morphologic differentiation of the microglial cell precursors appeared to start earlier than the morphologic and immunophenotypic differentiation of the astroglial cell precursors. These observations are discussed in relation to the origin of microglial cells and the potential functions of microglial cell precursors in the neurogenesis and gliogenesis within the developing hippocampus.

Classification of microglial cell precursors

Amoeboid microglial cells observed during brain development are considered to be the precursors of the ramified, resting microglia of the adult brain (Thomas, 1992; Ling and Wong, 1993). Morphologically, the amoeboid microglia, named brain macrophages by others (see Jordan and Thomas, 1988; Perry and Gordon, 1991), are round, macrophage-like cells with pseudopodia. In contrast, ramified or resting microglia have small, elongated somata, bearing two or more processes with a variable number of subsidiary branches, and populate the adult brain in a highly territorial manner. Between these two cell types are various transitional forms (Murabe and Sano, 1982; Perry et al., 1985; Cuadros et al., 1992; Wu et al., 1993). In the present study, we classified microglial cell precursors into two main groups, AM and PRM.

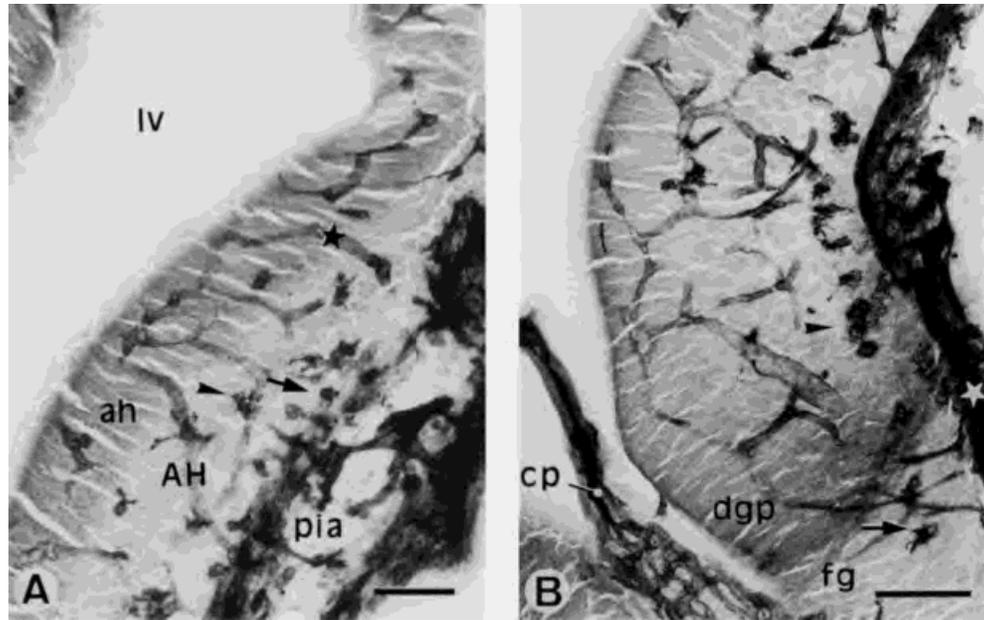


Fig. 6. Photomicrographs of the midposterior level of the hippocampus on E17. **A:** Ameboid microglia (arrow) adjacent to the pial limiting membrane in the differentiating field of the hippocampus. Ameboid microglia (arrowhead) are often observed tightly attached to the blood vessel walls (black star). **B:** Characteristically, ameboid microglia (AM) type 1 (arrowhead) are seen in the differentiating field of the

midposterior hippocampus close to the fimbrial glioepithelium (fg). In the fg, AM (arrow) not only locate adjacent to the pia (white star), but also deeply within this layer. ah, Ammonic (Ammon's horn) neuroepithelium; AH, Ammon's horn; cp, choroid plexus; dgp, primary dentate neuroepithelium; lv, lateral ventricle. Scale bars = 95 μ m.

