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Expression of Purine Metabolism-Related Enzymes by Microglial Cells in the Developing Rat Brain

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ABSTRACT

The nucleoside triphosphatase (NTPase), nucleoside diphosphatase (NDPase), 5′-nucleotidase (5′-Nase), and purine nucleoside phosphorylase (PNPase) activity has been examined in the cerebral cortex, subcortical white matter, and hippocampus from embryonic day (E)16 to postnatal day (P)18. Microglia display all four purine-related enzymatic activities, but the expression of these enzymatic activities differed depending on the distinct microglial typologies observed during brain development. We have identified three main morphologic typologies during the process of microglial differentiation: ameboid microglia (parenchymatic precursors), primitive ramified microglia (intermediate forms), and resting microglia (differentiated cells). Ameboid microglia, which were encountered from E16 to P12, displayed the four enzymatic activities. However, some ameboid microglial cells lacked 5′-Nase activity in gray matter, and some were PNPase-negative in both gray and white matter. Primitive ramified microglia were already observed in the embryonic period but mostly distributed during the first 2 postnatal weeks. These cells expressed NTPase, NDPase, 5′-Nase, and PNPase. Similar to ameboid microglia, we found primitive ramified microglia lacking the 5′-Nase and PNPase activities. Resting microglia, which were mostly distinguishable from the third postnatal week, expressed NTPase and NDPase, but they lacked or displayed very low levels of 5′-Nase activity, and only a subpopulation of resting microglia was PNPase-positive. Apart from cells of the microglial lineage, GFAP-positive astrocytes and radial glia cells were also labeled by the PNPase histochemistry. As shown by our results, the differentiation process from cell precursors into mature microglia is accompanied by changes in the expression of purine-related enzymes. We suggest that the enzymatic profile and levels of the different purine-related enzymes may depend not only on the differentiation stage but also on the nature of the cells. The use of purine-related histoenzymatic techniques as a microglial markers and the possible involvement of microglia in the control of extracellular purine levels during development are also discussed. J. Comp. Neurol. 398:333–346, 1998.

Indexing terms: nucleoside triphosphatase; nucleoside diphosphatase; 5′-nucleotidase; purine nucleoside phosphorylase; neuroglia

Extracellular purine nucleosides and nucleotides present in adult tissues are implicated in a variety of biological mechanisms such as the regulation of blood flow, cell growth, and immune reactions (Burnstock, 1990). In the nervous tissue, extracellular purines perform additional roles in neuromodulation and neurotransmission by acting as cotransmitters or as genuine neurotransmitters (Burnstock, 1993), and during central nervous system (CNS) development, they carry out trophic actions and participate in the regulation of different processes, including neural plasticity and the proliferation of glial and endothelial cells (Neary et al., 1996).

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The presence of a "cascade" of enzymes engaged in the extracellular hydrolysis of purine nucleotides is fundamental in the control of extracellular purine levels. Purine nucleotides are released in the CNS from a variety of cells and are rapidly hydrolyzed into nucleosides, which are taken up from the extracellular space by neurons and glial cells (Matz and Hertz, 1989). The enzymes involved in the sequential dephosphorylation of purine nucleotides into nucleosides are nucleoside triphosphatase (NTPase), nucleoside diphosphatase (NDPase), and nucleoside monophosphatase, frequently known as 5′-nucleotidase (5′-Nase). All three are enzymes whose enzymatic activities have been located on the plasma membrane of microglial cells (Ibrahim et al., 1974; Murabe and Sano, 1982; Vordrodt and Wisniewski, 1982; Kreutzberg et al., 1986; Kaur et al., 1987), thus suggesting the direct participation of microglial cells in the control of extracellular purines (Castellano et al., 1990, 1991a; Vela et al., 1995a).

NTPase activity has been detected in resting and activated microglia in the adult brain (Sjöstrand, 1966; Ibrahim et al., 1974) and in the ameboid type during the postnatal period of the developing brain (Ling, 1977). The expression of NDPase has been demonstrated by microglial cells, not only in vivo (Murabe and Sano, 1982; Vordrodt and Wisniewski, 1982; Sanyal and De Ruiter, 1985; Castellano et al., 1991a,b) but also in vitro (Ditrich, 1986; Castellano et al., 1991a; Norenberg et al., 1994; Dalmau et al., 1996), and it is known that microglial cells display NDPase activity during development (Fujimoto et al., 1987, 1989; Schnitzer, 1989; Dalmau et al., 1997a) and in the adult CNS, under both normal (Schnitzer, 1989; Castellano et al., 1991b; Vela et al., 1995a) and pathologic (Schnitzer and Sherer, 1990; Wisniewski et al., 1990; Finsen et al., 1993; Jørgensen et al., 1993; Jensen et al., 1994; López-García et al., 1994; Sørensen et al., 1996) conditions. The enzyme 5′-Nase has been found in cells from the microglial lineage in the early postnatal brain (Kaur et al., 1984, 1987) as well as in glial cells in the mature nervous tissue (Kreutzberg and Barron, 1978; Kreutzberg et al., 1978). However, the analyses of these purine-related ectoenzymes in relation to microglial cells during the prenatal period have been fewer (see Dalmau et al., 1997a).

Apart from NTPase, NDPase, and 5′-Nase, other enzymes such as the purine nucleoside phosphorylase (PNPase) play a key role in the purine metabolism. PNPase is an enzyme of the "inosinate cycle" that is crucial to the recycling of nucleosides (Simmonds, 1991). In the adult brain, PNPase has been demonstrated intracellularly in astrocytes and microglia (Castellano et al., 1990), and in vitro studies have demonstrated developmental changes of PNPase expression in astrocytes (Zoref-Shani, 1995), but there are no available in vivo studies focused on PNPase in the developing CNS.

The aim of this study was to analyze the distribution and time course of the appearance of the following purine-related enzymatic activities during the development of the rat brain: NTPase, NDPase, 5′-Nase, and PNPase. Special attention was paid to the activity of these enzymes in relation to cells from the microglial lineage. The following brain areas were examined: the cerebral cortex, subcortical white matter (the corpus callosum and the internal and external capsules), and the hippocampus, from embryonic day 16 to postnatal day 18.

MATERIALS AND METHODS

Tissue collection and fixation

A total of 40 Wistar rats of both sexes, ages ranging from embryonic day (E)16 to postnatal day (P)18, were used in this study. Animals were grouped as follows: E16, E18, E21, P0, P3, P6, P9, P12, P15, and P18.

The embryos were delivered by cesarean operation with the mothers in deep sodium pentobarbital anesthesia (50 mg/kg body weight). The fetal brains were carefully removed and fixed by immersion for 5–6 hours at 4°C in a 4% paraformaldehyde solution in 0.1 M cacodylate buffer (pH 7.4) containing 5% sucrose. The rat pups were anesthetized with sodium pentobarbital (50 mg/kg body weight) and killed by intracardiac perfusion with a 4% paraformaldehyde solution in 0.1 M cacodylate buffer (pH 7.4) containing 5% sucrose. The brains were then carefully removed and post-fixed for 2–3 hours at 4°C in the same fixative solution. After fixation, both fetal and postnatal brains were washed in cacodylate buffer (0.1 M cacodylate buffer, pH 7.4, with 7.5% sucrose) and coronally sliced with the aid of a Vibratome to obtain four parallel series of 50-μm-thick sections. The sections were collected in cacodylate buffer (0.1 M cacodylate buffer at pH 7.4 with 7.5% sucrose) and immediately processed for NTPase, NDPase, 5′-Nase, and PNPase histochemistry (see below).

All efforts were made to minimize animal suffering in all procedures. Protocols were approved by the appropriate animal care committee of the Autonomous University of Barcelona.

Histochemical demonstration of the NTPase, NDPase, 5′-Nase, and PNPase activities

NTPase enzymatic activity was demonstrated in accordance with the protocol reported by Wachstein and Meisel (1957). Incubation was carried out at 38°C for 25 minutes in a medium containing 12.5 mg of inosine 5′-triphosphate (Sigma; I-0885) diluted in 10 ml of distilled water, 1 ml of distilled water, 10 ml of 0.2 M Trizma maleate buffer, pH 7.4, 2.5 ml of 0.1 M $MgSO_4$ and 1.5 ml of 2% $(NO_3)_2Pb$.

The demonstration of NDPase enzymatic activity was performed as earlier reported (Castellano et al., 1991a). Sections were incubated at 38°C for 25 minutes in a medium (Novikoff and Goldfisher, 1961) containing 25 mg inosine 5′-diphosphate (Sigma; I-4375) diluted in 7 ml of distilled water, 10 ml of 0.2 M Trizma maleate buffer (Sigma; T-3128), pH 7.4, 5 ml of 0.5% $MnCl_2$, and 3 ml 1% $(NO_3)_2Pb$.

The 5′-Nase activity was demonstrated by incubating sections at 38°C for 50 minutes in the same medium described above for the NDPase histochemistry, but by using adenosine 5′-monophosphate (Sigma; A-1877) as substrate.

To demonstrate PNPase enzymatic activity (Castellano et al., 1990), the Vibratome sections were incubated at 38°C for 30 minutes in the following medium: 9.2 mg of ribose-1-phosphate (Sigma; H-R-9381), 13.6 mg of hypoxanthine (Sigma; H-9377), 6 ml of distilled water, 3 ml of 0.2 M Trizma maleate buffer (Sigma; T-3128, pH 7.4), and 1 ml of 1% $(NO_3)_2Pb$.

As a control for the enzyme histochemical NTPase, NDPase, and 5′-Nase reactions, some sections from each age were incubated in a medium lacking the substrates inosine 5′-triphosphate, inosine 5′-diphosphate, and adenosine 5′-diphosphate, respectively. As a control for the

PNPase staining, some sections of each age were incubated in a medium without hypoxanthine, ribose-1-phosphate, or both.

