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Development of Microglia in the Postnatal Rat Hippocampus

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ABSTRACT: During the prenatal development of the hippocampus, microglial cell precursors progressively occur in all subfields in accordance with known ontogenetic gradients of the region (Dalmau et al., J. Comp. Neurol. 1997a;377:70–84). The present study follows the regional distribution of these microglial cell precursors and their morphological differentiation in the rat hippocampus from birth to postnatal (P) day 18. The results demonstrate that the cellular differentiation and the subregional distribution of microglia follow the specific developmental gradients of the different parts of Ammon's horn and the dentate gyrus. Microglial cell distribution in the dentate gyrus is thus delayed compared with that in Ammon's horn. The appearance of microglia in the hippocampal subregions and differentiation of cell precursors into adult microglia occur earlier at temporal levels than at septal levels. Distribution of microglial cells follows an outside-to-inside pattern from the hippocampal fissure to the main cell layers in either Ammon's horn or the dentate gyrus. Meanwhile, the resident microglial cells located in the stratum oriens and dentate hilus at birth also increase in number and gradually disperse throughout the whole tissue of the two layers with age. In Ammon's horn, microglial differentiation occurs earlier in CA3 than in CA1. In the dentate gyrus, microglia appear earlier in relation to the external limb than to the internal limb, largely following a lateral-to-medial gradient. The differentiation and appearance of microglia in the various hippocampal and dentate subregions often correspond to the developmental stage of intrinsic and extrinsic afferent nerve fiber projections. Finally, in both Ammon's horn and the dentate gyrus, cells resembling reactive microglia are also observed and, in particular, in the perforant path projections from P9 to P18, suggesting their participation not only in phagocytosis of dead cells but also in axonal elimination and/or fiber reorganization. *Hippocampus 1998;8:458–474.*

KEY WORDS: nucleoside diphosphatase; neuroglia, fascia dentata; differentiaton; distribution

INTRODUCTION

Microglial cells of the adult vertebrates display a typical morphology with a highly territorial and regionally distinct distribution (Lawson et al., 1990; Castellano et al., 1991a; Davis et al., 1994; Streit, 1995; Vela et al., 1995). Most microglial cells are found in gray matter and have been estimated to comprise 5–20% of the central nervous system (CNS) cell population (Perry and Gordon, 1991; Peters et al., 1991). Microglia are mostly accepted to derive from mesodermal precursors such as infiltrating blood monocytes (Imamoto and Leblond, 1978; Ling, 1981; Perry et al., 1985; Chugani et al., 1991; Cuadros et al., 1994; Navascués et al., 1996; Dalmau et al., 1997a,b, 1998) or pial elements (Del Río Hortega, 1932; Cammermeyer, 1970; Boya et al., 1991; Cuadros et al., 1994; Navascués et al., 1996; Dalmau et al., 1997a, 1998). Although in vitro studies have reported that a number of compounds, such as extracellular matrix components (Chamak and Mallat, 1991), trophic factors (Giulian and Ingeman, 1988; Suzumura et al., 1991; Hagg et al., 1993), cytokines (Loughlin et al., 1992, 1993; Smith et al., 1993; Imamura et al., 1994; Panek and Benveniste, 1995), and neurotransmitters and glucocorticoids (Loughlin et al., 1993) can promote changes in microglial cell phenotype (see Thomas, 1992), the mechanisms regulating the differentiation of cell precursors into microglia in the developing brain are still not known.

Microglial development is not an isolated process and must take place with the microenvironmental changes associated with neurogenesis, gliogenesis, angiogenesis, and synaptogenesis, among others. Most of these pre- and postnatal developmental processes have been extensively studied in the hippocampus. In brief, all pyramidal cells of the hippocampus are formed before birth, whereas 80–90% of the dentate granule cells are formed postnatally (Altman and Das, 1965; Hine and Das, 1974; Schlessinger et al., 1975; Bayer, 1980a). Three

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well-defined gradients of neurogenesis have been described within the hippocampal region, namely deep to superficial, sandwich, and rhinal to dentate (Bayer, 1980a; Bayer and Altman, 1987). Within the dentate gyrus, there are also three morphogenetic gradients: from temporal to septal, from lateral to medial, and from outside to inside (see Zimmer, 1978; Bayer, 1980b; Gaarskjaer, 1986). During the postnatal period, the appearance of laminae corresponding to the terminal fields of the major afferent and intrinsic systems of the subiculum, dentate gyrus, and Ammon's horn have taken place by postnatal day (P) 9 (Zimmer and Haug, 1978). By P15, hippocampal histology is essentially in its mature form (see Hebel and Stromberg, 1986).

In a recent study of the prenatal rat hippocampus, we demonstrated that the microglial precursors gradually appear in all hippocampal subregions in accordance with the established ontogenetic gradients of the region (Dalmau et al., 1997a). The cells first appear in Ammon's horn and then in the dentate gyrus and invariably prefer differentiating rather than germinative zones. During the embryonic period, ameboid microglial cell precursors acquire a poorly ramified morphology, as so-called primitive ramified microglial cells, and maintain a close relationship to the vascular network. The analysis of the maturation and distribution of microglial cells in the developing postnatal hippocampus have not yet been defined.

The aim of the present study was focused on the differentiation and the temporospatial distribution of microglial cells in the early postnatal rat hippocampus by analyzing their possible correspondence to the histogenesis of the different subregions.

MATERIAL AND METHODS

Tissue Collection and Fixation

Fifty-six postnatal Wistar rats of both sexes and ranging in age from newborn (P0) to P18 were used. The rats were grouped according to age as follows: P0 (n = 10), P3 (n = 9), P6 (n = 9), P9 (n = 7), P12 (n = 7), P15 (n = 7), and P18 (n = 7). The rat pups were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and perfused for 10 min through the left ventricle with a solution of 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) with 5% sucrose. The brains were then removed and postfixed for an additional 2–3 h at 4°C in the same type of fixative, after which they were cut into parallel series of 50-μm-thick Vibratome sections. Sections were collected in cacodylate buffer (0.1 M cacodylate buffer at pH 7.4 with 7.5% sucrose) for immediate NDPase histochemical processing (see below).

Histochemical Demonstration of NDPase Activity

Labeling of microglial cells was carried out by histochemical demonstration of NDPase enzymatic activity as reported previously (Castellano et al., 1991b). Briefly, the Vibratome sections were incubated at 38°C for 25 min in a medium containing 25 mg sodium salt of inosine 5′-diphosphate (Sigma, St. Louis, MO; catalog no. I 4375) as the substrate, 7 ml of distilled water, 10 ml 0.2M Trizma maleate buffer (Sigma; T-3128, pH 7.4), 5 ml of 0.5% $MnCl_2$, and 3 ml 1% $(NO_3)_2Pb$. After incubation, the Vibratome sections were rinsed (3 × 10 min) in cacodylate buffer, treated in 2% ammonium sulfide for 2 min, rinsed in cacodylate buffer (2 × 10 min) and distilled water (2 × 1 min), and then immersed in 1% silver nitrate and rinsed again in distilled water (2 × 1 min). 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, 1996).

As a control for the histoenzymatic reaction, a few sections from each age group were incubated in a medium lacking the substrate inosine 5′-diphosphate.

RESULTS

The histochemical staining for NDPase activity visualized a population of positively stained cells in the hippocampus at all postnatal ages. These cells were identified as microglial cells on the basis of the morphological features (Murabe and Sano, 1982b; Castellano et al., 1991b). Additional staining of endothelial cells was observed but did not interfere with this identification. The terminology used in the present study for the different types of microglial cells is in accordance with that used in a previous study (Dalmau et al., 1997a) and the existing literature (Ling and Wong, 1993).

The main morphological features of the different types of NDPase-positive microglial cells are described, followed by a detailed description of the differentiation and spatiotemporal distribution of microglia.

Morphology of NDPase-Labeled Microglial Cells

Two main types of microglial cells were recognized in the postnatal hippocampus, the ameboid and the ramified microglial cells, and for each cell type different subtypes could be distinguished. Apart from these two cells, cells resembling the reactive type of microglia were also recognized. The most remarkable morphological features of microglial cell types are summarized in Figure 1.

Ameboid microglial cells (AM)

The ameboid cells in general displayed a remarkable diversity of shape and size but always with high NDPase activity. Compared with the ameboid cells in the prenatal hippocampus (Dalmau et

Type of cell	Shape	Cell processes	Diameter	NDPase activity	Time course of appearance	Cell morphology
Ameboid microglia type 2 [1]	Round	None, occasional filopodia	15-20 μm [2]	High	P0-P9, scarcely at P12	
Ameboid microglia type 3 [1]	Pleomorphic	Filopodia and/or Pseudopodia	15-50 μm [2]	Moderate	P0-P9, some at P15	
Primitive ramified microglia [1]	Oval to slightly elongated	Scantly developed processes showing a beaded shape	50-75/85 μm [3]	Low	P0-P12, some at P15 and rarely at P18	
Resting microglia	Oval to roundish	Fully developed processes	85-100 μm [3]	Low	Some at P12, P15-P18	
Reactive-like microglia	Large, plump, round to oval	Retracted, coarse processes	40/50-80 μm [3]	Very high	Mainly from P9 to P18	

[1] Terminology is based on Dalmau et al., 1997a.