Based on morphologic changes and regional differences to the developmental stage of the examined hippocampal areas, the ameboid microglial cells were further classified into three different subtypes. AM type 1 were observed from E14 with a preferential location next to the surrounding pia. Some type 1 cells with a vacuolated cytoplasm occasionally were blackberry-shaped with phagocytized Feulgen-stained pyknotic nuclei in their cytoplasm. In contrast, AM type 2 mainly occurred from E19 with a primary relation to blood vessels. These cells resemble the round microglia described during postnatal life by Murabe and Sano (1982). AM type 3 were essentially defined by their polymorphic shape bearing filopodia and/or pseudopodia. These cells occurred in parallel with type 1 and type 2 at the time when PRM began to appear. Primitive ramified microglia are considered to represent a transitional form between AM cells and resting microglia. In the literature, AM type 1 and type 2 have been classified as round cells or round microglial cells (Ashwell, 1991; Boya et al., 1991; Wu et al., 1992), whereas cells like AM type 3 have been named ameboid cells, brain macrophages, ameboid microglia, and pseudopodic or nascent ameboid microglia (Ling, 1981; Murabe and Sano, 1982; Ferrer et al., 1990; Ashwell, 1991; Boya et al., 1991; Wu et al., 1992).

The PRM is, in agreement with others (see Perry et al., 1985; Jordan and Thomas, 1988; Ling and Wong, 1993; Wu et al., 1993), considered to be a transitional morphologic type of microglia, preceding the typical ramified, resting microglial cell in the normal adult brain. We found PRM in late embryonic life coinciding with a progressive maturation of the hippocampus and the gradual disappearance of AM. We did not observe NDPase-positive cells with a morphology corresponding to mature, resting microglia at any of the prenatal ages studied. Although we have not

determined with the present material the proportion of AM cells that will mature to PRM, but both Imamoto and Leblond (1978) and Wu et al. (1992) reported that not all ameboid microglial cells may transform into ramified microglia.

Origin of microglial cells

Based on the initial location of cells, our observations suggest a dual pial and hematogenous origin of the microglial cell precursors within the hippocampus. This would be in accordance with the two major hypotheses of the origin of microglial cells: a monocytic origin (Imamoto and Leblond, 1978; Ling, 1981; Murabe and Sano, 1982; Perry et al., 1985; Hickey and Kimura, 1988; Chugani et al., 1991) and a mesodermal pial origin as first presented by del Río Hortega (1920b) and recently supported by Boya et al. (1991) and Cuadros et al. (1994).

Regarding the monocytic origin, it has unequivocally been demonstrated that hematogenously derived microglial cell precursors can infiltrate the brain parenchyma during the late embryonic period and the first postnatal week (Ling, 1981; Chugani et al., 1991). Applying NDPase histochemistry, Murabe and Sano (1982) reported that round microglial cells, which presumably originated from blood monocytes, differentiated into ramified microglial cells or brain macrophages (ameboid microglial cells) when they entered into the CNS. Our observations showed that NDPase-labeled microglial cell precursors were mostly related to the blood vessels. This is particularly evident in the highly vascularized hippocampal fissure from E19. Our findings accordingly suggest that monocytes may enter the hippocampus through the cerebral vascular network and gradually transform into microglial cell precursors later in embryonic life (Fig. 9). As morphologic