After incubations, sections were rinsed in cacodylate buffer, treated with 2% $(NH_4)_2S$ for 2 minutes, washed in distilled water (2 × 2 minutes), treated with 1% $AgNO_3$ for 1 minute, and rinsed again in distilled water (2 × 2 minutes) to visualize the reaction product. Finally, sections were mounted on gelatin-coated slides, dehydrated in alcohol, cleared in xylene, and cover-slipped with DPX synthetic resin.

Double-labeling technique. To test the cellular specificity of NTPase, NDPase, 5′-Nase, and PNPase stainings, some histochemically reacted sections from each age were additionally processed for double-labeling techniques according to the protocol described by Castellano et al. (1991a). The simultaneous demonstration of enzymatic activities and astrocytes or microglia was achieved by the sequential combination of enzyme histochemistries and selective markers for glial cells. Astroglial cells were demonstrated by immunocytochemical detection of glial fibrillary acidic protein (GFAP; Eng et al., 1971; Bignami et al., 1972), and microglial cells were demonstrated by tomato lectin histochemistry (Acarín et al., 1994).

The histochemical reaction for NTPase, NDPase, 5′-Nase, or PNPase was first performed as detailed previously but without the final ammonium sulfide/silver nitrate treatment. Next, sections were processed for GFAP immunocytochemistry or tomato lectin histochemistry. The GFAP immunohistochemical staining was performed by the avidin-biotin technique by using rabbit anti-GFAP antibody (Dako) as primary antibody (dilution 1:1800), anti-rabbit Ig-biotinylated (Amersham) as second antibody (dilution 1:300), and avidin labeled peroxidase (Sigma) as the third reagent (dilution 1:70). The tomato lectin histochemistry was carried out as detailed by Acarín et al. (1994), by using biotinylated tomato lectin (*Lycopersicon esculentum*; Sigma) diluted to 10 μg/ml and avidin-peroxidase in a 1:70 dilution.

The GFAP and tomato lectin reaction products were visualized by immersion of sections in 1-naphthol solution with 0.01% H_2O_2 for 15 minutes and then in 0.05% Azur A in 0.05 M TBS (pH 8.0) for 30 minutes. The naphthol solution was freshly prepared by dissolving 50 mg of 1-naphthol (Sigma) in 0.5 ml of ethanol and adding this, together with 10 ml 1% $(NH_4)_3CO_3$, to 89.5 ml of TBS. The solution was filtered before addition of the H_2O_2. Afterward, the NTPase, NDPase, 5′-Nase, and PNPase histochemistries were visualized by treatment of sections with 2% $(NH_4)_2S$ and 1% $AgNO_3$ as described before. Finally, sections were mounted on gelatin-coated slides and cover-slipped with Glycergel (Dako).

RESULTS

Microscopic examination of the NTPase, NDPase, 5′-Nase, and PNPase stainings in the developing rat brain revealed the presence of a heterogeneous population of glial cells belonging to the microglial lineage (Fig. 1). No reaction product was observed in control sections incubated without substrates. Positive cells for the different enzymatic markers were identified as microglial cells on the basis of their morphologic features and distribution pattern (Murabe and Sano, 1982; Perry et al., 1985; Ashwell, 1991; Dalmau et al., 1997a) during development (Figs. 1, 2). The microglial cell nature was further confirmed through double-labeling by combining these enzymatic techniques with tomato lectin histochemistry, a selective microglial marker (Acarín et al., 1994). By using light microscopy, the NTPase, NDPase, and 5′-Nase activities were located on the microglial plasma membrane, whereas the PNPase activity was observed intracellularly in both the nucleus and cytoplasm, although the nuclear location was predominant (Fig. 2I). As demonstrated through double-labeling techniques, NTPase and NDPase labeled all cells from the microglial lineage, whereas the 5′-Nase and PNPase labeling was restricted to some fractions of the microglial population observed during development (Figs. 1A,D, 2I). Apart from cells of the microglial lineage, the enzymatic techniques stained blood vessels. In this sense, the NTPase and NDPase histochemistries labeled more blood vessels than the 5′-Nase and PNPase histochemistries (Fig. 1). NTPase also stained some nerve fibers (Fig. 1C) and PNPase stained astrocytes and radial glia, as demonstrated by the double-labeling technique combining PNPase and GFAP immunostaining (Fig. 2J). In no case did we find GFAP-positive astroglial cells lacking PNPase staining.

Microglial cells, visualized with the different histoenzymatic techniques, were present at all ages considered in this study (from E16 to P18) and were distributed through the different brain areas selected for this study (cerebral cortex, subcortical white matter, and hippocampus). However, microglial cells showed different morphologic features with function of the age and the brain area examined. Their morphologies ranged from the roundish shape of the primitive microglial precursors to the typical ramified shape exhibited by adult microglial cells. According to the literature (Perry and Gordon, 1991; Thomas, 1992; Ling and Wong, 1993; Dalmau et al., 1997a,b), we have identified three main morphologic typologies during the process of differentiation of microglial cells: ameboid microglia (parenchymatic precursors), primitive ramified microglia (intermediate forms), and resting microglia (differentiated cells). Each microglial typology showed a differential time course of appearance and a specific pattern of distribution.

The expression of the different enzymatic activities differed depending on the distinct microglial typologies observed during brain development. Therefore, in the following paragraphs, the description of the different enzymatic activities has been detailed, taking into consideration the diverse microglial typologies. The main morphologic features and some relevant aspects of the temporospatial distribution of each microglial typology have also been included in the description. The expression of the four enzymatic activities, time course of appearance, and morphology of the different microglial typologies have been summarized in Figure 3.

Ameboid microglia

Morphology. Ameboid microglia (AM; Fig. 2A,C,F,H,I) showed a roundish shape. They generally had a soma ranging from 15 to 50 μm in diameter and often displayed thin filopodia, pseudopodia, or both. AM are the first cells from the microglial lineage appearing in the developmental brain parenchyma and are considered to be the precursors of the ramified, resting microglia of the adult brain (Ling and Wong, 1993).