[2] Diameter of cell body

[3] Diameter of territory occupied by individual microglia

FIGURE 1. Classification of the morphological types of NDPase-labeled microglial cells in the postnatal rat hippocampus.

al., 1997a), the AM cells observed in the postnatal hippocampus were type 2 (Fig. 2A,B) and type 3 (Fig. 2C,D) cells.

Ramified microglial cells (RM)

The ramified cells displayed a diversity of morphologies, with a variable number of cellular processes of different length and thickness. We distinguished two types, primitive ramified microglia (PRM; Fig. 3A–D) and resting microglia (ReM; Fig. 4A–D).

Microglial cells of the reactive type (reactive-like microglia)

These cells (Fig. 5A–D) were very similar to the various morphological types of activated microglial cells observed in the injured immature (Acarín et al., 1996) and adult (Finsen et al., 1993; Jørgensen et al., 1993; Sørensen et al., 1996) CNS. The reactive type were few in number, often in the pyramidal and granular cell layers, except for the perforant path projections and the CA3 stratum moleculare on P9, and mostly from P12, in which these cells were abundant.

Description of the Differentiation and Spatiotemporal Distribution of Microglia

Our results show that the maturation and spatial organization of microglia follow different developmental gradients of Ammon's horn and dentate gyrus development. The microglial cell differentiation and distribution occur earlier at temporal levels than at midposterior and septal levels. This temporoseptal gradient of microglial differentiation and distribution was particularly manifest in the dentate gyrus. Distribution of microglia first organized in relation to the external limb and then to the internal limb of the dentate gyrus, largely following a lateral-to-medial gradient. Moreover, microglial cells appeared within the subregions of the dentate gyrus, with a delay of some days as compared with the appearance of these cells in Ammon's horn. Microglial differentiation often occurred earlier in CA3 than in CA1. Distribution of microglial cells follows an outside-to-inside pattern from the hippocampal fissure to the main cell layers in either Ammon's horn or the dentate gyrus. Meanwhile, the resident microglial cells located in the stratum oriens and dentate hilus at birth gradually

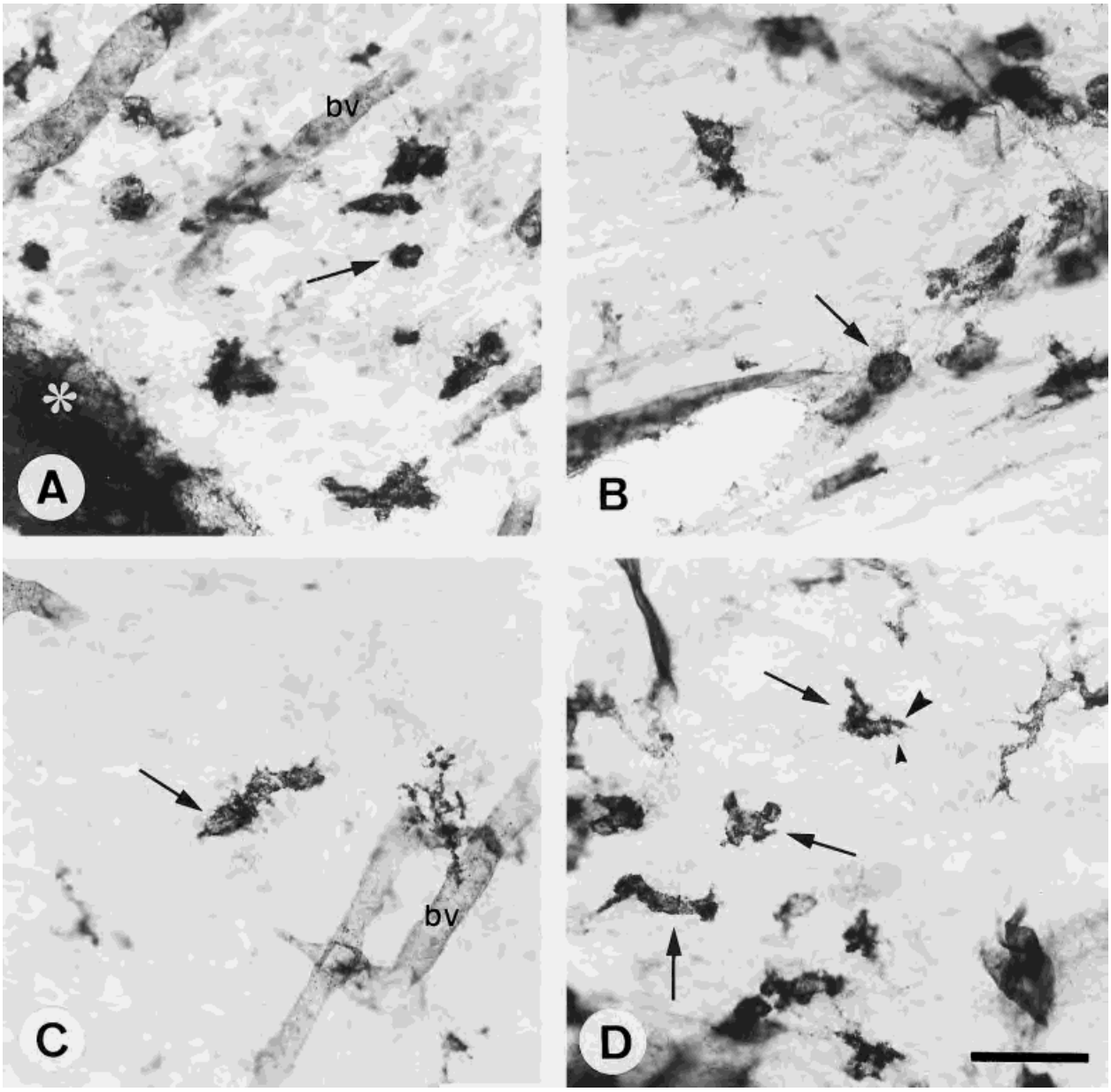

FIGURE 2. Ameboid microglial cells. A: Ameboid microglia type 2 cell (arrow) next to the tertiary dentate matrix close to the pial surface (white asterisk) at P0. The cell displays an intense NDPase staining and is generally devoid of well-defined cellular processes. B: Ameboid microglia type 2 (arrow) in the hippocampal commissure at P0. C: Ameboid microglia type 3 (arrow) in the striatum radiatum of CA3 at midposterior level of the hippocampus on P0. D: Ameboid microglia type 3 cells (arrows) with fine filopodia (small arrowhead) and pseudopodia (large arrowhead) arranged in parallel with axons in fimbria. bv, blood vessel. Scale bar = 40 μm.

increase in number and disperse throughout the whole tissue of the two layers with age. The arrangement of microglia in the different subregions of Ammon's horn and the dentate gyrus was often related to the development of intrinsic and extrinsic afferent nerve fiber projections.

To make data more accessible to the reader, the detailed description of the appearance and distribution of microglia is given for the midposterior levels and, when necessary, for the septal and temporal levels. We first report the ealiest ontogenetically formed part of the hippocampus (Ammon's horn and subicular area) and then a similar description on the latest formed part (dentate gyrus). The nomenclature and analysis of the developing postnatal hippocampus are based on those of Zimmer and Haug (1978), Bayer (1980b), and Altman and Bayer (1990a–c) and the atlas of Paxinos and Watson (1986) and Paxinos et al. (1991).

Development of Microglia in the Hippocampal Subregions CA1/CA3, Fimbria, and the Subicular Area

At birth, AM and PRM were already observed in fimbria and almost all subregions of Ammon's horn and subicular area except