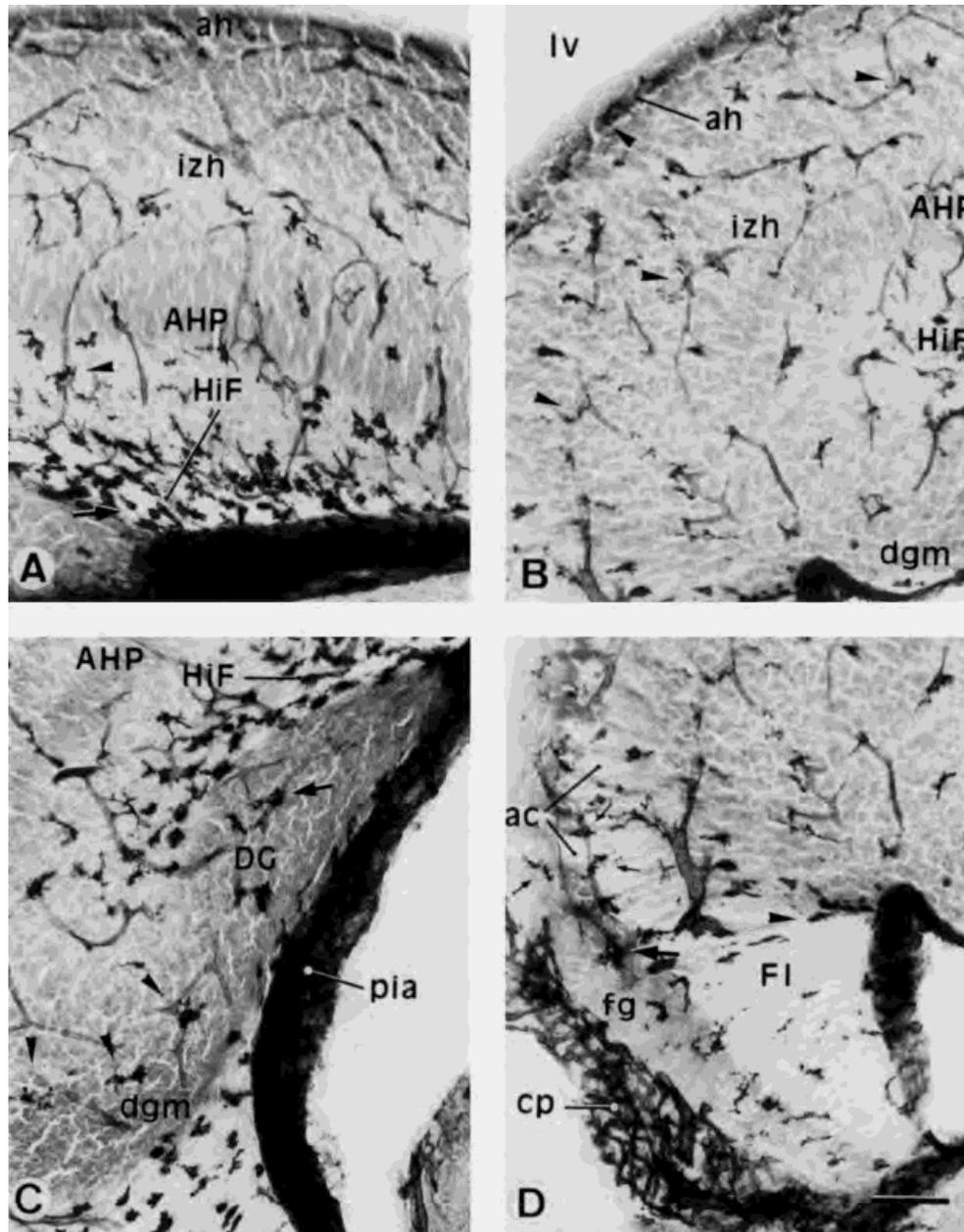


Fig. 7. Hippocampus on E19. **A:** Ameboid microglia (arrow) and primitive ramified microglia (arrowhead) are mainly apparent around the hippocampal fissure (HiF). **B:** Microglial cell precursors (arrowheads) are often seen next to blood vessels related to the Ammon's horn neuroepithelium (ah) and in the hippocampal intermediate zone (izh). This latter area is also referred to as the Ammon's horn migration. **C:** By contrast, microglial cell precursors (arrow) scarcely populate the dentate gyrus (DG) and they arrange in the developing dentate hilus nearby or adjacent to pia. Note that these cells (arrowheads) do not

locate within the dentate migration (dgm) but flank this field. **D:** Microglial cell precursors in the fimbria (FI) are arranged in parallel to axons but also found next to blood vessels (arrow) and the pia (arrowhead). In the area with the alveolar channels (ac), microglial cell precursors (small arrows) are generally situated in the vicinity of the alveolar channels. AHP, Ammon's horn, stratum pyramidale (pyramidal cell layer); cp, choroid plexus; fg, fimbrial glioepithelium; lv, lateral ventricle. Scale bar = 95 μ m.

