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Microglial and astroglial cells in the rat paraclaustral reservoir during postnatal development: an immunohistochemical study

Beata Ludkiewicz, Beata Domaradzka-Pytel and **Janusz Moryś**

Department of Anatomy and Neurobiology, Medical University of Gdańsk, 1 Dębinki St., 80-211 Gdańsk, Poland

Abstract. A immunohistochemical study of postnatal development of the paraclaustral reservoir of migrating cells in the rat brain was performed using anti-GFAP (for astroglia), ED1 and OX-42 (for microglia) antibodies. From birth to the 4th day of postnatal life most GFAP-positive cells in the paraclaustral reservoir are similar to transitional astroglia. From the end of the first postnatal week they have the morphology of mature astrocytes, although during the next week, their density was a slightly higher than in neighboring structures. On the 21st day, the morphology and density of astroglial cells in the ventral part of the external capsule did not differ from the surrounding regions. ED1/OX-42- positive microglial cells present in the paraclaustral reservoir during the first postnatal week represented ameboid microglia; their density was clearly higher than in the neighboring structures. During the second week they began to transform into ramified microglia and from the 21st day on, only OX-42 positive resting microglial cells were observed in the ventral part of the external capsule. We suggest that the paraclaustral reservoir is a place of accumulation of astroglia and microglia during brain development and may possibly serve as source of glial cells for neighboring structures. Alternatively, these glial populations may perform local developmental functions.

Correspondence should be addressed to J. Moryś, Email: jmorys@amedec.amg.gda.pl

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INTRODUCTION

A distinct group of small cells lying in the ventral part of the external capsule of the rat brain is clearly visible at birth. On the basis of its location (medially to the prepiriform claustrum) and function probable as a source of neurons for adjacent structures, we suggest naming this transient nucleus "paraclaustral reservoir" (Maciejewska et al. 1999).

During brain development, neuronal cells generated in the neocortical ventricular zone use two migratory paths: (1) the radial - to the dorsal cortical plate and (2) the lateral (usually called the lateral cortical stream) - to the lateral and ventrolateral cortical plate (Bayer and Altman 1991). Some of the cells generated in the neocortical neuroepithelium, which migrate in the lateral cortical stream for more than four days, accumulate in the paraclaustral reservoir (Bayer and Altman 1991). From there, they penetrate into the piriform cortex and probably into the intercalated masses of the amygdaloid body (Bayer 1980), as well as into the areas of the basal thelencephalon (Bayer et al. 1993). Our previous morphometric investigations showed that the paraclaustral reservoir is most prominent between the 3rd and 5th days of postnatal life and it disappears at the end of the first postnatal week (Maciejewska et al. 1999).

Because the lateral cortical steam represents a pathway for both migrating neuroblasts and glial cells (Bayer and Altman 1991), we can assume that two cell populations, neurons and glia, coexist in the paraclaustral reservoir.

Astrocytes represent the most abundant cell type in the CNS. They are a highly heterogeneous group of cells and their morphology depends on their brain localization, profile of enzymes, antigenic markers as well as on their receptors (Wilkin et al. 1990). Their shape can be modified by physiologic and pathologic stimuli (Guillemin et al. 1997). Astrocytes have diverse functions. The radial cells (developmental type of astroglia) form a network that serves as a scaffolding for nerve cells migrating from their germinal zones towards the developing cortex (Cameron and Rakic 1991, Compston et al. 1997). Astrocytes contribute to the cellular architecture of the CNS and act as a source of energy, nutrient and growth factors for neurons (Tsacopoulos and Magistretti 1996). They have influence on the guidance of nerve impulses, neurite outgrowth, synaptogenesis and synaptic plasticity (Muller et al. 1995), as well as the regulation of the extracellular concentration of ions (Syková et al. 1992). They also participate in the response to injury (Compston et al. 1997).

Microglia, the second group of glia cells, as immunocompetent cells of the CNS play an active role in brain inflammatory, immune and degenerative processes (Minghetti and Levi 1998). In the immature brain, microglial cells show a rounded and simple morphology and are often referred to as ameboid microglia; they are thought to be involved in phagocytosis and removal of degenerating cells during development (Minghetti and Levi 1998). At the next stage of the brain maturation, ameboid cells change their morphology, acquire branched processes and evolve into resting microglia, which are present in the adult brain. These cells "penetrate" the CNS and can be rapidly activated in response to pathological events (Perry 1994, Kreutzberg 1996a,b).