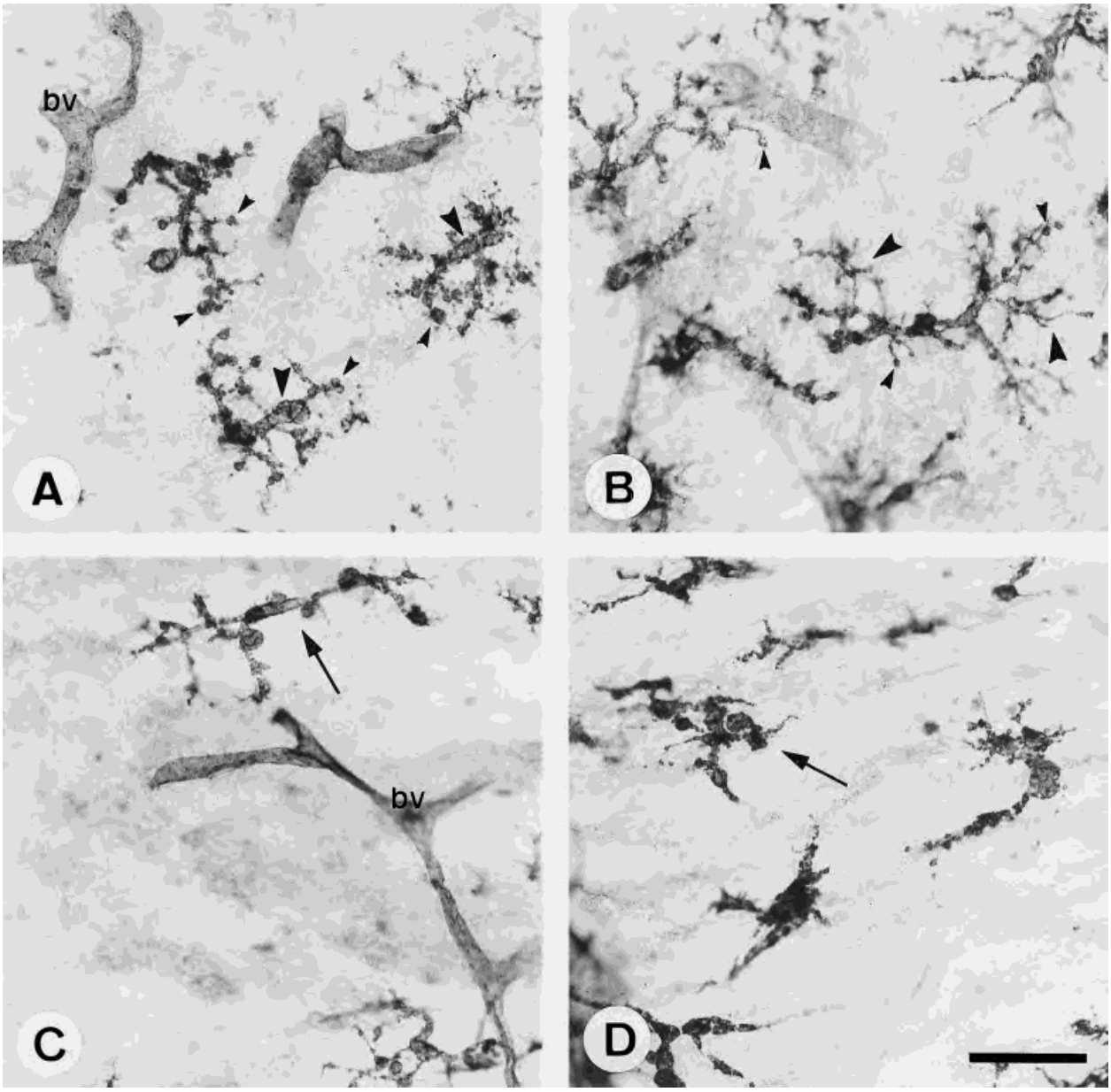

FIGURE 3. Primitive ramified microglia. A: Primitive ramified microglia in the stratum radiatum of CA3 at midposterior hippocampal level at P3. The cells have oval to elongated cell bodies (large arrowheads) and short processes with small swellings (small arrowheads). B: Primitive ramified microglial cells from the stratum radiatum of CA3 at midposterior hippocampal level at P9 with long cellular processes (large arrowheads) and small-caliber swellings (small arrowheads). C, D: Primitive ramified microglial cells (arrows) in the hippocampal commissure (C) at P0 and in the fimbria (D) at the midposterior level at P3. bv, blood vessel. Scale bar = 40 μm.

for the stratum pyramidale. In Ammon's horn, these cells were found predominantly in the CA1 stratum lacunosum-moleculare and the CA3 stratum moleculare. During the first week, AM transformed into PRM at the midposterior and temporal levels and occurred without remarkable changes in distribution. During this period, microglial cells showed a close relationship with the NDPase-labeled vascular network from birth to P6. From the second postnatal week, the population of microglial cells increased, gradually dispersed over the different hippocampal subregions, losing their close relationship with blood vessels, and transformed into ReM.

Temporal and regional distribution of microglia from P0 to P6

Between P0 and P3, microglial cells in Ammon's horn, mostly PRM, were often located next to the hippocampal fissure (Fig. 6A). In the stratum oriens of CA1 and CA3, microglia were present preferentially as AM cells, whereas the proportion of PRM cells increased from the temporal to the midposterior levels. Microglia were throughout this layer, with some accumulations in the deepest parts toward the alveus (Fig. 6A,B). These cells were especially numerous in CA1a. In this area, NDPase-reacted

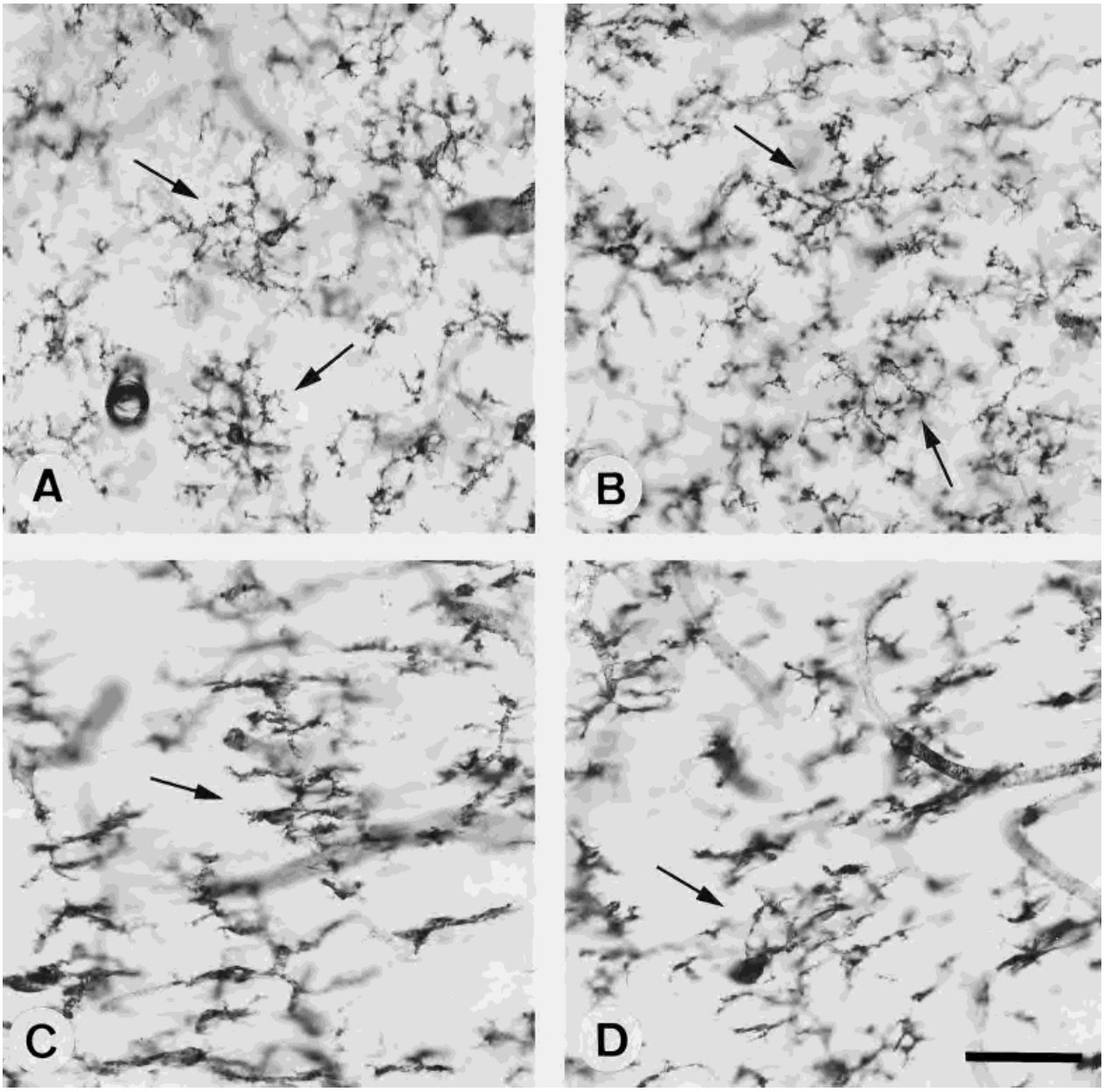

FIGURE 4. Resting microglia. A: Resting microglial cells (arrows) with extensively ramified processes and round to slightly elongated cell bodies in the dentate hilus at the midposterior level on day P15. B: Resting microglial cells (arrows) in the stratum radiatum of CA3 at the midposterior level on day P18 with cell processes displaying a crenulated form. C, D: Resting microglial cells (arrows) in the fimbrial white matter (C) are similar to those in the gray matter (D) at day P18. These cells in the white matter often display shorter cell processes than do their counterparts in gray matter. Scale bar = 40 μm.

sections counterstained with the Feulgen nuclear reaction often showed type 3 AM engulfing pyknotic cells. In the adjacent alveus, AM cells were observed in CA1 and CA3 at P0 and PRM cells from P3. Microglia were rare in the remaining Ammon's horn neuroepithelium.

At P6, the density of PRM cells in the stratum lacunosum-moleculare of CA1 and the stratum moleculare of CA3 were higher than that in the CA1–CA3 stratum radiatum (Fig. 7A). In contrast to the homogeneous distribution of microglia in the stratum radiatum of CA1, microglia in the CA3 stratum radiatum were absent in the adjacent area of stratum pyramidale, which corresponds to stratum lucidum (Fig. 7B). In the stratum oriens, most of the AM cells transformed into PRM cells, which were widely distributed throughout this layer (Fig. 7A,B).

In the fimbria from P0 to P6, most microglial cells were distributed along the course of axons. Dorsally, microglia were located next to the ventricular lumen and subjacent to the pia (Figs. 6D, 7D), and AM cells found from P0 to P3 and differentiated into PRM cells at P6. Ventrally, both AM and PRM cells were seen on P0 and from P3 PRM cells were only present.