signs of blood-brain barrier establishment are present from E16–E17 (Yoshida et al., 1988), the mechanism by which blood-borne cells enter the nervous tissue during development is still poorly known. It has been demonstrated in certain pathologic and experimental states in adult animals (Sloan et al., 1992; Fabry et al., 1994) that blood cell migration into the neural tissue through an

intact blood-brain barrier is mediated through a transient upregulation of cell adhesion molecules. Moreover, comparing the microglial cell populations in the developing hippocampus at days E17 and E19, we observed an increased number of NDPase-labeled cells at the later age. This increase coincides with a peak in the hematopoietic activity in the liver and an intensified myeloid development at

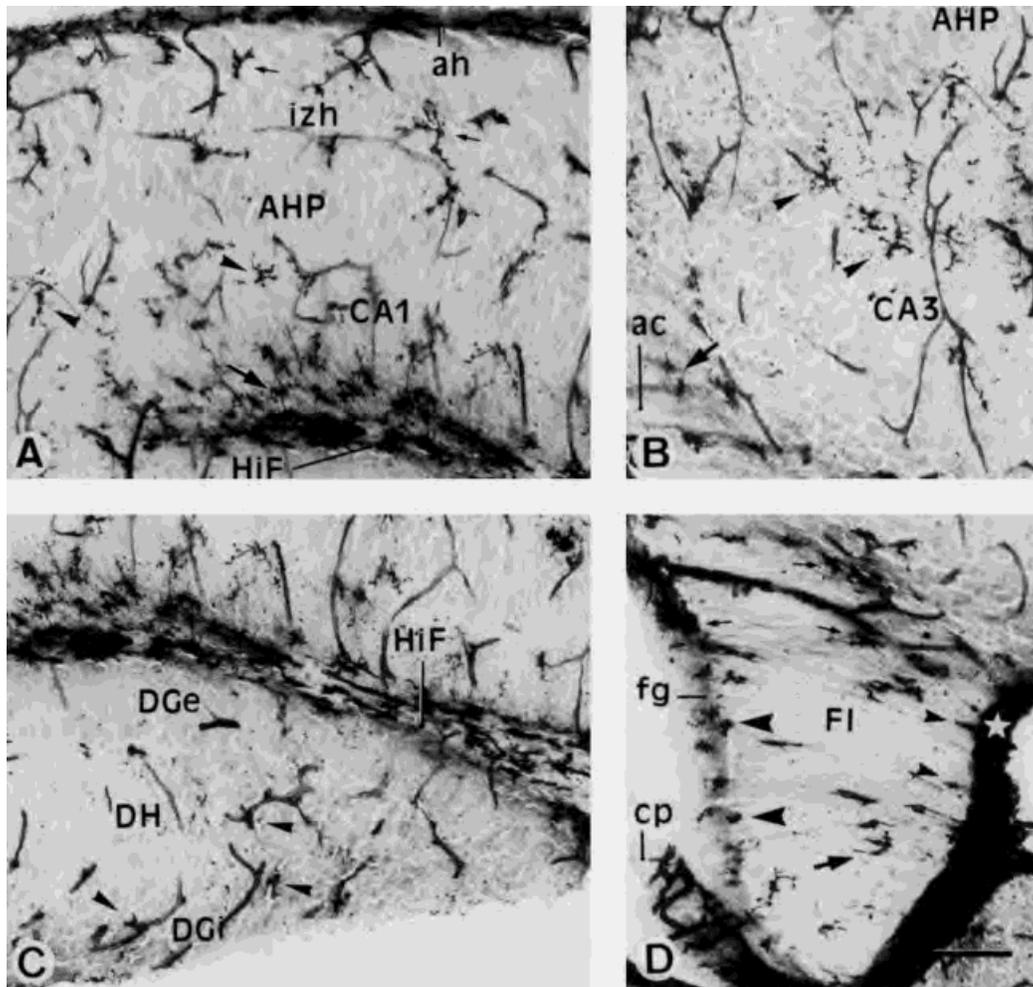


Fig. 8. Hippocampus on E21. **A:** Similar to E19, AM (arrow) and primitive ramified microglia (arrowheads) mainly situate around the hippocampal fissure (HiF). Primitive ramified microglia are also observed next to blood vessels in the thinner Ammon's horn neuroepithelium (ah) and in the hippocampal intermediate zone (izh) (small arrows). **B:** Primitive ramified microglia (arrowheads) are specially ramified in the stratum radiatum of the developing CA3. In the area with the alveolar channels (ac) and similar to E19, microglial cell precursors (arrow) flank the alveolar channels. **C:** The dentate gyrus is considerably more vascularized than at the previous aged studied.