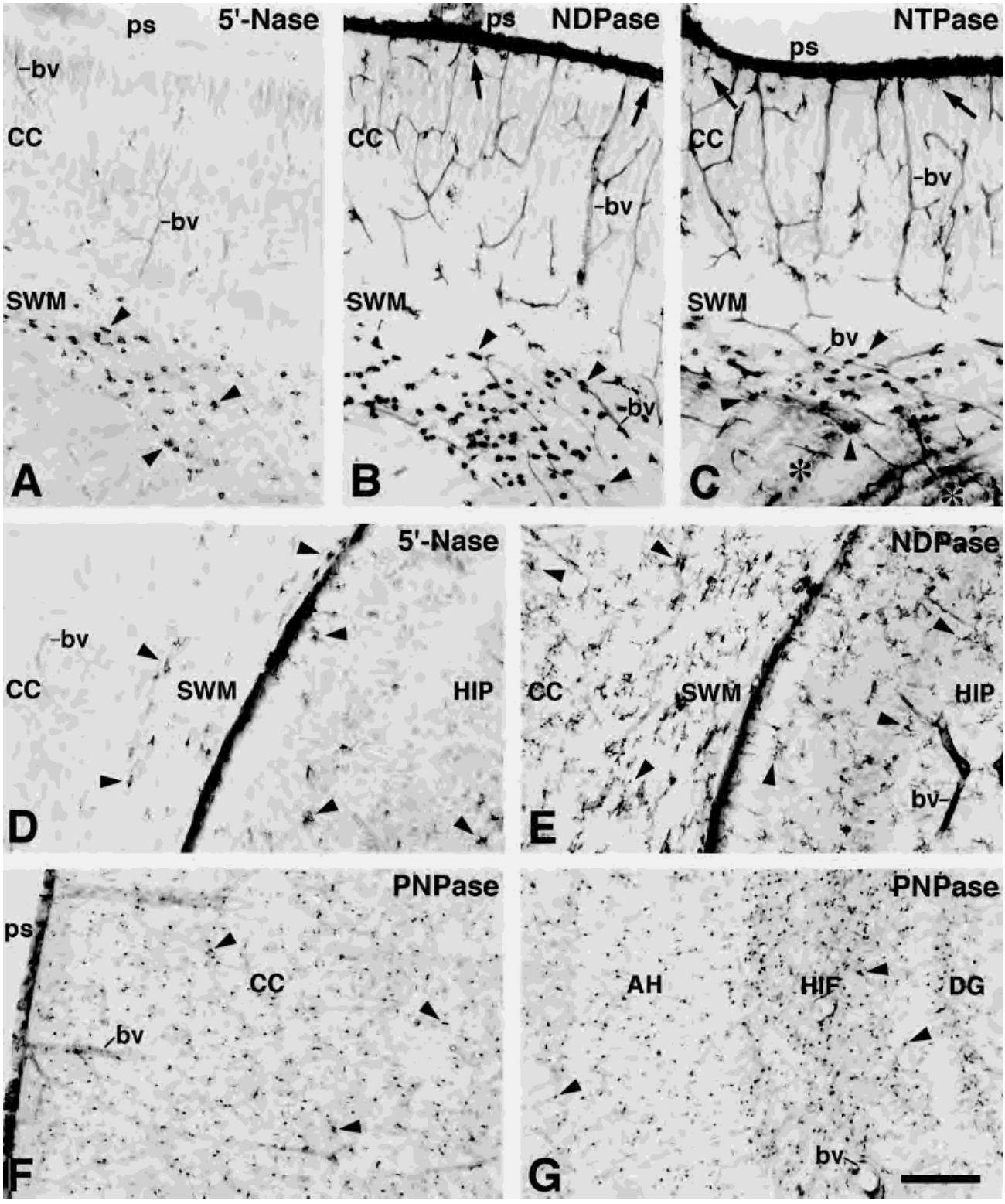

Fig. 1. Expression of purine-metabolizing enzymes at different stages of rat brain development. **A–C:** Cerebral cortex (CC) at embryonic day 21. By means of 5′-Nase histochemistry (A), cells from the microglial lineage are basically observed in the subcortical white matter (SWM; arrowheads) and in deep cortical layers. Note that microglial elements are not visualized subjacent to the pial surface (ps) after 5′-Nase staining. In contrast, microglia are demonstrated not only in white matter (arrowheads) but also in the different cortical layers, including the subpial molecular layer (arrows), by means of nucleoside diphosphatase (NDPase; B) and nucleoside triphosphatase (NTPase; C) histochemistries. Blood vessels (bv) display intense NDPase and NTPase activities, but only a few blood vessels display apparent 5′-Nase activity. Note that a considerable number of microglial elements are found next to the blood vessel walls, particularly in the subcortical white matter. The NTPase, but not the 5′-Nase and the NDPase, stains some nerve fibers (asterisks in C). **D,E:** At postnatal day (P)12, cells of the microglial population (arrowheads) are found in both gray (CC, cerebral cortex; HIP, hippocampus) and white matter (SWM, subcortical white matter) by means of 5′-Nase (D) and NDPase (E) histochemistries. Microglial cells expressing 5′-Nase are less abundant than microglial cells expressing NDPase, especially in gray matter areas. **F,G:** PNPase staining of the cerebral cortex (F) and hippocampus (G) at P15. PNPase activity is displayed by a large number of cells (arrowheads) that are distributed through all layers of the cerebral cortex (CC) and hippocampus (AM, Ammon's horn; HiF hippocampal fissure; DG, dentate gyrus). Some blood vessels (BV) displaying PNPase activity are also found. Scale bar = 150 µm (applies to all).

Distribution. AM were the only microglial cell type observed in the rat brain on E16, although they were gradually replaced by primitive ramified microglia with age. In the gray matter, this cell type was scarce (Fig. 1A–C). In the cerebral cortex on E16–E21, AM were observed next to the surrounding pia and the intermediate zone. These cells were rarely observed in the cortical plate before E21. In the hippocampus, this cell type also arranged subjacent to pia on E16 and were mostly gathered next to the hippocampal fissure on days E18 and E21. In the dentate gyrus, AM were rarely seen during the prenatal period and were located in the dentate hilus during the first postnatal days. By contrast, AM in the white matter were abundantly encountered (Fig. 1A–C) and were demonstrated not only during the prenatal period but also during the first (fimbria, external and internal capsule, and corpus callosum) and second (external capsule and corpus callosum) postnatal weeks. A significant number of AM in white matter areas were found closely attached to or in the immediate vicinity of the vascular capillary wall (Fig. 1A–C).

Enzymatic activities. AM displayed all four enzymatic activities, although the NTPase and NDPase stainings were displayed at higher levels than the 5′-Nase and PNPase stainings. However, the activity of these enzymes was not generalized in all cells of the AM population. AM were always NTPase- (Fig. 2A) and NDPase-positive (Fig. 2C) cells. AM were often 5′-Nase-positive (Fig. 2F,H), but we also demonstrated AM cells lacking 5′-Nase activity in the developing gray matter of the cerebral cortex and hippocampus subjacent to the pia (Fig. 1A–C). AM cells showing 5′-Nase activity in gray matter were predominantly located next to the blood vessel walls. Regarding PNPase, we found some AM cells in both gray and white matter (Fig. 2I) that were PNPase-negative. In addition, PNPase-positive AM cells found in white matter expressed higher levels of activity than their counterparts in gray matter.

Primitive ramified microglia

Morphology. Primitive ramified microglia (PRM; Fig. 2A,D,G,I) were sparsely ramified and showed a slightly elongated to oval cell body. The processes of PRM, initially poorly developed, became longer and thinner with age and typically displayed small swellings or dilations of variable diameter. They are considered intermediate forms in the process of transformation of AM into mature microglia (Murabe and Sano, 1982; Dalmau et al., 1997a).

Distribution. PRM were first demonstrated on E18, being the most extensively recognized microglial cell type from birth until P9–P12 in the gray matter and until P12–P15 in the white matter (Fig. 1). During the embryonic period, PRM mainly distributed in the differentiating fields rather than the germinative neuroepithelium and subventricular zone. In the cerebral cortex on E18–E21, they were arranged in the developing molecular layer and intermediate zone. At birth, PRM were seen in all cortical layers except for the external granular layer, which was poorly populated by microglia. From P3, PRM cells gradually distributed in a homogenous pattern through all cortical layers. In the hippocampus, PRM were predominantly arranged in the vicinities of the hippocampal fissure from E18 until P9 and in the hilus of the dentate gyrus on P6, distributing later through all layers except for the pyramidal and granular layers, in which PRM appeared to be at lower levels.

Enzymatic activities. PRM were stained by NTPase, NDPase, 5′-Nase, and PNPase histochemistries. In general, PRM displayed the NTPase, NDPase and 5′-Nase activities at lower levels than AM. PRM were NTPase (Fig. 2A) and NDPase (Fig. 2D) positive, and, as in the case of AM, we found some PRM lacking 5′-Nase activity, which were specifically found in gray matter of the cerebral cortex (Fig. 1A–C) and hippocampus (Fig. 1D,E). In addition, 5′-Nase-positive PRM cells in white matter expressed higher levels of activity than their counterparts in gray matter (Fig. 2G). The 5′-Nase labeled PRM cells in the embryonic period were predominantly located next to the blood vessel walls. Finally, some PNPase-negative PRM cells were also found in both gray and white matter during the prenatal and postnatal period (Fig. 2I).

Resting microglia

Morphology. Resting microglia (ReM; Fig. 2B,E) have a round to slightly elongated cell body with several long, fine, tortuous, crenulated primary processes that subsequently divide into a variable number of numerous, but shorter, subsidiary branches. These cells correspond to the typical ramified microglial cells observed in the adult brain (Lawson et al., 1990; Castellano et al., 1991b).

Distribution. ReM were first observed in the second postnatal week and represented the predominant microglial cell type from the third postnatal week. In particular, ReM were extensively distributed in the gray matter from P12 to P15 and in the white matter from P15 to P18.

Enzymatic activities. ReM expressed NTPase, NDPase, and PNPase, although the NTPase and NDPase activities at lower levels than PRM and AM (Fig. 2B,E). Some ReM lacking PNPase activity were found in both gray and white matter. With regard to 5′-Nase, there were only some ReM in both gray and white matters displaying this activity. The expression of 5′-Nase by ReM in the white matter was observed at higher levels than in the gray matter.

DISCUSSION

As shown by our results, the differentiation process of microglial cell precursors into mature microglia is accompanied by changes not only in the morphology but also in the expression of purine-related enzymes. In fact, both the occurrence and levels of the different enzymatic activities differed depending on the stage of differentiation of the microglial cells. The NTPase and NDPase activities are preserved during the differentiation of microglia, although they are down-regulated: NTPase and NDPase are found at higher levels in AM than PRM and in ReM. The 5′-Nase is also down-regulated, but unlike NTPase and NDPase, its activity is not completely preserved through the microglial development, and the great majority of ReM have either lost the 5′-Nase activity, or it is shown at low levels. Similar changes in the expression of other enzymes and membrane receptors have also been described during the process of differentiation of microglial cells (Thomas, 1992; Ling and Wong, 1993; Streit, 1995) and may be considered as a part of the physiologic mechanism of adaptation of microglial cells to the variable developmental conditions