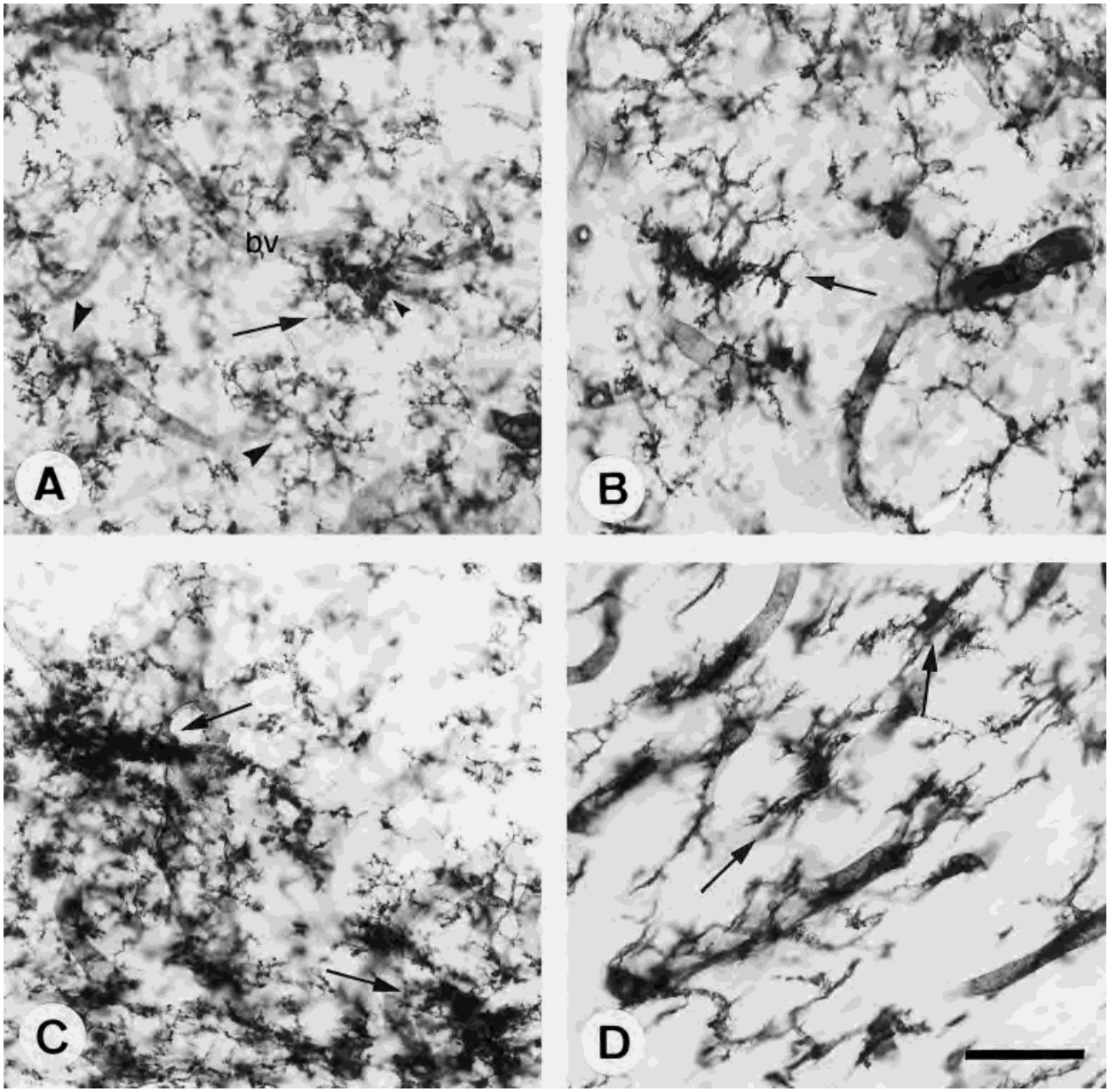

FIGURE 5. Microglia of the reactive cell type. A: Reactive-like microglia (arrow) in the stratum radiatum of CA3 at the temporal level at P15 are characterized by a larger cell body (small arrowhead), with shorter and stouter processes than cell processes of resting microglia (large arrowheads). B: Reactive-like microglia (arrow) in the dentate hilus at the midposterior level of the hippocampus on P15 with an intense NDPase staining. C: Reactive-like microglia (arrows) in the stratum lacunosum-moleculare of CA1 at P12. D: Reactive-like microglia (arrows) in the fimbria at day P18. bv, blood vessel. Scale bar = 40 μm.

In the subiculum at P0, microglia and AM and PRM cells were located in the stratum moleculare next to the pia and scarcely in the stratum pyramidale. From P3 to P6, there were more microglial cells than at P0 and AM cells had transformed into PRM cells.

The differentiation of AM into PRM at septal levels was delayed when compared with the differentiation at the midposterior and temporal levels. From P0 to P3, microglia in the ventral hippocampal commissure and fimbria often arranged in parallel to axons. These cells were AM type 3 and PRM, with a predominance of the latter. In the fimbria, AM type 2 cells were also found and were located mostly in the vicinities of the lateral ventricle. In this area, type 2 AM cells formed clusters in association with blood vessels. From birth to P3, microglial cells in CA3 were particularly prominent in the stratum oriens. Scattered cells were also found within the pyramidal cell layer. Between the pyramidal cell layer and the lateral ventricle, microglia were often located in relation to a prominent NDPase-labeled vascular network. Medially to CA3, microglia, mostly of the AM cell type, were especially abundant next to the pyramidal cell layer and in the dorsal hippocampal commissure. Significant numbers of AM cells were seen in the remaining Ammon's horn neuroepithelium and in the

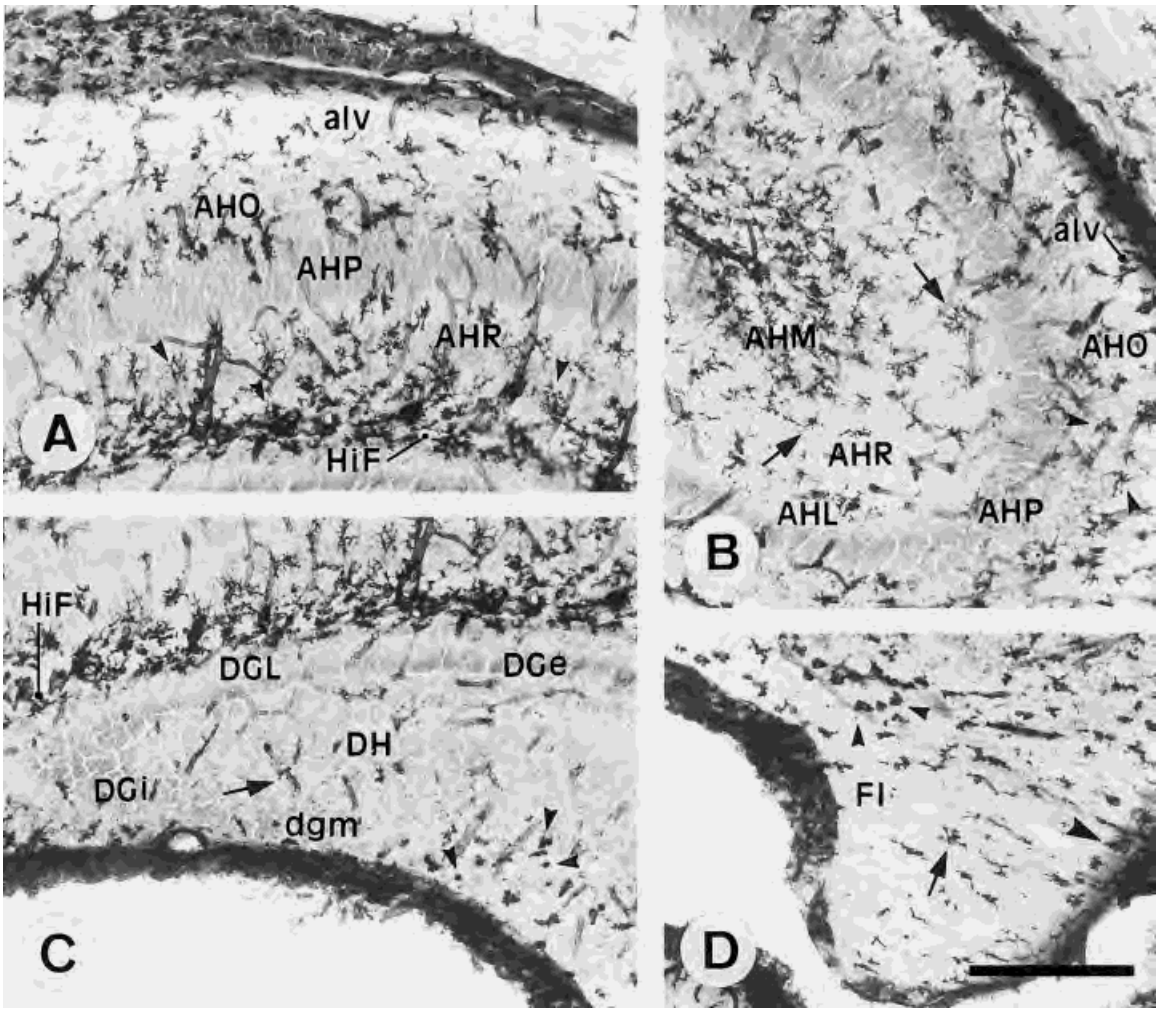

FIGURE 6. Distribution of microglial cells at the midposterior level of the hippocampus at day P0. A: Microglial cells (arrowheads) in CA1 are predominantly located near the obliterated hippocampal fissure (HiF). B: Microglial cells are absent in the stratum lucidum of CA3 (AHL) and sparse (arrow) in the stratum radiatum (AHR) of CA3 as compared with the stratum moleculare of CA3 (AHM). At this stage, microglial cells also populate the stratum oriens (AHO; arrowheads). C: In the dentate gyrus, there are fewer microglial cells than in Ammon's horn, and they are predominantly ameboid microglia (arrowheads) located next to the tertiary dentate matrix (dgm) below the pial surface. Only scattered primitive ramified microglia (arrow) are found in the dentate hilus (DH). D: In the fimbria (FI), ameboid microglia tend to be located near the lateral (large arrowhead) and the medial surface (small arrowheads), and primitive ramified microglia (arrow) are located in the middle. alv, alveus. Scale bar = 250 μm.

subependymal layer and next to the subfornical organ, in which they were often related to the vascular network. PRM cells were the predominant cell type at P6, being distributed within all laminae, with the exception of the pyramidal cell layer of CA3, where they were fewer.