Microglial cell precursors (arrowheads) located in the developing dentate hilus (DH) next to blood vessels and bordering the well-defined external limb of DG (DGe) and the less-distinguished internal limb of the DG (DGi). **D:** Ameboid microglia distribute next to the dorsal area of the fimbrial glioepithelium (arrowheads) and blood vessels (small arrows). Moreover, AM (small arrowheads) are also observed close to the pia (white star). However, PRM (arrow) mostly arrange in the ventral area of fimbria (FI) parallel to developing axons. AHP, Ammon's horn, pyramidal cell layer. Scale bar = 95 μ m.

E18 (Cline and Moore, 1972; Crocker and Milon, 1992). One may therefore speculate that during late embryonic life, monocytes originating mostly in the liver may migrate into the hippocampus through the vascular network by active and regulated mechanisms.

The mesodermal pial origin of microglial cell precursors is supported by our results at the earliest ages (E14–E17), during which time we observed NDPase-labeled cells both between the blood vessels outside the hippocampal primordium and along the neuroepithelial basement membrane. Scarce microglial cell precursors were located in the vicinity of the developing cerebral blood vessels and in the depth of the relatively avascular neuroepithelium and the surrounding pia. By using GSA I-B4 lectin histochemistry to identify macrophages and microglia, Sorokin et al. (1992) found GSA I-B4-labeled cells in the surrounding

mesenchyme of the rat brain at E11 and in the relatively avascular neuroepithelium at E12. These cells were identical to the "primitive" or "fetal" macrophages of the yolk sac. Apart from this origin, the embryonic mesodermal tissue has been proposed to be another source of macrophages and/or microglial precursor cells. Similarly, Ashwell (1991) demonstrated occasional GSA I-B4-stained ameboid microglia in the rat brain on E11 and next to the medial walls of the telencephalon and in the connective tissue immediately external to the neuroepithelium from E14 to E16. Therefore, it seems plausible that the adult microglial cell population may also derive from cell precursors in the yolk sac or the embryonic mesenchyme (Merrill, 1992) that may migrate from the surrounding mesenchyme into the relative avascular hippocampal primordium (Fig. 9).