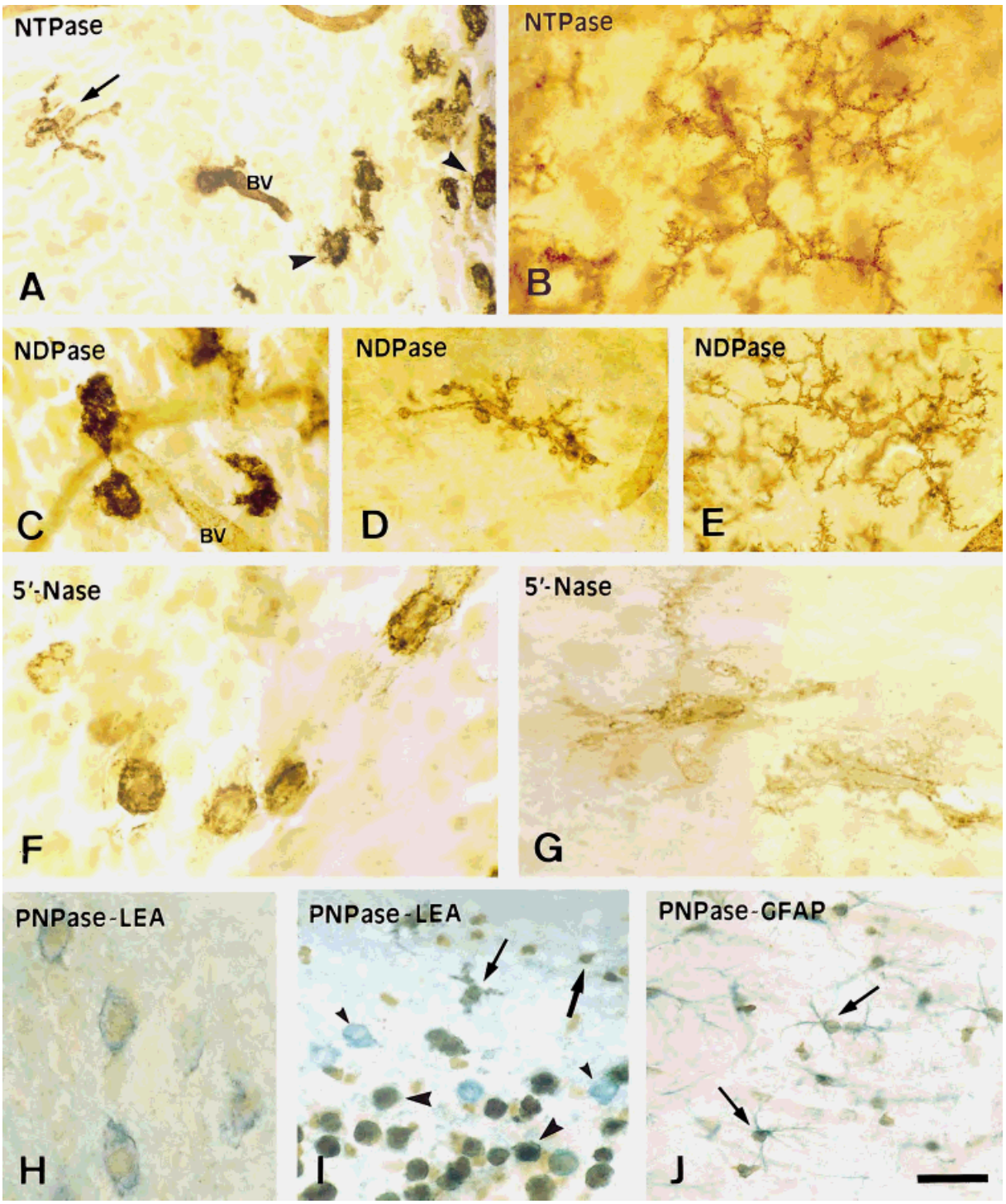

Figure 2

(Wang et al., 1990). Signals arising from the microenvironment, presumably related to the local presence/absence of substrates or to the deficit/excess of products, may stimulate/inhibit the expression and activity of these hydrolytic enzymes. This possibility could explain, at least partially, the temporal and regional differences in the regulation of purine-related enzymes during microglial development. However, the enzymatic profile of a given differentiating cell must also be in accordance with those of its specific ontogenic lineage.

Cellular classification	Enzymatic activities				Time course of appearance and morphology of microglial cells
	NTPase	NDPase	5'-Nase	PNPase	
Ameboid microglia (AM)	+	+	+ and -	+ and -	*G.M.*: E16-P6. *W.M.*: E16-P12. Rarely at P15.
Primitive ramified microglia (PRM)	+	+	+ and - *G.M.*: Mostly during the postnatal period *W.M.*: E18-P18	+ and -	*G.M.*: E18-P12. Rarely at P15. *W.M.*: E18-P15. Rarely at P18.
Resting microglia (ReM)	+	+	- *	+ *	Some at P9/P12, but mostly from P15.

+ Cells that display the enzymatic activity.

+ * Some cells do not display the enzymatic activity.

- Cells that do not display the enzymatic activity.

- * Some cells display low levels of the enzymatic activity.

+ and - Existence of two cell subpopulations, one that displays the enzymatic activity and other that does not display the enzymatic activity.

G.M.: Gray matter.

W.M.: White matter.

Fig. 3. Classification and enzymatic profile of microglial cells in the developing prenatal and postnatal brain.

Fig. 2. Expression of purine-metabolizing enzymes by cells of the microglial lineage. **A:** Developing white matter of the cerebral cortex and its surrounding gray matter at embryonic day (E)21. Nucleoside triphosphatase (NTPase) activity in embryos is expressed by two types of microglia, the ameboid (arrowheads) and the primitive ramified (arrow). Observe that ameboid microglia display higher levels of activity than primitive ramified microglia. Apart from microglia, blood vessels (BV) also display NTPase activity. **B:** Cerebral cortex on postnatal day (P)18. NTPase-stained microglial cell of the resting type. **C–E:** Photomicrographs showing microglial cells visualized by means nucleoside diphosphatase (NDPase) staining in the developing corpus callosum at E21 (C), CA3 stratum radiatum of the P3 hippocampus (D), and the cerebral cortex on P18 (E). Similar to NTPase, NDPase labels ameboid microglia (C), primitive ramified microglia (D), and resting microglia (E). Note that NDPase levels displayed by ameboid microglia are higher than those displayed by primitive ramified and resting microglial cells. Blood vessel (BV) also shows NDPase activity. **F,G:** Microglial cells visualized by 5′-Nase staining in the external capsule at E21 (F) and corpus callosum on P9 (G). These photomicrographs show ameboid microglial cells (F) and primitive ramified microglial cells (G) expressing 5′-Nase activity. Compared with ameboid microglia, 5′-Nase activity is down-regulated in primitive ramified microglia. **H:** External capsule at E21. The double-labeling technique enables us to visualize 5′-Nase-positive ameboid microglia (brownish color), which simultaneously display tomato lectin (LEA) staining (bluish color), thus confirming the microglial nature of these 5′-Nase-positive cells. **I:** Corpus callosum at P6. The combination of PNPase (brownish color) and tomato lectin (bluish color) histochemistries enables us to visualize double-labeled ameboid (big arrowheads) and primitive ramified (small arrow) microglial cells. Note, however, that there are tomato lectin-labeled cells with the typical morphology of ameboid microglia which are PNPase-negative (small arrowheads) and PNPase-positive cells that lack tomato lectin labeling (big arrow). **J:** Cerebral cortex at P18. The double-labeling technique combining PNPase histochemistry (brownish color) and GFAP immunohistochemistry (bluish color) demonstrates that a subpopulation of PNPase-positive cells are astrocytes (arrows). No GFAP-positive astrocytes lacking PNPase activity are found. In contrast, PNPase-positive cells lacking GFAP immunostaining, presumably belonging to the microglial lineage, are found. Scale bars = 35 μm in A,I,J, 15 μm in B,G,F, 25 μm in C,E, 60 μm in D, 13 μm in H.

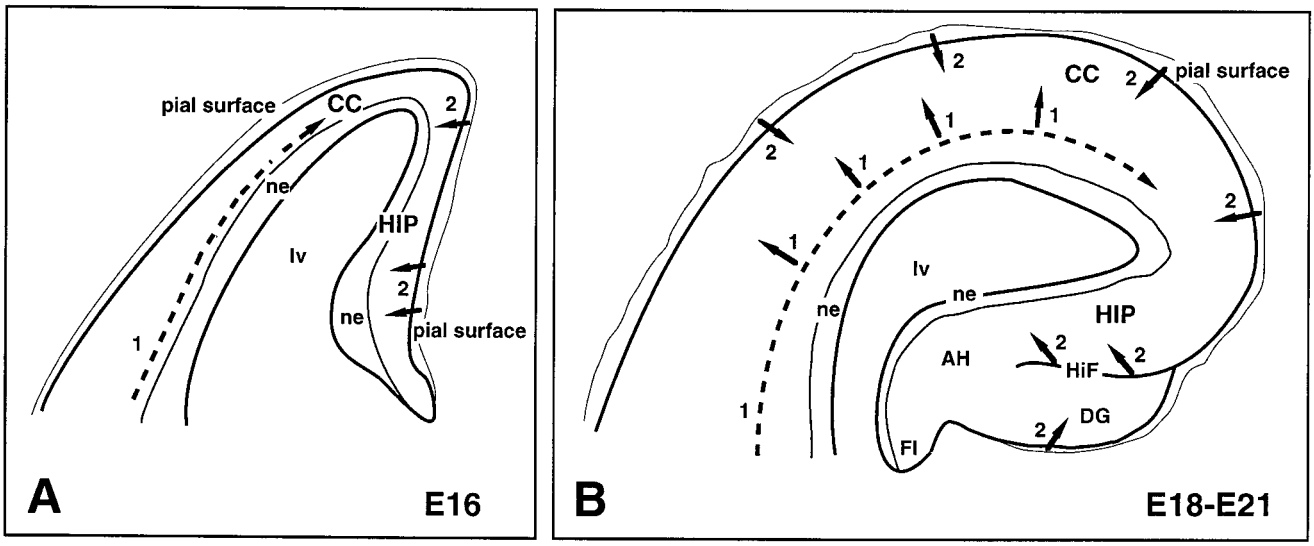

Fig. 4. Distribution and presumable traffic routes of microglial cells during the prenatal development of the rat brain. **A:** Microglial cell precursors in the cerebral cortex on E16 are concentrated in the developing subcortical white matter (between the intermediate zone and the neocortical neuroepithelium) and subpially in the medial telencephalon. In the hippocampus (HIP), microglial cells are found subjacent to pia. The dashed arrow (1) represents ameboid microglial precursors that, according to the literature, arise from blood monocytes that extravasate and disseminate through the developing white matter following a tangential pattern. The arrows (2) represent ameboid microglial precursors arising from pial elements, probably fetal macrophages, that penetrate the differentiating subpial gray matter. **B:** On embryonic day (E)18–E21, microglial cells are mostly located in the subcortical white matter, in the deep layers of the cerebral cortex (over the white matter), and beneath the pial surface (molecular layer). Microglia in the hippocampus are mostly gathered next to the hippocampal fissure and in the fimbria. The arrows (1) represent microglial cells of monocytic origin that accumulate in white matter and migrate centrifugally (radial pattern in accordance with Navascués et al., 1996) toward the cortical layers. The arrows (2) represent microglial cells coming from pial elements that accumulate in the subpial gray matter and centripetally invade the nervous tissue. See references in the text. AH, Ammon's horn; CC, cerebral cortex; DG, dentate gyrus; FI, fimbria; HiF, hippocampal fissure; HIP, hippocampus; lv, lateral ventricles; ne, neuroepithelium.