Temporal and regional distribution of microglia from P9 to P18

At P9, PRM cells were the principal microglia cell type present in Ammon's horn. Microglia of the reactive type were also seen at this age in the stratum lacunosum-moleculare of CA1 and stratum moleculare of CA3 (Figs. 5C, 8A). In the stratum radiatum of both CA1 and CA3, microglia were homogeneously distributed throughout this layer, except for the stratum lucidum, in which there were only microglial cell processes (Fig. 8C).

From P12, PRM cells gradually transformed into ReM cells. In stratum lacunosum-moleculare of CA1 and stratum moleculare of CA3 at P12 and onward, the cell density of the microglial reactive type (Fig. 9A,B) increased. In the stratum radiatum of CA1, microglial cell processes radiated up among the individual neuronal cell bodies of the stratum pyramidale. In the CA3, a new layer of microglial cells became discernible between the apical margin of the stratum pyramidale and the stratum lucidum, which still remained almost free of microglial cell bodies until P18 (Fig. 9B). In the stratum oriens of CA1, microglial cells were arranged in a stratified manner, with more cells next to the alveus and to the stratum pyramidale. This stratified distribution was particularly prominent at P15 and P18 (Fig. 9A), when the microglial cells close to the stratum pyramidale constituted a cellular wall flanking the pyramidal cells. At P18, some microglial cell bodies were visualized within stratum pyramidale of CA3.

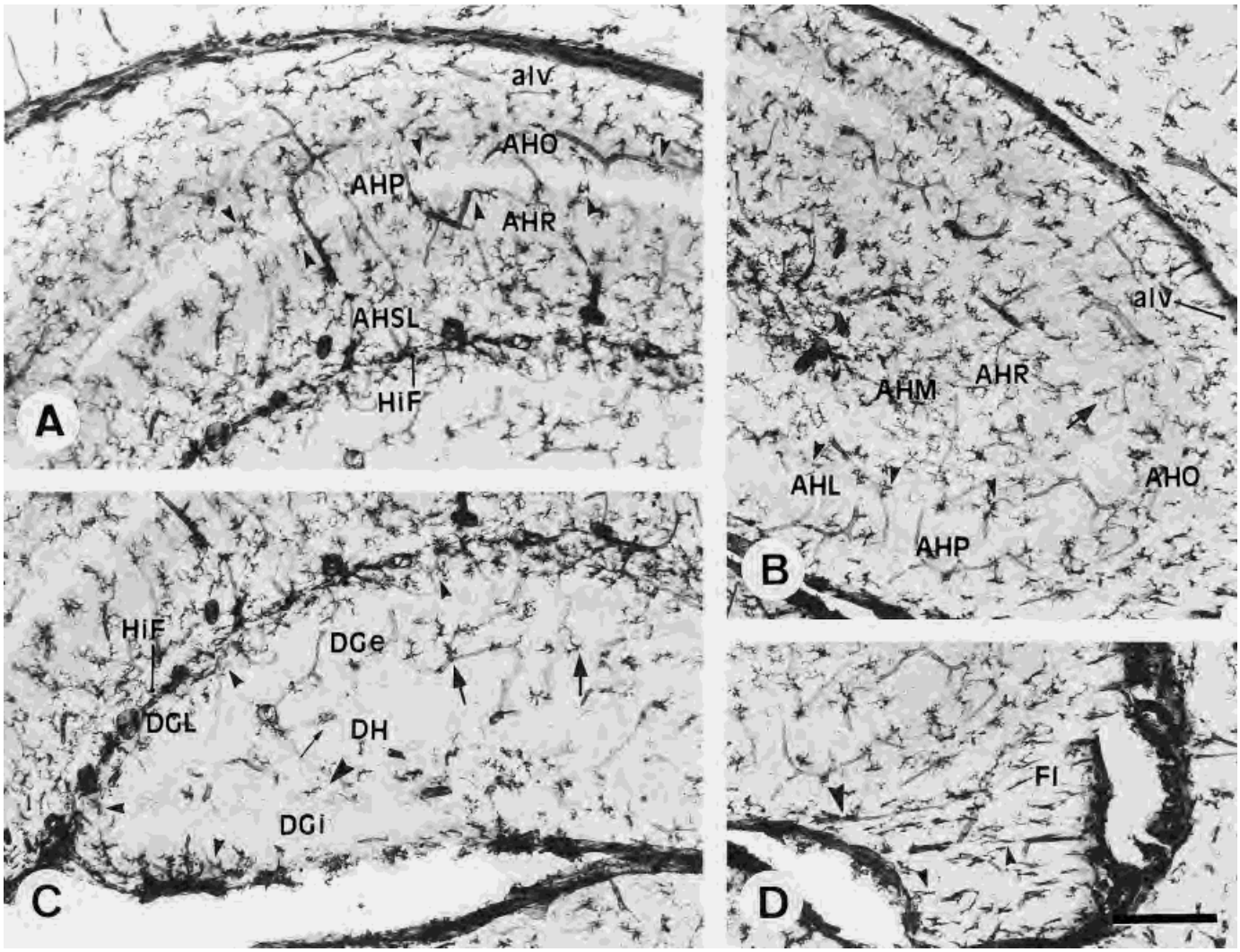

FIGURE 7. Microglial distribution at the midposterior hippocampal level at P6. A: Microglia (arrowheads) are located bordering the pyramidal cell layer in CA1 and in the neuropil layers of the stratum oriens (AHO), stratum radiatum (AHR), and stratum lacunosum-moleculare (AHSL). B: In CA3, primitive ramified microglia (arrows) are infrequent along the apical part of the stratum pyramidale, and farther apically microglial cells (arrowheads) are found along the transition between the stratum lucidum (AHL) and stratum radiatum. C: In the dentate gyrus, microglial cells (small arrowheads) start to appear in the stratum moleculare (DGL) at this age. In the dentate hilus, primitive ramified cells are present below the granule cells of the external limb (DGe; arrows), and in the middle zone (small arrow), but fewer cells are located beneath the internal limb (large arrowhead) of the granule cell layer (DGi). D: Almost all microglial cells in the fimbria (FI) are of the primitive ramified type (small arrowhead). Except for cells in the subpial area, which still harbors ameboid microglia (large arrowhead), the primitive ramified microglia are arranged parallel to the fibers. alv, alveus; DH, dentate hilus; HiF, hippocampal fissure. Scale bar = 250 μm.

In the fimbria (Figs. 8C, 9D), PRM cells were distributed along the fibers and from P15 gradually transformed into ReM cells. However, in contrast to Ammon's horn, some PRM cells were still encountered in the fimbria at P18.

In the subiculum, PRM cells were in both the stratum moleculare and stratum pyramidale from P9, progressively transforming into ReM cells with age.

At the septal level, PRM cells were the main cell type seen, although AM cells were still found until P15 at low density. From P12, the majority of these cells transformed gradually into ReM cells. In the ventral and dorsal hippocampal commissure and in the subjacent fimbria, microglia were arranged preferentially in parallel with fibers through all ages of this period. In the CA3 field, microglia were widely distributed throughout all layers, except for stratum pyramidale, in which they were fewer.

Development of Microglia in the Dentate Gyrus

At birth, the dentate gyrus contained much fewer microglial cells than did Ammon's horn. During the first postnatal week, AM cells progressively transformed into PRM cells. From the second postnatal week, the number of PRM cells increased, gradually arranged in laminae in almost all dentate subregions, and progressively transformed into ReM cells.