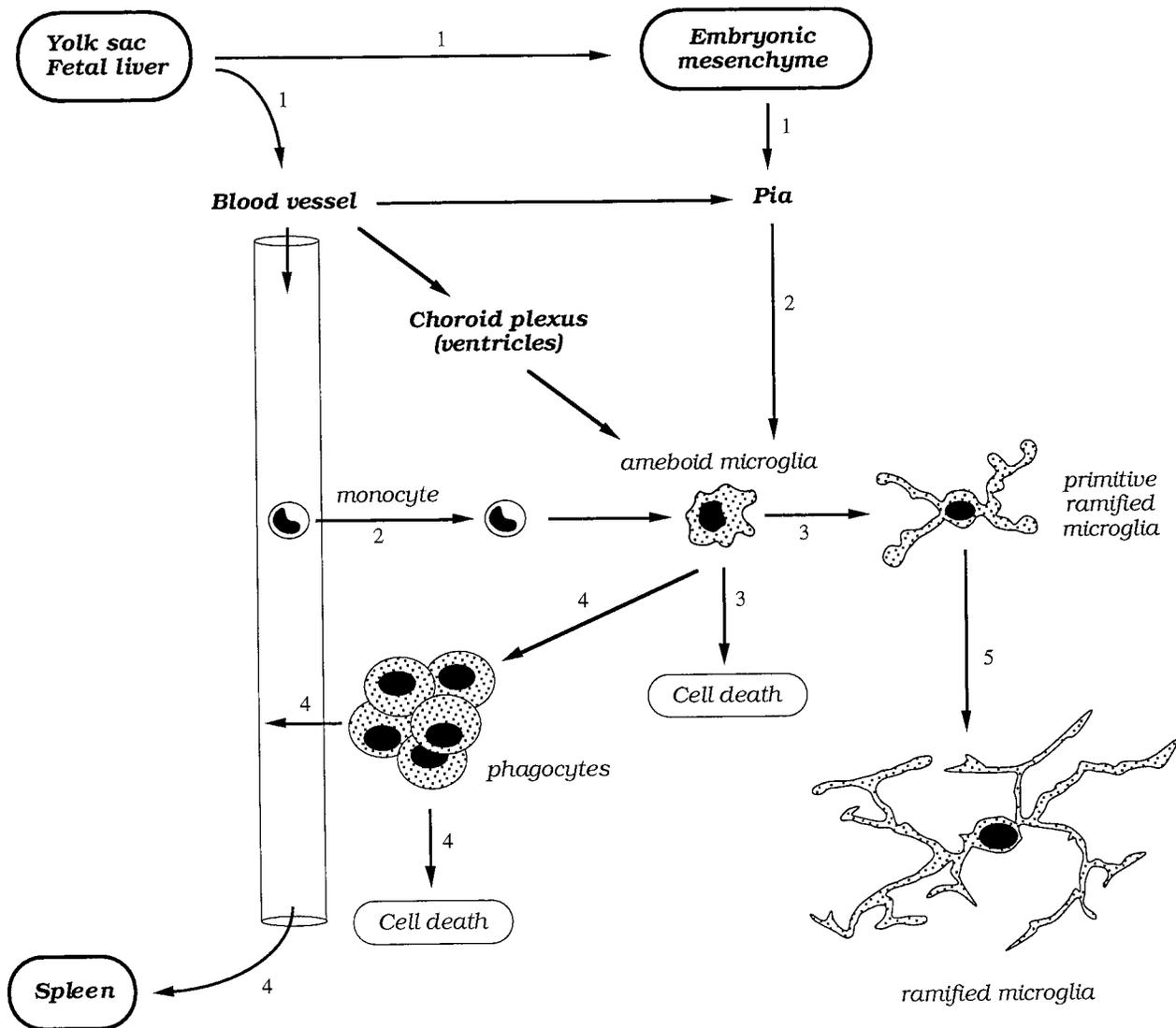


Fig. 9. Microglial cell precursor sources in the developing hippocampus. **1:** Cell precursors of microglia in the hippocampus may originate in the yolk sac, liver, spleen, and later in the bone marrow or surrounding mesenchyme depending on the embryonic age. **2:** Two pathways may serve cell precursors to migrate into the hippocampal parenchyma in the prenatal period. First, pial elements cross the neuroepithelium basal lamina and enter into a relatively avascular hippocampus. Second, from the blood-stream, monocytes pass into the hippocampus through the developing vascular network. However, the two paths could also coincide in time. Moreover, cell precursors from the ventricles should be also considered, as discussed in the text, just

Finally, other studies have claimed that microglial cell precursors may enter the hippocampal primordium from the choroid plexus and the ventricles by crossing the ependymal cell layer (Sturrock, 1978). Possibly some of the AM cells located in the periventricular region at E14 may enter into the hippocampal primordium from the ventricles. Moreover, the occasional observation of pairs of microglial cells suggests that the increase of the microglial precursor cell population may also occur by local proliferation, as demonstrated in pathologic and experimental states in adult animals (Miyake and Kitamura, 1991; Giordana et al., 1994).

as proliferation of cell precursors found in the hippocampus may contribute to the final number of microglia. **3:** Once in the hippocampus, the cell precursors, now termed ameboid microglia (AM), may differentiate into primitive ramified microglia (PRM). However, it is not clear whether all AM may mature into PRM or if some may die like other CNS cells by naturally occurring cell death phenomena. **4:** Furthermore, AM may also transform into phagocytic cells without a later maturation into PRM, dying or leaving the CNS after having accomplished their role as phagocytes. **5:** Finally, PRM will mature into the resting microglia as shown by other studies.