Microglia: Origin, differentiation, and purine-related enzymatic activities

Ameboid microglial cells are the first cells from the microglial lineage appearing in the developmental brain parenchyma and are considered to be the precursors of the ramified, resting microglia of the adult brain (Thomas, 1992; Ling and Wong, 1993). It has been suggested by a number of studies that the ameboid microglial precursors may derive either from pial elements that migrate into the brain from the surrounding mesenchymal tissue or from blood monocytes that enter the nervous tissue through extravasation from blood vessels (Del Río Hortega, 1920, 1932; Imamoto and Leblond, 1978; Ling, 1981; Boya et al., 1991; Chugani et al., 1991; Sorokin et al., 1992; Cuadros et al., 1993; Cossmann et al., 1997; Dalmau et al., 1997b). Thus, similar to macrophage populations in other organs like the liver (Naito et al., 1990, 1996), microglia in the brain may have a double origin: fetal macrophages and blood monocytes. Pial elements presumably penetrate the subpial gray matter and centripetally invade the nervous tissue (Del Río Hortega, 1920, 1921, 1932; Boya et al., 1991; Sorokin et al., 1992; Navascués et al., 1996), whereas blood monocytes are mainly recruited in white matter and centrifugally invade the brain tissue (Ling, 1979; Ling et al., 1980; Murabe and Sano, 1982; Navascués et al., 1996; Dalmau et al., 1997b). This finding is in accordance with the observation that microglial cells (AM and PRM) found in the brain during the embryonic period are initially concentrated in the subpial gray matter and at the vicinity of blood vessels in white matter areas. Afterward, during the late embryonic and early postnatal period, microglial cells progressively distribute throughout the brain parenchyma. The distribution and presumable traffic routes of microglial precursors have been summarized in Figure 4.

The two putative microglial cell sources have a different ontogeny. Pial fetal macrophages arise from yolk sac or embryonic mesenchyme (Takahashi and Naito, 1993) whereas blood monocytes mostly originate in fetal liver during the embryonic period or in bone marrow from the perinatal time (Cline and Moore, 1972; forreviews, see Auger and Ross, 1992; Fedoroff, 1995). The fetal macrophages develop from hematopoietic stem cells that bypass the promonocyte and monocyte stages and appear in the mesenchyme before the occurrence of monocytic cells (Takahaschi and Naito, 1993; Takahaschi et al., 1989, 1996). Unfortunately, tissue macrophages arising from mesenchymal fetal macrophages have a similar morphology to those coming from monocytes. These two subpopulations, however, may be distinguished on the basis on their enzymatic profile because blood monocytes and differentiating monocyte-derived macrophages, but not mesenchymal fetal macrophages, express 5′-Nase activity (Sunderman, 1990; Takahaschi et al., 1989).

In the developing brain parenchyma, the two precursors are indistinguishable on the basis on their morphologic features and are identified as a whole as ameboid microglia. In addition, both pial fetal macrophage- and monocyte-

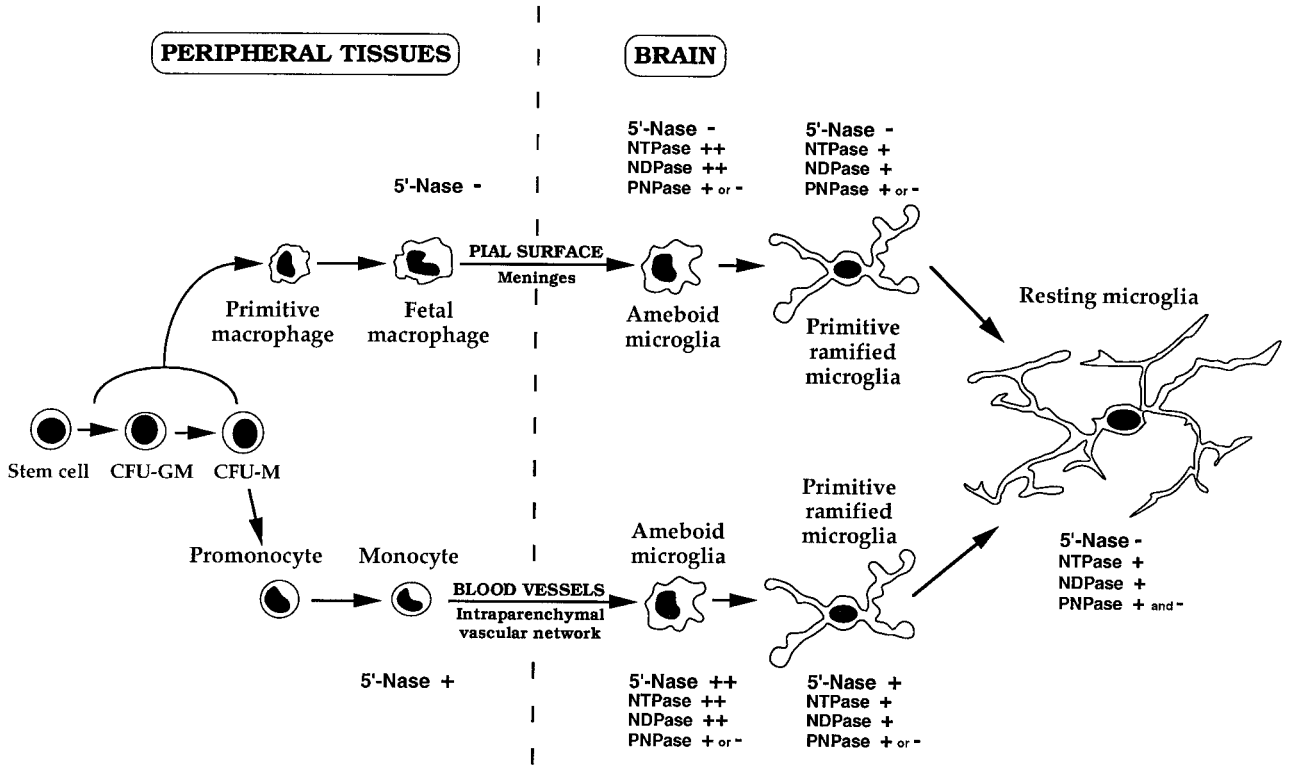

Fig. 5. Proposed scheme for the ontogenesis of microglial cells. This scheme is based on the present study and other available studies on macrophage/microglia ontogenesis (Ling, 1981; Auger and Ross, 1992; Naito et al., 1996; Navascués et al., 1996; Dalmau et al., 1997a,b). CFU-GM, colony forming unit for granulocyte/macrophage cell lines; CFU-M, colony forming unit for macrophage cell lines.

derived microglial precursors show NTPase and NDPase activities because all ameboid precursors found in the developing brain seem to display these two enzymatic activities. However, we have observed that 5′-Nase in the developing brain parenchyma is predominantly expressed by microglial precursors (AM and PRM) present in white matter areas, whereas a considerable number of microglial precursors lacked 5′-Nase activity in the differentiating subpial gray matter. Note that the distribution of 5′-Nase-positive cells closely correlates with the presumable distribution of monocyte-derived microglial precursors, whereas microglial precursors lacking 5′-Nase were mostly distributed in the expected, pial macrophage-derived, subpial gray matter. This finding also agrees with the observation in other tissues that 5′-Nase activity is related to differentiating monocyte-derived macrophages (Sunderman, 1990; Auger and Ross, 1992), whereas fetal macrophages are devoid of 5′-Nase activity (Takahashi et al., 1989). Thus, it is suggested that fetal macrophage- and monocyte-derived microglial precursors show the same morphology and express NTPase and NDPase, but only the cell subpopulation arising from blood monocytes express 5′-Nase. Afterward, 5′-Nase is down-regulated through the differentiation of microglia and the mature forms (ReM), including those coming from blood monocytes, may lack this enzymatic activity. Figure 5 summarizes the proposed differentiation pattern and enzymatic profile of the microglial lineage.

The specific contribution to microglial homing of these two mechanisms remains to be elucidated. However, on the basis of our observations and those of other authors (Sorokin et al., 1992), fetal macrophage entrance seems to be restricted to the early embryonic period, when the brain is not or it is poorly vascularized, and these pial elements may be only responsible for the production of a small number of subpial precursors. In contrast, monocytes may enter the brain during both the embryonic and postnatal period as the expansion and vascularization of the brain proceed, and may generate the major part of microglial cells. In addition, monocytes may be responsible not only for the production of microglial precursors that migrate across the developing white matter tracts and extend to gray matter areas, but also for microglia that come from the discrete extravasation of blood monocytes throughout the developing brain (Dalmau et al., 1997b).

Similar to 5′-Nase, the PNPase activity is related to a subpopulation of microglial cells. However, PNPase-negative microglial cells were encountered in gray matter and white matter, and we were unable to detect any clear correspondence between the distribution of PNPase-positive microglial cells and the presumable distribution of fetal macrophage- or monocyte-derived microglial cells. In addition, the PNPase activity has been observed in relation to cells from the astroglial lineage, not only during development, but also in the adult brain (Castellano et al., 1990). The accurate significance of PNPase activity in astrocytes and a subpopulation of microglia is presently unknown, but it may be related to the metabolic requirements of purine nucleobases (Castellano et al., 1990).

Purine-related enzymes as microglial markers

The histochemical demonstration of different enzymatic activities related to the metabolism of purines, in particular NTPase, NDPase, 5′-Nase, and PNPase, have been successfully applied to visualize microglia in the adult brain (Ibrahim et al., 1974; Kreutzberg and Barron, 1978; Kaur et al., 1984; Castellano et al., 1990; Schoen et al., 1992; Jensen et al., 1994; Sørensen et al., 1996). In the present study, we show that all four histoenzymatic techniques are valid to label cells from the microglial lineage during development. There are, however, some substantial differences.

The NTPase and NDPase histochemistries provided the labeling of all cells from the microglial lineage present in the developing rat brain during both the prenatal and postnatal period. The NTPase and NDPase stainings were both located in the plasma membrane of microglial cells and both techniques allowed us the detailed visualization of the different microglial morphologies. However, the NTPase labeling differed somehow to that shown by NDPase, because NTPase also labeled some axons in different brain regions, and this hindered the clear visualization of microglia, especially during the postnatal period. The 5′-Nase staining was also located in the microglial plasma membrane, but it did not allow all microglial cell typologies to be visualized.

The 5′-Nase technique labeled an important fraction of microglial cell precursors (AM and PRM), but the more noticeably differentiated cells found during the postnatal period (ReM) generally lacked this enzymatic activity.