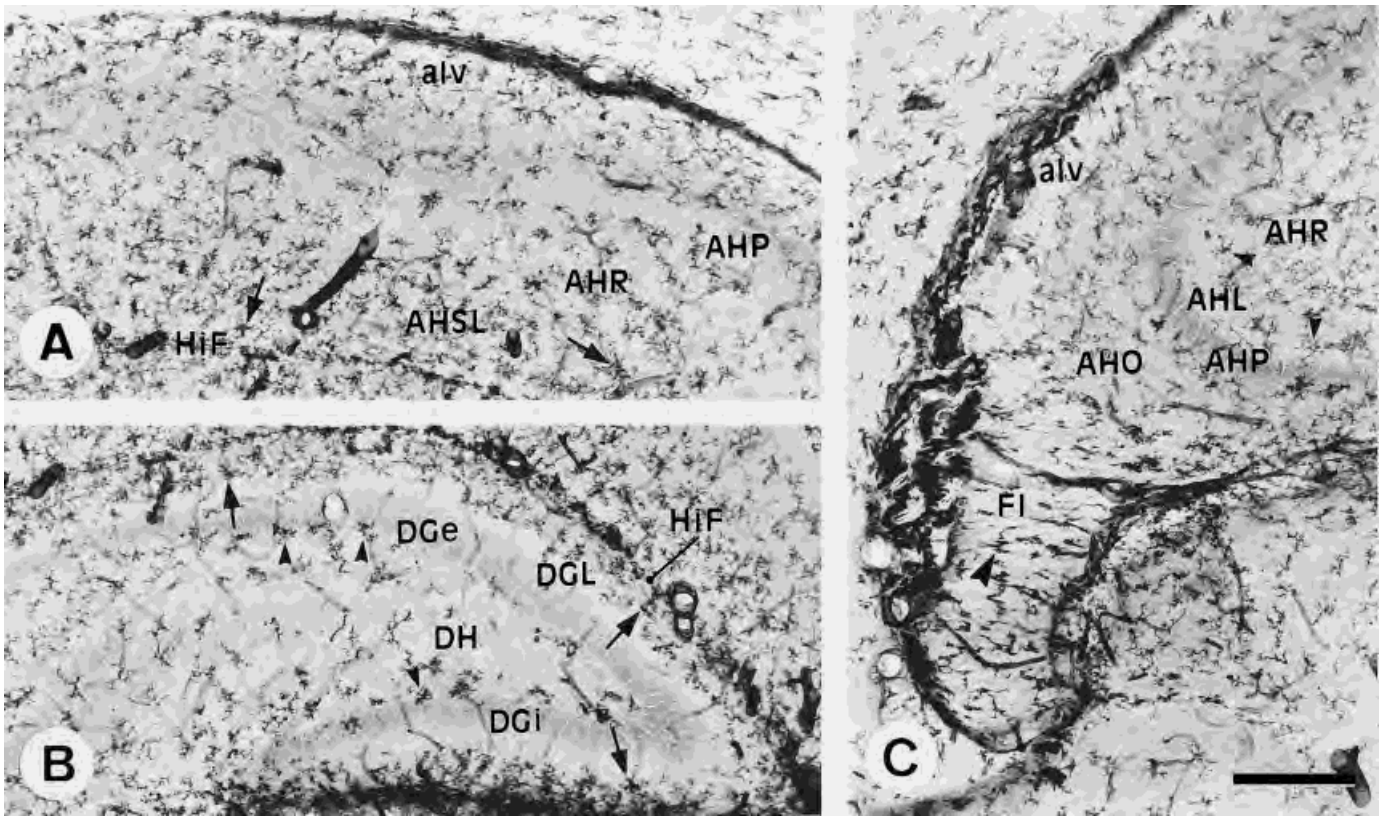

FIGURE 8. Microglial distribution at the midposterior levels of hippocampus on P9. A: At P9, microglial cells of the reactive type (arrows), or reactive-like microglia, appear in the stratum lacunosum-moleculare of CA1 (AHSL) and the stratum moleculare of CA3, and this, with the increase of microglial cell population, makes the border between these two layers and the stratum radiatum slightly easier to distinguish. B: In the dentate gyrus, resting microglia (arrowheads) are located in the subgranular zone of both the external (DGe) and internal (DGi) limbs of the stratum granulare. Reactive-like microglial cells (arrows) are also found in the stratum moleculare of the dentate gyrus (DGL). C: Microglial cells (small arrowheads) bordering the stratum lucidum (AHL). In the fimbria (FI), primitive ramified microglial cells (large arrowhead) are located in parallel between axons. alv, alveus; DH, dentate hilus; HiF, hippocampal fissure. Scale bar = 250 μm.

Temporal and regional distribution of microglia from P0 to P6

At P0, microglia were confined to the dentate hilus (Fig. 6C). The majority of cells were AM cells, although some PRM cells were also seen in this areas and next to the edge of the external limb of granule cell layer (Fig. 6C). At P3, the outer part of the stratum moleculare was invaded by cell processes from microglia distributed in the stratum lacunosum-moleculare of CA1. On P6, PRM cell bodies were encountered for the first time in the dentate molecular layer (Fig. 7C).

Temporal and regional distribution of microglia from P9 to P18

At P9, in the dentate hilus, microglia showed a remarkable association with blood vessels (Fig. 8B). Microglia were also seen lining up along the deep side of the dentate granule cell layer and preferentially in the external limb of the stratum granulare. In the stratum moleculare at this age, PRM cells and microglia of the reactive type were seen in the outer two-thirds but rarely seen in the inner one-third (Fig. 8B).

From P12, PRM cells gradually transformed into ReM cells, and the microglial population of the reactive cell type increased in the stratum moleculare. In the dentate hilus, microglial cells spread out in a pattern throughout almost all of this area. Between P15 and P18, microglial cells were found along the basal part of both the external and internal limbs of stratum granulare (Fig. 9C). Microglial cell processes were observed among the granular cell bodies of the external limb from P12 and the internal limb from P15. The inner one-third of stratum moleculare gradually became populated with more microglia, which were gathered mainly in the supragranular zone at P18 (Fig. 9C). On P18, in the stratum granulare, microglial cell bodies were rarely seen, although some cell processes from microglial cells located along the basal and apical parts of the stratum granulare were observed.

DISCUSSION

Microglia and Hippocampal Fiber Connections

The present study compared the structural differentiation and distribution of microglial cells in relation to morphogenetic changes in the postnatal rat hippocampus. Microglial cells were