Microglia and neurogenesis

At all prenatal stages examined, microglial cell precursors were located mainly in the Ammon's horn, with much lower density in the dentate gyrus. Provided that the microglial cell precursors, as discussed below, mainly locate themselves to differentiating rather than proliferative neuronal areas, this correlates well with the fact that Ammon's horn neurons are produced before birth, whereas approximately 85% of the rat granule cells form postnatally during the first 3 weeks (Bayer, 1980a,b). Similarly, microglial cell precursors in the dentate hilus were almost

exclusively found at temporal levels at E19, possibly as a reflection of the existing temporoseptal gradient in dentate development (Bayer, 1980a). These findings are also in agreement with observations from other brain regions with protracted development such as the cerebellum (see Jacobson, 1991), in which the number of ameboid microglial cells significantly increases after birth (Matsumoto and Ikuta, 1985; Ashwell, 1990; Milligan et al., 1991).

Within the Ammon's horn, it was also apparent that the microglial cell precursors at all septotemporal levels were preferentially located in the differentiating fields and not in the germinative zones. This was particularly evident at E17, when the microglial precursor cells were located in the differentiating matrix corresponding to the stratum radiatum and stratum oriens (Bayer, 1980a) but were virtually absent from the proliferative neuroepithelium. In contrast, radial astroglial processes extended through both the germinative and migratory zones and the developing cell and neuropil layers. Taken together, this is in accordance with the fact that the astroglial cells play a significant role in the migration and axonal guidance of the newly formed neurons (Rakic, 1971; Hatten, 1990). In contrast, the additional presence of microglial cell precursors only in the developing cell and neuropil layers suggests that these cells alone or in combination with the astroglial cells (Merrill, 1992; Müller et al., 1995) may play a role in regulating the survival and neurite outgrowth but, apparently, not the migration of the young neurons settled in these areas. The influence of microglial precursor cells on the neurons could be mediated through some of the neurotoxic agents, which microglia are known to be able to produce *in vitro* (Streit, 1993; Mallat and Chamak, 1994), or through release of substances with direct or indirect neurotrophic activities. The latter type of substances might include elastase (Nakajima et al., 1993), urokinase-type plasminogen activator (Nakajima et al., 1992b), and plasminogen (Nakajima et al., 1992a; Nagata et al., 1993) as well as more classical neurotrophic factors such as NGF (Mallat et al., 1989) and FGF (Shimojo et al., 1991) and cytokines like IL-1, IL-6, and TGF β (Giulian et al., 1986; Righi et al., 1989; Lehrmann et al., 1995), which all have been shown to influence the survival or neurotogenic potential of different types of neurons (Plata-Salamán, 1991; Merrill, 1992).

Microglia and radial glial cells

Throughout the embryonic stage, microglial cell precursors had a more restricted distribution than the VIM- and GFAP-immunoreactive cell processes of the hippocampal radial glial cells. In accordance with other studies in rodent brains (Woodhams et al., 1981; Rickman et al., 1987; Rami and Rabié, 1988), we found that the radial glia in general were arranged perpendicular to the principal cell layers and extended through the entire width of the subregions. From the analysis of the double-stained material combining tomato lectin (Acarin et al., 1994) and GFAP/VIM, we found that microglial cell precursors were closely related to the radial glial cell processes. This was particularly the case in the fimbria (unpublished results), supporting the view that substances released by the astroglial cell lineage not only may be implicated in neuronal migration and nerve pathway formation (Müller et al., 1995), but also in the migration and differentiation of microglial cells (Chamak and Mallat, 1991; Cuadros et al., 1994). In particular, the colony-stimulating factors