Because we did not find in the literature any information on the developmental changes in the glia organization of the paraclaustral reservoir, the present study considers the morphological changes of the microglial and astroglial cells during maturation in this structure using an immunohistochemical method.

METHODS

The material consisted of 28 rat brains from the Wistar strain, of postnatal ages: P0, P4, P7, P10, P14, P21 and P30. In each group, four animals were studied.

Care and treatment of the animals were in accordance with the guidelines for laboratory animals established by the National Institutes of Health as well as by the Local Ethical Committee of the Medical University of Gdańsk. All animals were deeply anaesthetized with intraperitoneal injection of lethal doses of Nembutal (80 mg/kg of body weight) and perfused using 0.9% solution of NaCl with 1,000 units of heparin (50 ml), followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4; 50-250 ml) at room temperature. The brains were removed immediately from the skull and postfixed in the same solution for up to 24 h. The brains were then kept in 0.1 M phosphate buffer containing 10% sucrose (overnight at 4°C) and 30% sucrose (until sunk). Coronal 50-μm-thick serial sections of the brain were cut on a JUNG 1800 cryostat (Leica, Germany). Every 5th section was stained with cresyl violet. Additional sections were stained with antibodies OX-42 and ED1 (microglial cell detection) and anti-GFAP (astrocyte detection) for 18-20 h at room temperature. Specifications and dilutions of the antibodies are

shown in Table I. The antibody OX-42 was used for detection of complement type 3 receptor. ED1 is a specific macrophage marker of unknown function. Antibody anti-GFAP recognizes glial fibrillary acidic protein. Before incubation with the respective primary antibodies, the sections were pretreated with 1% H_2O_2 for 1 h to block any possible endogenous peroxidase and then with 5% normal serum and 0.2% Triton X-100 in 0.01M PBS for 1 h. After multiple rinses in PBS, sections were incubated with biotinylated horse anti-mouse IgG (1:200) diluted in PBS and 0.2% Triton X-100 for 1.5 h, followed by avidin-biotin peroxidase complex (1:100; Vector Labs, Burlingame, CA) for 1h at room temperature. The bound peroxidase was revealed by incubating the sections in a medium containing 0.05% 3,3'-diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2 for 10 min at room temperature. Rinsing the sections in H_2O stopped the reaction. Sections were finally counterstained with cresyl violet, dehydrated in alcohol, cleared in xylene, and cover-slipped with DPX synthetic resin. Control sections were processed with the omission of primary antibodies.

The number of astroglial and microglial cells in the paraclaustral reservoir was estimated semi quantitatively by counting the number of immunopositive cells in the testing frame.

RESULTS

Astroglial cells in the paraclaustral reservoir during postnatal development

From birth day (P0) to P4 many of the GFAP-immunopositive cells observed in the paraclaustral reservoir possess both long, radial glia-like processes oriented parallel to the white matter fibers of the external capsule as well as shorter astrocytic processes close to the cell body (Fig. 1B and C). Some of the GFAP-immunopositive cells at these stages were similar either to well-differentiated radial glia or to the astrocytes present in the adult. The latter occurred mainly near the boundary of the paraclaustral reservoir. The number of GFAP-positive cells and their fibers was clearly higher than in the surrounding structures (particularly at P0; Fig. 1A).

On P7 and P14, the number of GFAP-immunopositive cells was still somewhat larger than in surrounding structures. They resembled adult astroglia, but their morphology differed slightly from one another. On P7 GFAP-positive cells were stellate and possessed short, stocky processes (Fig. 1D); on P14 they were more elongated with longer, slender processes (Fig. 1E).

After P21 both density and distribution of GFAP-immunopositive cells observed in the ventral part of the external capsule did not differ from the other parts of the external capsule. They showed the typical morphology of fibrillary astroglia and were characterized by a relatively small cell body and slender ramified processes (Fig. 1F). They were elongated with their longitudinal axis lying parallel to the nerve fibers (Fig. 1F).

Microglial cells in the paraclaustral reservoir during postnatal development

From birth (P0) to P4 ameboid microglial cells in the paraclaustral reservoir stained selectively both with ED1 and OX-42 and resembled macrophages with mostly oval and round shapes; their processes resembled filopodia or occasional pseudopodia (Fig. 2B and C). The longitudinal axis of these cells lay parallel to the white matter fibers. At these stages, there were no cells with the morphology of adult microglia. The number of the ameboid microglia cells of the paraclaustral reservoir was clearly higher than in the surrounding white and gray matter (Fig. 2A).