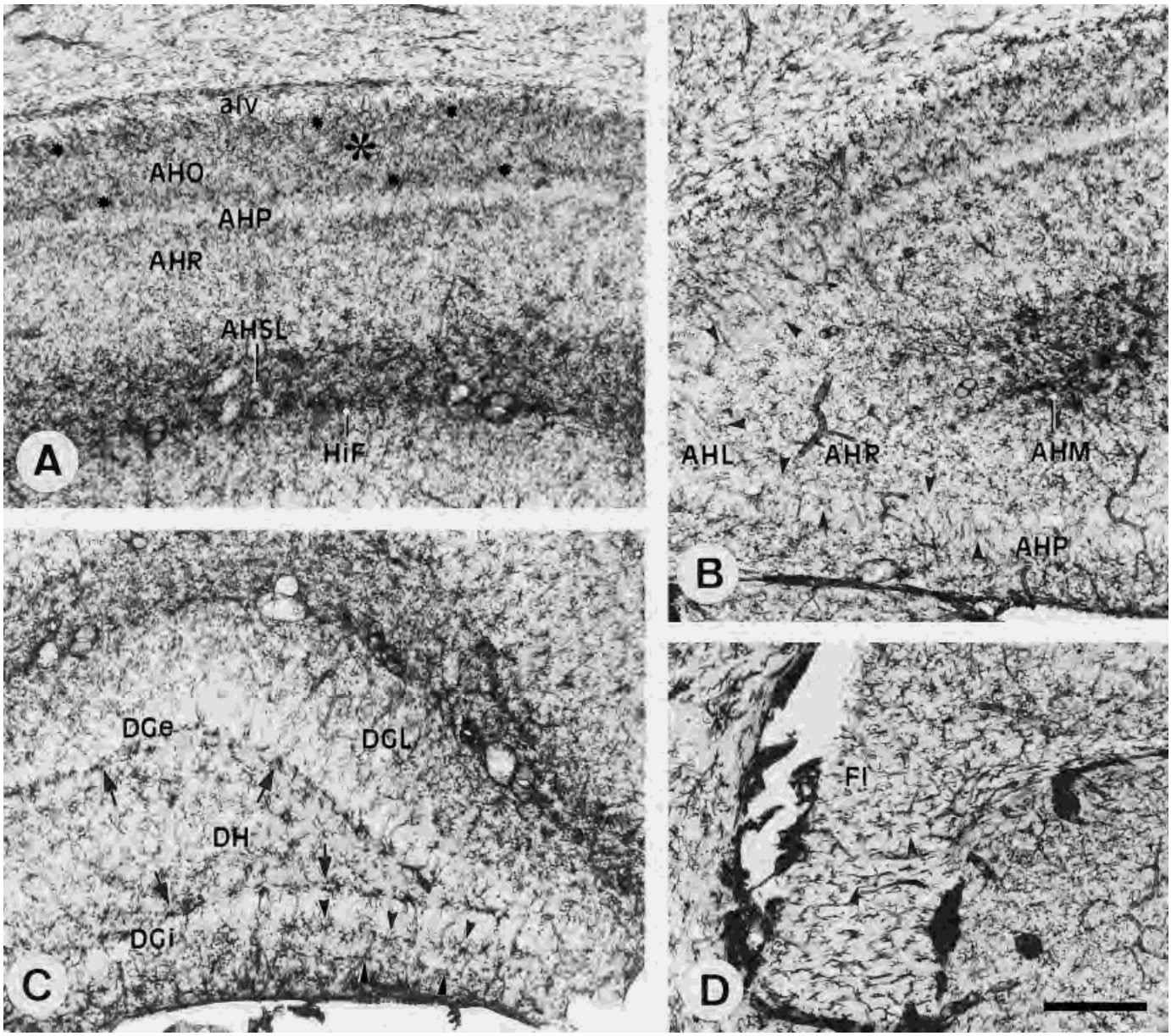

FIGURE 9. Microglial distribution at the midposterior level of the hippocampus on P18. A: Due to differences in the densities of cells and labeling of NDPase in the stratum radiatum (AHR) and the stratum lacunosum-moleculare of CA1 (AHSL) and stratum moleculare of CA3, the border between these layers is clearly discernible at this age. In the stratum oriens (AHO) of CA1, the microglial cells are arranged in a stratified manner, with more cells toward the alveus (alv) and the pyramidal cell layer (AHP; small asterisks) and fewer microglia in the middle part of the stratum oriens (large asterisk). B: In CA3, the border between the stratum moleculare (AHM) and stratum radiatum (AHR) is less easily distinguishable than in CA1. Due to the presence of more microglial cells in the stratum lucidum (AHL), there are now almost even densities of cells in this layer and the stratum radiatum. C: In the dentate gyrus, microglial cells (arrowheads) now also occupy the inner parts of the stratum moleculare (DGL), although the occurrence of cells still increases superficially toward the hippocampal fissure or the pial surface. In the dentate hilus (DH), resting microglia are almost uniformly distributed, with some lining up immediately subjacent to the granule cell layer (arrows). D: In the fimbria (FI), microglial cells (arrowheads), mainly the resting cell type, are distributed in parallel with axon bundles. Scale bar = 250 μm.

distributed in an almost adult-like organotypic fashion on P18, reflecting the histoarchitecture of the different hippocampal subregions. Two hallmarks during this period were (1) that the microglial cells appeared throughout the different hippocampal subregions from P9 and onward and (2) that the differentiation of microglial precursor cells into the resting type of microglial cells took place mainly after P15.

In the hippocampus, laminae corresponding to the terminal fields of the major extrinsic and intrinsic afferent fiber systems of the subiculum, dentate gyrus, and Ammon's horn have appeared by P9 of age (Zimmer and Haug, 1978), and the basic neuronal and connective organization of the rat hippocampus histology is essentially laid out by P15 in the rat (Hebel and Stromberg, 1986). Although inherent genetic factors may be considered in

relation to the gradient of differentiation of microglia (Wu et al., 1993), the structural differentiation and the distribution of the microglial cell population appeared to relate closely to the maturation of the hippocampal cell and neuropil layers. In the following sections, we correlate the structural differentiation of the microglial cells and their pattern of arrangement with the development of specific fiber systems in the hippocampus based on the use of Timm staining according to Zimmer and Haug (1978).

Commissural and ipsilateral association systems to the Ammon's horn

The hippocampal commissural and ipsilateral association systems (CA3–CA1 Schaffer collaterals and the longitudinal associational path in CA3) of the developing hippocampus display a temporal-to-septal gradient, coinciding with the earlier maturation of microglia at the temporal levels of CA1 and CA3, in particular in the stratum radiatum but also in the stratum oriens. Furthermore, the earlier morphological differentiation of microglia in CA3, and mostly in CA3c vs. CA3ab, seems to correspond with an earlier differentiation of axonal terminals in this specific area (Loy et al., 1977; Loy, 1980). In both CA1 and CA3, microglia spread gradually over time from stratum lacunosum-moleculare and stratum moleculare, next to the hippocampal fissure, toward the stratum radiatum, stratum pyramidale, and lastly the stratum oriens, which was also populated from its superficial layers subjacent to the alveus. These spatiotemporal gradients of microglial cells in the stratum oriens were observed later in the infrapyramidal area than in the superficial part of this layer. With Timm staining, the apical parts of the stratum radiatum of both CA1 and CA3 display a terminal-like staining earlier than do the more basal parts, which corresponds to the apical to basal sequence of the microglial distribution in the stratum radiatum. Moreover, although few microglia were seen in the infrapyramidal part of stratum oriens on P6, they were mostly observed from the second postnatal week. This observation agrees with the observation that the differentiation of this part of stratum oriens of CA1 and CA3 occurs relatively late, around days 5–7 (Zimmer and Haug, 1978).

Crossed and uncrossed temporoammonic tracts and the lateral perforant path projection in Ammons' horn

The Timm staining of the crossed and uncrossed temporoammonic tracts in the stratum lacunosum-moleculare of CA1 by days 1–5 is rather pale. At this time, microglial cells were especially observed in this area and in stratum moleculare of CA3. Around P9, the adult lamination of stratum lacunosum-moleculare of CA1 is first attained, coinciding with an increase of the microglial cell population on P9 but more noticeable from P12 onward and with the maturation of primitive ramified microglia into resting microglia. Therefore, microglial cells seemed to become distributed organotypically and then completed their differentiation into resting microglial cells from P9 in the stratum lacunosum-moleculare of CA1 and stratum moleculare of CA3 (and in the stratum radiatum) after the crossed and uncrossed temporoammonic tracts and the perforant path had attained a pattern similar to that of the adult brain.

The perforant path and the commissural and ipsilateral association systems to the dentate gyrus

The perforant path consists of two separate lateral and the medial perforant pathways. In the stratum moleculare of the dentate gyrus, the perforant path terminates in its outer two-thirds, demonstrating in Timm-stained material (Zimmer, 1974, 1978) a distinctive lateral perforant path terminal field that appears in the outer one-third of stratum moleculare of the dentate gyrus around P5, preceded by the presence of fibers at P3 (Fricke and Cowan, 1977; Loy et al., 1977). In addition, a distinctive medial perforant path zone first begins to appear within the medial third of the stratum moleculare of the dentate gyrus from P5. Interestingly, microglial cell bodies are not visualized in the outer one-third part of the stratum moleculare of the dentate gyrus until P6, although some microglial cell processes are already demonstrated on P3, thus coinciding with the appearance of the arrival of fibers from the lateral and the medial perforant paths.

Microglial cells in the inner part of the dentate molecular layer are not clearly demonstrated until P12. In this zone, fibers belonging to the associational system are evident at P3, whereas afferents belonging to the commissural system become present from P3 to P6 (Loy et al., 1977). Cholinergic fibers originating in the medial septal nucleus begin to arrive within the supragranular zone of the stratum moleculare at P10, along with afferents from other sources (Loy et al., 1977). Therefore, the delay of microglial cells in the inner one-third of the stratum moleculare of the dentate gyrus may be explained by the later arrival of fiber systems into this zone and to the ongoing remodeling and competition for synaptic sites by commissural and associational afferents (Fricke and Cowan, 1977), which is believed to result from the sequential development of the granule cells.

All in all, microglia seem to settle and differentiate first in the stratum moleculare of the dentate gyrus after the afferent fibers, as visualized by the Timm sulphide silver method, have arrived and the pattern of fibers have stabilized within their respective terminal fields.

Mossy fiber system

In the dentate hilus and the prospective mossy fiber zone of CA3, the presence of Timm-stained mossy fiber terminals is suggested at P1 and unequivocal by P3, not completed by P12, and shows a mature staining on day P18. In addition, the Timm staining develops later in the medial than in the lateral part of the dentate hilus. At P0, microglial cells of the ameboid and the primitive ramified types were rarely observed in the dentate hilus. At the same age, however, primitive ramified microglia were frequently found in the proximal part of the external limb of the stratum granulare, and ameboid microglia were found in the vicinity of the developing internal limb of the stratum granulare. By P15, resting microglia were present in the entire dentate hilus

subjacent to the granule cell layer, showing a gradient from the external limb to the internal limb. On P18, however, the density of microglial cells was similar in both limbs of the stratum granulare. In CA3, microglial cells were scarce in the suprapyramidal layer until P9, when microglial cell processes, but not cell bodies, were present in the mossy fiber zone. On P12, microglia lined up in a three-layered arrangement in association with the stratum lucidum. This arrangement was transient and had disappeared by P18, when microglia in this layer showed a pattern similar to that of the adult brain.

As demonstrated by these observations, the spread and differentiation of microglial cells from the lateral to the medial part of the dentate hilus may occur in parallel with the lateral-to-medial development of the Timm staining of mossy fiber terminals in the dentate hilus. In addition, the later appearance of microglia in the less-developed medial limb of the stratum granulare and the protracted maturation of the microglial arrangement in the stratum lucidum may coincide with the maturation of the mossy fiber system (Gaarskjaer, 1986).

Microglia and Synaptogenesis

In Ammon's horn, the most intense spine formation takes place in the second and third postnatal weeks (Zimmer, 1978; Zimmer and Haug, 1978). The generation of spines on the hippocampal pyramidal cells occurs on the apical dendrites before the basal dendrites. Similar to the rat dentate gyrus, where Golgi-impregnated spines can be recognized on the granule cell dendrites by P7, a substantial number of spines are present by P12 (Fricke and Cowan, 1977), and they continue to develop after P25 (Cotman et al., 1973). Interestingly, the initial phase of the morphological differentiation of microglial cells into the resting type began in the second postnatal week, when the most striking spine formation occurs. In addition, we observed that microglia preferentially distributed in an outside-to-inside gradient in both Ammon's horn and the dentate gyrus. Thus, microglial cells in Ammon's horn initially were found mostly along the stratum lacunosum-moleculare of CA1 and stratum moleculare of CA3 harboring the apical pyramidal cell dendrites.