The PNPase staining was located in the nucleus and in the cytoplasm and was displayed by a fraction of, but not all, microglial cells present during the prenatal and postnatal periods. In addition, the PNPase activity was displayed not only by microglia but also by astroglial cells, and this made difficult the study of the microglial morphology and distribution in the different brain regions, mostly in the postnatal period. In this sense, double-labeling techniques combining PNPase histochemistry and markers for microglia and astroglia were needed for the unambiguous identification of PNPase-positive cells. Lastly, it must be noted that all four histoenzymatic techniques labeled blood vessels, the intensity of staining being higher for NTPase and NDPase than for 5′-Nase and PNPase.

In agreement with our light microscopy observations during development, studies in the adult brain have located the NTPase (Ibrahim et al., 1974), NDPase (Murabe and Sano, 1982; Vordrodt and Wisniewski, 1982; Vela et al., 1995b), and 5′-Nase (Kreutzberg and Barron, 1978; Kreutzberg et al., 1978) enzymatic activities on the outer plasma membrane of microglial cells. Also in agreement with our observations, NTPase activity has been shown in the axon, the terminal axon region, and the postsynaptic membrane (Grondal et al., 1988), and the PNPase has been found in the nucleus and cytoplasm of astroglia and a subpopulation of microglia (Castellano et al., 1990). The 5′-nucleotidase has been found to exist in microglia not only as a membrane-bound ectoenzyme (Kreutzberg et al., 1986), but also as a cytosolic form associated with lysosomes and vacuoles (Kaur et al., 1984). In addition, ecto-5′-Nase activity has been found in relation to the plasma membrane of reactive astrocytic processes that are frequently found engulfing synaptic terminals (Kreutzberg et al., 1986), and it has been located in some neurons at certain sites (Kreutzberg and Hussain, 1984; Kreutzberg et al., 1986). In the developing brain, apart from microglia (Kaur et al., 1987), the ectoenzyme 5′-Nase marks some maturing synapses (Schoen et al., 1990, 1991, 1993), and it is present on migrating neuroblasts (Schoen et al., 1988; Heilbronn et al., 1995). Finally, different authors have reported purine-related enzymatic activities in relation to endothelial cells (Ibrahim et al., 1974; Vordrodt and Wisniewski, 1982; Castellano et al., 1990).

Possible role of microglia in the control of extracellular brain purine levels

In contrast to other tissues (Muller et al., 1983), the de novo biosynthetic capacity of purines is reduced at low levels in the brain, and an important mechanism for obtaining purines from nerve cells is recycling, by means of the uptake of extracellular purines (Sperlágh and Vizi, 1996). The first step in this salvage pathway is the transportation of purines across the plasma membrane, and it is known that purine nucleosides, but not the nucleotides, are taken up from the extracellular space by nerve cells (Griffith and Jarvis, 1996). The extracellular transformation of purine nucleotides into their dephosphorylated nucleosides is, thus, a key event for the uptake and subsequent reutilization of purines. Microglial cells display on their plasma membrane the degradative enzymes needed for the dephosphorylation in cascade of nucleotides: nucleoside triphosphatase (NTPase), nucleoside diphosphatase (NDPase), and 5′-nucleotidase. Therefore, microglial cells may be directly involved in the dephosphorylation of nucleotides present extracellularly (Fig. 6). The resulting nucleosides may thus be taken up by neurons and glial cells (Matz and Hertz, 1989).

Microglial cells may also have a particular significance in relation to the synaptic function. Purine nucleotides and their dephosphorylated nucleosides (i.e., adenosine, guanosine, and inosine) are released from neurons in a number of regions of the CNS and act as neurotransmitters or neuromodulators (Fredholm et al., 1993). In particular, there is now considerable evidence that ATP has excitatory effects on the activity of neurons by acting by means of extracellular P_2-purinergic receptors (Zimmermann, 1994). Extracellular ATP released from stimulated neurons is rapidly metabolized by ectonucleotidases into AMP or adenosine, which are taken up by means of extracellular P_1-purinergic receptors by neurons and astrocytes (Wu and Phillis, 1984; Hösli and Hösli, 1988). Microglial cells display ectonucleotidase activity and may, therefore, be the cells responsible for the sequential dephosphorylation of purine nucleotides and consequent inactivation of these purinergic excitatory neurotransmitters.

The correct regulation of the purine metabolism is essential for the normal development and function of tissues, because purines are constituents of the genetic material, DNA and RNA, and are thus necessary for cell replication and differentiation. They also act as the "energy currency," which is required, among other vital functions, for the synthesis of growth factors regulating cell proliferation and differentiation. Interestingly, in addition to its widespread intracellular functions, purines in the nervous system play a number of specific roles in development by acting as extracellular diffusible factors. Extracellular purine nucleosides and nucleotides have mitogenic

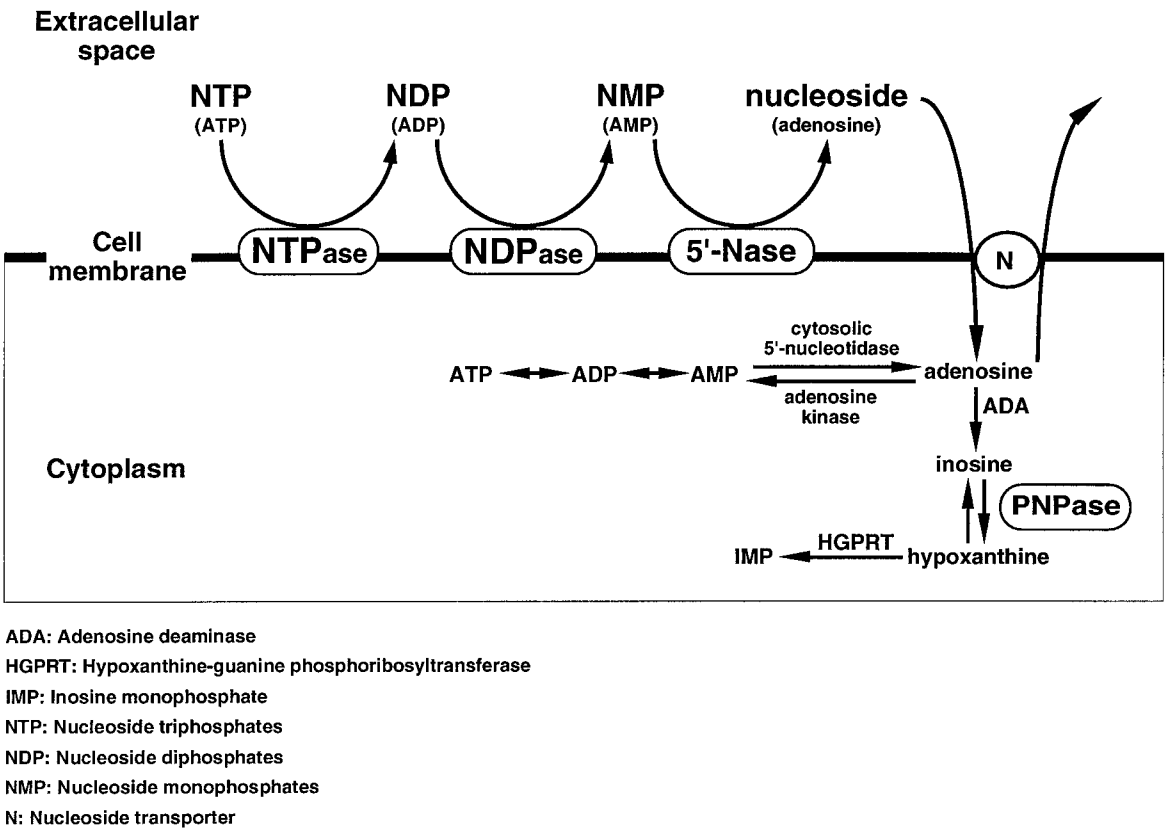

Fig. 6. Purine metabolism-related enzymes associated with microglial cells.

and morphogenic properties in the CNS (Neary et al., 1996). Compounds like adenosine and ATP, when released, directly exert different effects on cells of the nervous tissue through their interaction with the cell surface purinoceptors P_1 and P_2 (Dalziel and Westfall, 1994; Fredholm et al., 1994), respectively. Both glia and neurons possess purinoceptors (Neary et al., 1996). In particular, microglia possess a P_2 purinoceptor linked to an ion channel, and an ATP-microglial interaction is likely to deliver the transmembrane signal for the transition for one microglial functional state to another (Kettenmann et al., 1993). Interestingly, these purines can act synergistically with other growth factors and mitogens (Wang et al., 1990; Abbracchio et al., 1995b), promoting differentiation and proliferation of astroglial cells (Kim et al., 1991; Rathbone et al., 1992b,c; Abbracchio et al., 1994), and they can inhibit the proliferation of microglial cells in vitro (Qiu-Sheng et al., 1996). These compounds also promote neurite extension, at least in NGF-responsive cells (Guroff et al., 1981; Abbracchio et al., 1989), and appear to influence the process of neurogenesis (Weaver, 1996). Moreover, extracellular purines have been involved in the regulation of apoptosis (Abbracchio et al., 1995a; Bronte et al., 1996), adherence of leukocytes to endothelial cells (Parker et al., 1996), and angiogenesis (Rathbone et al., 1992a), all outstanding biological processes in the developing brain.

PNPase is an enzyme of the "inosinate cycle" (Simmonds, 1991) that catalyzes the conversion of the nucleosides inosine and guanosine and their deoxy-analogues to the corresponding bases, hypoxanthine and guanine. PNPase, together with hypoxanthine-guanine phosphoribosyltransferase (HGPRT), which converts inosine and guanosine into IMP or GMP, respectively, are both necessary for the recycling of nucleosides (Fig. 6). In fact, the deficiency of PNPase results in a double disorder, because HGPRT cannot function without its substrates, hypoxanthine and guanine, which are normally provided by PNPase (Simmonds, 1991). This defect is directly associated with immunodeficiency and indirectly with neurologic abnormalities affecting the CNS. We do not yet know, however, if PNPase activity associated with microglia has a specific significance in the function of the nervous system in addition to playing a major role in the turnover and metabolic control of purines.