(CSF), some of which are synthesized and secreted by cultivated astrocytes (Théry et al., 1990) and are present as early as E13 in the brain (Mehler and Kessler, 1994), have attracted interest. Thus, there is convincing evidence that CSF may act as a chemoattractant for the initial migration of ameboid microglial cells during development (Mizuno et al., 1994). CSF may also act in concert with other glioblast-derived factors promoting proliferation and differentiation of microglia (Frei et al., 1986; Giulian and Ingeman, 1988; Sawada et al., 1990; Suzumura et al., 1990; Théry et al., 1990; Chamak and Mallat, 1991; Giulian et al., 1991; Mizuno et al., 1994). Our finding that the differentiation of the microglial precursor cells in all subregions of the developing hippocampus appears to be initiated before the differentiation of the astroglial cell population is based on the shift in the immunoreactivity of the astroglial cell population from being exclusively VIM-positive to displaying a dual immunoreactivity for VIM and GFAP (Dalmau et al., 1992). This may be taken as additional support for the existence of a fine regulation of the intercellular signaling between the differentiating microglial and astroglial cell populations.

Microglia and naturally occurring cell death

Cell death is a naturally occurring event during normal CNS histogenesis (for review see Oppenheim, 1991; Clarke, 1994). Microglial cells and macrophages have often been suggested as being actively involved in the elimination of exuberant neurons and glial cells and axonal projections (Hume et al., 1983; Innocenti et al., 1983a,b; Perry et al., 1985; Ashwell et al., 1989; Schnitzer, 1989; Ashwell, 1990; Ferrer et al., 1990; Caggiano and Brunjes, 1993). Macrophages phagocytosing pyknotic cells have been convincingly demonstrated in areas of the developing nervous system with naturally occurring cell death (Perry et al., 1985; Ashwell, 1990, 1991; Ferrer et al., 1990; Cuadros et al., 1993). It has been suggested (Hume et al., 1983; Perry et al., 1985) that such cell death attracts blood-borne mononuclear phagocytes into the nervous tissue. Other authors have argued that microglial cell precursors arrive before the period of cell death and that other types of stimuli are involved (Ashwell et al., 1989; Ashwell, 1991). By counterstaining the NDPase-stained sections with the Feulgen nuclear reaction, we found pyknotic nuclei in the cytoplasm of AM cells in the developing fornix at E17 and in the subicular complex at E21. Ashwell (1991) also observed an accumulation of microglial cells in the developing fornix from E15 to E19, during which period they may clear debris from fornical and callosal projections. Ashwell (1991) did not, however, observe pyknotic figures until E19, suggesting that cellular degeneration is an unlikely stimulus for the invasion of this region. In contrast, we observed pyknotic nuclear figures already engulfed by AM and E17, still leaving the possibility open that cell degeneration within the developing white matter might have acted as a chemotactic signal. Alternatively, the Feulgen-reactive AM might have phagocytosed the nuclear debris in neighboring regions and then migrated into the fornix.

The observation by Ferrer et al. (1992) of cellular degeneration at E21 and E22 in the border region between the retrosplenial cortex and the subicular complex corresponds with our observations of pyknotic Feulgen-stained structures within AM at E21 in the subicular complex, near the developing cortical white matter. The AM type 1 cells were mainly located next to the pia. As discussed

previously, these cells may have entered from the meninges. Removal of cellular debris in the embryonic hippocampus might accordingly be carried out by phagocytes recruited from the surrounding connective tissue. The fact that we could not identify pyknotic Feulgen-stained figures in other hippocampal subregions populated with microglial cell precursors does, of course, suggest that signals other than cellular degeneration induce microglial cell migration into these regions.

CONCLUSION

The morphologic differentiation and distribution of microglial cell precursors within the developing, prenatal hippocampus was followed from E14 to E21. The intraparenchymal occurrence and distribution of the microglial precursor cells was in accordance with both a monocytic and a pial origin of the cells, but proliferation of intraparenchymal microglial precursors might also add to the microglial cell population. The microglial differentiation in the developing hippocampus paralleled the differentiation of the hippocampal cell and neuropil layers. The location of the microglial precursor cells to the differentiating cell and neuropil layers, rather than the active germinative zones, suggests that the microglial cells may have an active role in the histogenesis of the hippocampus by influencing the survival and neurite outgrowth of young neurons and eliminating cell debris and nonfunctional projections.

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