With time, microglial cells populated the stratum radiatum of the CA1 and CA3, the deepest suprapyramidal part of the stratum radiatum in CA1, and somewhat later in CA3, where the stratum lucidum with the developing mossy fiber tract can be distinguished. In the same way, the cellular processes but not the cell bodies of the microglial cells were observed in relation to the distal part of the dendrites of the granular cells in the outer one-third of the stratum moleculare of the dentate gyrus on P3, although the first microglial cells did not appear in this area until P6. From the second postnatal week, microglial cells gradually were distributed through this area, following a largely outside-to-inside gradient. The finding that the morphological maturation of microglial cells takes place during the period of spine formation suggests that these cells may be actively involved in synaptogenesis, which in turn may also influence the arrangement of microglial cells in the different subregions. In line with our results, Murabe and Sano (1982a) reported that the microglial cell processes often encircle axodendritic synapses, and they postulated that microglia may play an integral role in the formation and maintenance of the structure of certain types of dendritic synapses. Others have made the same correlation for other brain areas and have proposed a similar hypothesis (Caggiano and Brunjes, 1993).

Microglia and Phagocytosis

Developmental phenomena such as naturally occurring cell death have been associated with the appearance of ameboid microglia in the CNS (Hume et al., 1983; Perry et al., 1985; Ashwell, 1990; Cuadros et al., 1993). Such cells have been demonstrated to be active phagocytes that remove dead cells and eliminate exhuberant axonal projections (Hume et al., 1983; Innocenti et al., 1983; Ferrer et al., 1990b; Moujahid et al., 1996). Naturally occurring cell death in the subicular complex and hippocampus in the rat has been reported during the first three postnatal weeks (Ferrer et al., 1990a). In particular, cell death appears not to be evenly distributed through layers of Ammon's horn, starts at birth in the stratum oriens, and extends within the stratum pyramidale from P3 to P5. Dead cells are not observed in the stratum radiatum and stratum lacunosum-moleculare (Ferrer et al., 1990a). In the dentate gyrus, cell death has been reported in the hilar region (Ferrer et al., 1990a) and in the outer molecular layer (Soriano et al., 1994; Super and Soriano, 1994; Del Río et al., 1995, 1997). Large numbers of dead cells in the subicular complex are found randomly distributed in the subcortical plate, are grouped into small clusters in the cortical plate at P0–P1, and are more uniformly found between P3 and P5 (Ferrer et al., 1990a). In the present study, we often demonstrated the presence of Feulgen-stained pyknotic nuclei engulfed by ameboid microglia type 3 in the stratum oriens and in the subicular area, where these cells mostly associated with the stratum pyramidale. However, not only ameboid microglia but also activated ramified microglia may act as a macrophage (Finsen et al., 1993, 1996; Jensen et al., 1994; Sørensen et al., 1996).

Eighty-five percent of the dentate granule cells are formed postnatally during the first three weeks (Altman and Das, 1965; Hine and Das, 1974; Schlessinger et al., 1975; Bayer, 1980a). This phenomenon implies that the dendritic tree of the newly formed granule cells situated in the basal part of the granule cell layer must extend up among the granule cells and up through the already formed stratum moleculare of the dentate gyrus to establish synapses with the perforant path axons. Moreover, the lateral perforant path is definitively present on P7, whereas the medial perforant path begins to be present on P5. All in all, the afferent fibers and/or the synapses within stratum moleculare of the dentate gyrus may be subject to a prolonged intrinsic reorganization. This fact presumably includes the component of a selective axonal elimination, similar to what happens in other areas (Jacobson, 1991b). Because there are no longer ameboid microglia present in this area, the cells that act as a phagocyte may be the primitive ramified microglia (Murabe and Sano, 1982b) or astrocytes. The presence of reactive-like microglia in the perforant path zones during development may be compared with the activation of resting microglia in the perforant path lesion model

(Fagan and Gage, 1994; Jensen et al., 1994, 1997), in which there is no entry of blood-borne macrophages. In this lesion, activated resident microglia are responsible for the elimination of the degenerating axons and, along with the astrocytes, are responsible for restructuring other, still intact afferent fiber systems in the area. This interpretation accords with the finding by Ferrer et al. (1990a) who found no cell death in the stratum lacunosum-moleculare and the stratum moleculare of the dentate gyrus at any age, and with other findings, i.e., that the presence of similar reactive-like microglia is promoted by axonal and terminal axonal degeneration rather than by actual cell death (Sørensen et al., 1996). However, the recent studies reporting a transiently appearing neurons in the outer molecular layer of the dentate gyrus (Soriano et al., 1994; Super and Soriano, 1994; Del Río et al., 1995, 1997) that undergo cell death during the postnatal time suggest that reactive-like microglia may also participate as active phagocytes removing dead cells. Thus, some primitive ramified microglia, possibly as a consequence of ongoing axonal degeneration and plasticity and/or cell death, may transform into microglial cells of the reactive-like type, whereas other primitive ramified microglia may differentiate directly into resting microglia.

Microglia and Blood Vessels

By taking advantage of the NPDase labeling of endothelial cells, we were able to observe an outstanding association between microglial cells and the vascular network in the developing hippocampus. We previously noticed a frequent perivascular location of microglial cells in the developing embryonal hippocampus (Dalmau et al., 1997a), which was still discernible on P6 and less evident on P9. Due in part to the general increase of the microglial cell population, microglia lose their special association with the vascular walls from P12. Nevertheless, the physical contact between some microglial cells and blood vessels was maintained through the processes of the perivascular microglial cells. Previous developmental studies have also reported the presence of intraparenchymal microglial cells next to blood vessels during fetal and early postnatal life (Murabe and Sano, 1982b; Ashwell, 1991). These cells may be reminiscent of the perivascular microglial cells observed in the adult CNS (Graeber et al., 1989; Graeber and Streit, 1990) and which incorporate their cellular processes into the glia limitans of 4–13% of cerebral blood vessels (Lassmann et al., 1991). In the rat brain, the period of maximal capillary proliferation occurs between P6 and P8–P9 (Robertson et al., 1985) and continues up to P14, coinciding with the period of dendritic growth and glial cell proliferation (see Jacobson, 1991a), and is the period during which we found a remarkable increase in the microglial cell density. In accord with this, Wu et al. (1993) reported a similar relationship in the cerebral cortex.

Microglia and brain macrophages produce angiogenic factors (Giulian et al., 1988), vasoregulatory factors such as thromboxane (Giulian and Corpuz, 1993), and nitric oxide (for review, see Murphy et al., 1993). Moreover, they release cytokines such as tumor necrosis factors (Frei et al., 1987) that influence the blood–brain barrier permeability (Megyeri et al., 1992) and that with interleukin-1 (IL-1; Giulian et al., 1986) modulate the expression of endothelial surface molecules (see Rössler et al., 1992; Fabry et al., 1994). Taken together, these observations indicate that microglial cells in the developing brain may endow the physiology of blood vessels. However, the vascular network in the CNS may also influence microglial cell proliferation and differentiation. In this sense, brain microvessel endothelial cells produce cytokines such as IL-1 and IL-6 (Fabry et al., 1993) and granulocyte-macrophage colony-stimulating factor (Hart et al., 1992), which all are important growth factors for microglial cells (Giulian and Ingeman, 1988; Benveniste, 1995).

Microglial–Astroglial Cell Interaction

During prenatal life, the differentiation of microglial cell precursors into more mature forms occurs earlier than for astroglial cells (Dalmau et al., 1997a). Mature astrocytes begin to appear throughout the hippocampal formation at the end of the first postnatal week (Rickmann et al., 1987; Rami and Rabié, 1988; Dalmau, unpublished observations), extending into the period (P9–P18) when primitive ramified microglial cells transform into resting microglia (present study). During astroglial differentiation, the expression of the intermediate filament protein vimentin changes to expression of glial fibrillary acidic protein (GFAP; Bignami and Dahl, 1974; Dahl et al., 1981), with a caudal-to-rostral gradient of GFAP expression (Landry et al., 1990). Corresponding to the developmental gradient among astrocytes, we have observed that microglial cell differentiation occurs earlier at temporal levels than at midposterior and septal levels. Although the differentiation of microglia begins earlier than than that of astrocytes, the astroglial maturation may catch up with microglial development during the postnatal period. The differentiation of microglia and astroglia may be closely related and consistent with overall brain maturation, either by the release of substances (Benveniste, 1993) or cell-to-cell contact (Dalmau et al., 1996; Tanaka and Maeda, 1996).

CONCLUSION

The present study demonstrates that the differentiation of microglial cell precursors, which first appeared in the hippocampal primordium on embryonic day 14 (Dalmau et al., 1997a), follows established gradients and time courses for the development of the rat hippocampus and dentate gyrus during the first three postnatal weeks. The arrangement of microglial precursor cells in the hippocampal subregions and layers thus relates closely to the neuronal differentiation and the development of intrinsic and extrinsic afferent nerve fiber projections during the postnatal period. This special pattern of appearance and differentiation of microglial cell precursors in the developing hippocampus may be interpreted in terms of either interaction with the microenvironment or acquisition of a shape and spatial localization that will enable these cells to exert their normal functions in the adult CNS. The data suggest that primitive ramified microglia, together

with ameboid microglia, play a role in the elimination of cellular debris, synaptogenesis, and angionesis. Observations reported in the present study may improve our understanding of the reaction pattern of microglial cells in experimental injury of the fetal and perinatal CNS.

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