Taken together, our results suggest that microglia play a crucial function in the developing brain by controlling the extracellular purine levels and the purine metabolism. Thus, the regulation of extracellular levels of purines by microglial cells could be crucial for normal CNS development. This function, however, may be shared with other components of the immature tissue because we observed NTPase, NDPase, 5′-Nase, and PNPase labeling in blood vessels, NTPase in some nerve fibers, and PNPase in cells from the astroglial lineage, suggesting the existence of a complex mechanism of control. Finally, we want to emphasize that the enzymatic profile and levels of the different purine-related enzymes may also depend on the differentiation stage and the changing microenvironment that take place during the formation of the nervous tissue.

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LITERATURE CITED

Abbracchio, M.P., F. Cattabeni, F. Clementi, and E. Sher (1989) Adenosine receptors linked to adenylate cyclase activity in human neuroblastoma cells: Modulation during cell differentiation. Neuroscience *30:*819–825.

Abbracchio, M.P., M.J. Saffrey, V. Hopker, and G. Burnstock (1994) Modulation of astroglial cell proliferation by analogues of adenosine and ATP in primary cultures of rat striatum. Neuroscience *59:*67–76.

Abbracchio, M.P., S. Ceruti, D. Barbieri, C. Franceschi, W. Malorni, L. Biondo, G. Burnstock, and F. Cattabeni (1995a) A novel action for adenosine: Apoptosis of astroglial cells in rat brain primary cultures. Biochem. Biophys. Res. Commun. *213:*908–915.

Abbracchio, M.P., S. Ceruti, R. Langfelder, F. Cattabeni, M.J. Saffrey, and G. Burnstock (1995b) Effects of ATP analogues and basic fibroblast growth factor on astroglial cell differentiation in primary cultures of rat striatum. Int. J. Dev. Neurosci. *13:*685–693.

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

Auger, M.J. and J.A. Ross (1992) The biology of the macrophage. In C.E. Lewis and J.O.D. McGee (eds): The Natural Immune System. The Macrophage. New York: Oxford University Press, pp. 1–74.

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

Bignami, A., L.F. Eng, D. Dahl, and T. Uyeda (1972) Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. Brain Res. *43:*429–435.

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

Bronte, V., B. Macino, A. Zambon, A. Rosato, S. Mandruzzato, P. Zanovello, and D. Collavo (1996) Protein tyrosine kinases and phosphatases control apoptosis induced by extracellular adenosine 5'-triphosphate. Biochem. Biophys. Res. Commun. *218:*344–351.

Burnstock, G. (1990) Overview. Purinergic mechanisms. Ann. NY Acad. Sci. *603:*1–17.

Burnstock, G. (1993) Physiological and pathological roles of purines: An update. Drug Dev. Res. *28:*195–206.

Castellano, B., B. González, B.R. Finsen, and J. Zimmer (1990) Histochemical demonstration of purine nucleoside phosphorylase (PNPase) in microglial and astroglial cells of adult rat brain. J. Histochem. Cytochem. *38:*1535–1539.

Castellano, B., B. González, M.B. Jensen, E.B. Pedersen, B.R. Finsen, and J. Zimmer (1991a) A double staining technique for simultaneous demonstration of astrocytes and microglia in brain sections and astroglial cell cultures. J. Histochem. Cytochem. *39:*561–568.

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

Cline, M.J. and M.A.S. Moore (1972) Embryonic origin of the mouse macrophage. Blood *39:*842–849.

Chugani, D.C., N.L. Kedersha, and L.H. Rome (1991) Vault immunofluorescence in the brain: New insights regarding the origin of microglia. J. Neurosci. *11:*256–268.

Cossmann, P.H., P.S. Eggli, B. Christ, and H. Kurz (1997) Mesoderm-derived cells proliferate in the embryonic central nervous system: Confocal microscopy and three-dimensional visualization. Histochem. Cell Biol. *107:*205–213.

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

Dalmau, I., B. Castellano, E.B. Pedersen, B. Finsen, J. Zimmer, and B. González (1996) Reduction of the microglial cell number in rat primary glial cell cultures by exogenous addition of dibutyryl cyclic adenosine monophosphate. J. Neuroimmunol. *70:*123–129.

Dalmau, I., B. Finsen, N. Tønder, J. Zimmer, B. González, and B. Castellano (1997a) Development of microglia in the prenatal rat hippocampus. J. Comp. Neurol. *377:*70–84.

Dalmau, I., J.M. Vela, B. González, and B. Castellano (1997b) Expression of LFA-1α and ICAM-1 in the developing rat brain: A potential mechanism for the recruitment of microglial cell precursors. Dev. Brain Res. *103:*163–170.

Dalziel, H.H. and D.P. Westfall (1994) Receptors for adenine nucleotides and nucleosides: Subclassification, distribution, and molecular characterization. Pharmacol. Rev. *46:*449–466.

Del Río Hortega, P. (1920) El tercer elemento de los centros nerviosos. III.-Naturaleza probable de la microglía. Bol. Soc. Esp. Biol. *8:*154–166.

Del Río Hortega, P. (1921) El tercer elemento de los centros nerviosos. Histogénesis y evolución normal; éxodo y distribución regional de la microglía. Mem. Real Esp. Hist. Nat. *11:*213–268.

Del Río Hortega, P. (1932) Microglia. In W. Penfield (ed): Cytology and Cellular Pathology of the Nervous System. Vol 2. New York: Paul B. Hoeber, pp. 481–534.

Ditrich, H. (1986) Brain macrophages in cerebellar cell cultures. Tissue Cell. *18:*645–658.

Eng, L.F., J.J. Vanderhaeghen, A. Bignami, and B. Gerstl (1971) An acidic protein isolated from fibrous astrocytes. Brain Res. *28:*351–354.

Fedoroff, S. (1995) Development of microglia. In H. Kettenmann and B.R. Ransom (eds): Neuroglia. New York: Oxford University Press, pp. 162–181.

Finsen, B., N. Tønder, G.F. Xavier, J.C. Sørensen, and J. Zimmer (1993) Induction of microglial immunomolecules by anterogradely degenerating mossy fibers in the rat hippocampal formation. J. Chem. Neuroanat. *6:*267–275.

Fredholm, B.B., B. Johansson, I. van der Ploeg, P.S. Hu, and S. Jin (1993) Neuromodulatory roles of purines. Drug Dev. Res. *28:*349–353.

Fredholm, B.B., M.P. Abbracchio, G. Burnstock, J.W. Daly, T.K. Harden, K.A. Jacobson, P. Leff, and M. Williams (1994) Nomenclature and classification of purinoreceptors. Pharmacol. Rev. *46:*143–156.

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

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

Griffith, D.A. and S.M. Jarvis, (1996) Nucleoside and nucleobase transport systems of mammalian cells. Biochim. Biophys. Acta *1286:*153–181.

Grondal, E.J., A. Janetzko, and H. Zimmermann (1988). Monospecific antiserum against 5'-nucleotidase from torpedo electric organ: Immunocytochemical distribution of the enzyme and its association with Shwann cell membranes. Neuroscience *24:*351–363.

Guroff, G., G. Dickens, D. End, and C. Londos (1981) The action of adenosine analogs on PC12 cells. J. Neurochem. *37:*1431–1439.

Heilbronn, A., V. Maienschein, K. Carstensen, W. Gann, and H. Zimmermann (1995) Crucial role of ecto-5'-nucleotidase in differentiation and survival of developing neural cells. Neuroreport *7:*257–261.

Hösli, E. and L. Hösli (1988) Autoradiographic studies on the uptake of adenosine and on binding of adenosine analogues in neurones and astrocytes of cultured rat cerebellum and spinal cord. Neuroscience *24:*621–628.

Ibrahim, M.Z.M., Y. Khreis, and D.S. Koshayan (1974) The histochemical identification of microglia. J. Neurol. Sci. *22:*211–233.

Imamoto, K. and C.P. Leblond (1978) Radioautographic investigation of gliogenesis in the corpus callosum of young rats. II. Origin of microglial cells. J. Comp. Neurol. *180*:139–163.

Jensen, M., B. González, B. Castellano, and J. Zimmer (1994) Microglial and astroglial reactions to anterograde axonal degeneration: A histochemical and immunocytochemical study of the adult rat fascia dentata after entorhinal path lesions. Exp. Brain Res. *98:*245–260.

Jørgensen, M.B., B.R. Finsen, M.B. Jensen, B. Castellano, N. Diemer, and J. Zimmer (1993) Microglial and astroglial reactions to ischemic and kainic acid-induced lesions of the adult rat hippocampus. Exp. Neurol. *120*:70–88.

Kaur, C., E.A. Ling, and W.C. Wong (1984) Cytochemical localization of 5'-nucleotidase in ameboid microglial cells in postnatal rats. J. Anat. *139:*1–7.

Kaur, C., E.A. Ling, and W.C. Wong (1987) Origin and fate of neural macrophages in a stab wound of the brain of the young rat. J. Anat. *154:*215–227.

Kettenmann, H., R. Banati, and W. Walz (1993) Electrophysiological behavior of microglia. Glia *7:*93–101.

Kim, J.K., M.P. Rathbone, P.J. Middlemiss, D.W. Hughes, and R.W. Smith (1991) Purinergic stimulation of astroblast proliferation: Guanosine

and its nucleotides stimulate cell division in chick astroblasts. J. Neurosci. Res. *28:*442–455.

Kreutzberg, G.W. and K.D. Barron (1978) 5'-nucleotidase of microglial cells in the facial nucleus during axonal reaction. J. Neurocytol. *7:*601–610.

Kreutzberg, G.W. and S.T. Hussain (1984) Cytochemical localization of 5'-nucleotidase activity in retinal photoreceptor cells. Neuroscience *11:*857–866.

Kreutzberg, G.W., K.D. Barron, and P. Shubert (1978) Cytochemical localization of 5'-nucleotidase in glial plasma membranes. Brain Res. *158:*247–257.

Kreutzberg, G.W., D. Heymann, and M. Reddington (1986) 5'-nucleotidase in the nervous system. In GW. Kreutzberg, M. Reddington, and H. Zimmermann (eds): Cellular Biology of Ectoenzymes. Berlin, Heidelberg, New York: Springer-Verlag, pp. 147–164.

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

Ling, E.A. (1977) Light and electron microscopic demonstration of some lysosomal enzymes in the amoeboid microglia in neonatal rat brain. J. Anat. *123:*637–648.

Ling, E.A. (1979) Transformation of monocytes into amoeboid microglia and into microglia in the corpus callosum of postnatal rats, as shown by labelling monocytes by carbon particles. J. Anat. *128:*847–858.

Ling, E.A. (1981) The origin and nature of microglia. In S. Federoff and L. Hertz (eds): Advances in Cellular Neurobiology. Vol II. New York: Academic Press, pp. 33–82.

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

Ling, E.A., D. Penney, and C.P. Leblond (1980) Use of carbon labelling to demonstrate the role of blood monocytes as precursors of the "amoeboid cells" present in the corpus callosum of postnatal rats. J. Comp. Neurol. *193:*631–657.

López-García, C., J. Nacher, B. Castellano, J.A. Luís de la Iglesia, and A. Molowny (1994) Transitory disappearance of microglia during the regeneration of the lizard medial cortex. Glia *12:*52–61.

Matz, H. and L. Hertz, (1989) Adenosine metabolism of neurons and astrocytes in primary cultures. J. Neurosci. Res. *24:*260–267.

Muller, M.M., M. Kraupp, P. Chiba, and H. Rumpold (1983) Regulation of purine uptake in normal and neoplastic cells. Adv. Enzyme Regul. *21:*239–256.

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

Naito, M., K. Takahashi, and S. Nishikawa (1990) Development, differentiation, and maturation of macrophages in the fetal mouse liver. J. Leukoc. Biol. *48:*27–37.

Naito, M., S. Umeda, T. Yamamoto, H. Moriyama, H. Umezu, G. Hasegawa, H. Usuda, L.D. Shultz, and K. Takahashi (1996) Development, differentiation, and phenotypic heterogeneity of murine tissue macrophage. J. Leukoc. Biol. *59:*133–138.

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

Neary, J.T., M.P. Rathbone, F. Cattabeni, M.P. Abbracchio, and G. Burnstock (1996) Trophic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. Trends Neurosci. *19:*13–18.

Norenberg, W., P. Illes, and P.J. Gebicke-Haerter (1994) Sodium channel in isolated human brain macrophages. Glia *10:*165–172.

Novikoff, A.B. and S. Goldfisher (1961) Nucleoside diphosphatase activity in the Golgi apparatus and its usefulness for cytological studies. Proc. Natl. Acad. Sci. USA *47:*802–810.

Parker, A.L., L.L. Likar, D.D. Dawicki, and S. Rounds (1996) Mechanism of ATP-induced leukocyte adherence to cultured pulmonary artery endothelial cells. Am J. Physiol. *270:*659–703.

Perry, V.H. and S. Gordon (1991) Macrophages and the nervous system. Int. Rev. Cytol. *125:*203–243.

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

Qiu-Sheng, S., Y. Nakamura, P. Shubert, K. Rudolphi, and K. Kataoka (1996) Adenosine and propentofylline inhibit the proliferation of cultured microglial cells. Exp. Neurol. *137:*345–349.

Rathbone, M.P., S. DeForge, B. DeLuca, B. Gabel, C. Laurenssen, P. Middlemiss, and S. Parkinson (1992a) Purinergic stimulation of cell division and differentiation: Mechanisms and pharmacological implications. Med. Hypotheses *37:*213–219.

Rathbone, M.P., L. Christjanson, S. DeForge, B. DeLuca, J.W. Gysbers, S. Hindley, M. Jovetich, P.J. Middlemiss, and S. Takhal (1992b) Extracellular purine nucleosides stimulate cell division and morphogenesis: Pathological and physiological implications. Med. Hypotheses *37:*232–240.

Rathbone, M.P., P. Middlemiss, J.K. Kim, J.W. Gysbers, S. DeForge, R.W. Smith, D.W. Hughes (1992c) Adenosine and its nucleotides stimulate proliferation of chick astrocytes and human astrocytoma cells. Neurosci. Res. *13:*1–17.

Sanyal, S. and A. De Ruiter (1985) Inosine diphosphatase as a histochemical marker of retinal microvasculature, with special reference to transformation of microglia. Cell Tissue Res. *241:*291–297.

Schnitzer, J. (1989) Enzyme-histochemical demonstration of microglial cells in the adult and postnatal rabbit retina. J. Comp. Neurol. *282:*249–263.

Schnitzer, J. and J. Sherer (1990) Microglial cell responses in the rabbit retina following transection of the optic nerve. J. Comp. Neurol. *302:*779–791.

Schoen, S.W., M.B. Graeber, L. Toth, and G.W. Kreutzberg (1988) 5'-nucleotidase in postnatal ontogeny of rat cerebellum: A marker for migrating nerve cells? Dev. Brain Res. *39:*125–136.

Schoen, S.W., B. Leutenecker, G.W. Kreutzberg, and W. Singer (1990) Ocular dominance plasticity and developmental changes of 5'-nucleotidase distributions in the kitten visual cortex. J. Comp. Neurol. *296:*379–392.

Schoen, S.W., M.B. Graeber, L. Toth, and G.W. Kreutzberg (1991) Synaptic 5'-nucleotidase is transient and indicative of climbing fiber plasticity during the postnatal development of rat cerebellum. Dev. Brain Res. *61:*125–138.

Schoen, S.W., M.B. Graeber, and G.W. Kreutzberg (1992) 5'-nucleotidase immunoreactivity of perineuronal microglia responding to rat facial nerve axotomy. Glia *6:*314–317.

Schoen, S.W., G.W. Kreutzberg, and W. Singer (1993) Cytochemical redistribution of 5'-nucleotidase in the developing cat visual cortex. Eur. J. Neurosci. *5:*210–222.

Simmonds, H.A. (1991) Purine metabolic disorders and neurological dysfunction. In T. Stone (ed): Adenosine in the Nervous System. London: Academic Press, pp. 247–265.

Sjöstrand, J. (1966) Changes of nucleoside phosphatase activity in the hypoglossal nucleus during nerve regeneration. Acta Physiol. Scand. *67:*219–228.

Sorokin, S.P., R.F. Hoyt, Jr., D.G. Blunt, and N.A. McNelly (1992) Macrophage development: II. Early ontogeny of macrophage populations in brain, liver, and lungs of rat embryos as revealed by a lectin marker. Anat. Rec. *232:*527–550.

Sørensen, J.C., I. Dalmau, J. Zimmer, and B. Finsen (1996) Microglial reactions to tracer identified thalamic neurons after frontal motocortex lesions in adult rats. Exp. Brain Res. *112:*203–212.

Sperlágh, B. and S. Vizi (1996) Neuronal synthesis, storage and release of ATP. Semin. Neurosci. *8:*175–186.

Streit, W.J. (1995) Microglial cells. In H. Kettenmann and B.R. Ransom (eds): Neuroglia. New York: Oxford University Press, pp. 85–96.

Sunderman, F.W. Jr., (1990) The clinical biochemistry of 5'-nucleotidase. Ann. Clin. Lab. Sci. *20:*123–139.

Takahashi, K. and M. Naito (1993) Development, differentiation, and proliferation of macrophages in the ra yolk sac. Tissue Cell *25:*351–362.

Takahashi, K., F. Yamamura, and M. Naito (1989) Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: A light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. J. Leukoc. Biol. *45:*87–96.

Takahashi, K., M. Naito, and M. Takeya (1996) Development and heterogeneity of macrophages and their related cells through their differentiation pathways. Pathol. Int. *46:*473–485.

Thomas, W.E. (1992) Brain macrophages: Evaluation of microglia and their functions. Brain Res. Rev. *17:*61–74.

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

Vela, J.M., I. Dalmau, L. Acarín, B. González, B. Castellano (1995b) Microglial cell reaction in the gray and white matter in spinal cords from jumpy mice. An enzyme histochemical study at the light and electron microscope level. Brain Res. *694:*287–298.

Vordrodt, A.W. and H.M. Wisniewski (1982) Plasmalemma-bound nucleoside diphosphatase as a cytochemical marker of central nervous system (CNS) mesodermal cells. J. Histochem. Cytochem. *30:*418–424.

Wachstein, M. and E. Meisel (1957) Histochemistry of hepatic phosphatases at a physiological pH. Am. J. Clin. Pathol. *27:*13–23.

Wang, D.J., N.N. Huang, and L.A. Heppel (1990) Extracellular ATP shows synergistic enhancement of DNA synthesis when combined with agents that are active in wound healing or as neurotransmitters. Biochem. Biophys. Res. Commun. *166:*251–258.

Weaver, D.R. (1996) A_1-adenosine receptor gene expression in fetal rat brain. Dev. Brain Res. *94:*205–223.

Wisniewski, H.M., A.W. Vordrodt, J. Wegiel, J. Morys, and A.S. Lossinsky (1990) Ultrastructure of the cells forming amyloid fibers in Alzheimer disease and scrapie. Am. J. Med. Genet. Suppl. *7:*287–297.

Wu, P.H. and J.W. Phillis (1984) Uptake by central nervous tissues as a mechanism for the regulation of extracellular adenosine concentrations. Neurochem. Int. *6:*613–1824.

Zimmermann, H (1994) Signalling via ATP in the nervous system. Trends Neurosci. *17:*420–426.

Zoref-Shani, E., Y. Bromberg, G. Lilling, I. Gozes, S. Brosh, Y. Sidi, and O. Sperling (1995) Developmental changes in purine nucleotide metabolism in cultured rat astroglia. Int. J. Dev. Neurosci. *13:*887–896.