On P7 ED1/OX-42-immunopositive ameboid cells were still oval or round but many of them began to emit short pseudopodial processes (Fig. 2D). The number of these cells in the paraclaustral reservoir was high and

Table I

Specifications and dilutions of the primary antibodies

Antibodies	Clone	Species of immunogens	Manufacturers	Dilution
OX-42	MCA275G	Rat	Serotec	1:100
ED1	MCA341	Rat	Serotec	1:500
GFAP	Polyclonal	Cow	DAKO	1:250

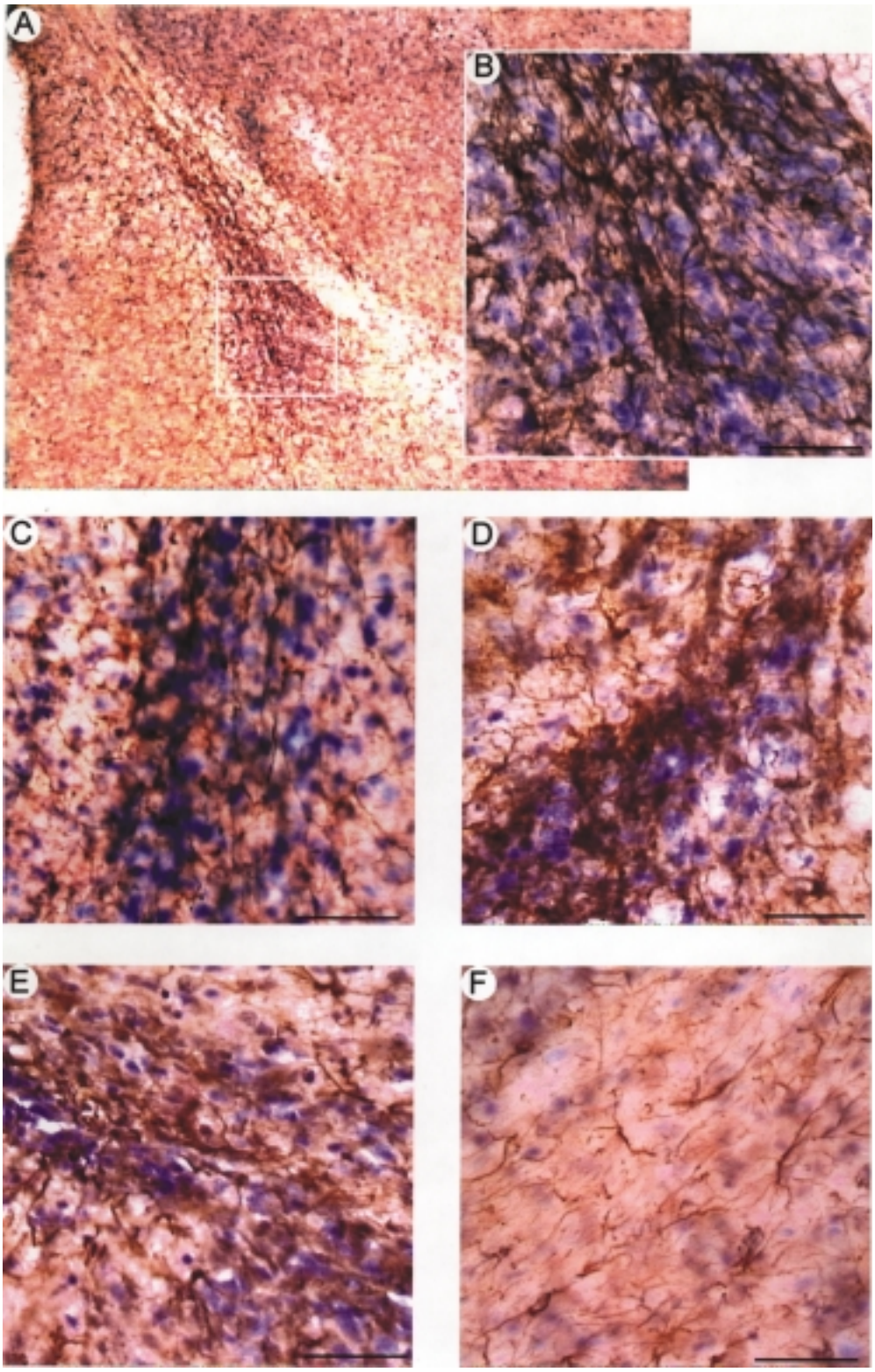

Fig. 1. GFAP-positive cells of the paraclaustral reservoir during postnatal development of the rat brain. A, B (day of the birth; P0) and C, (P4) – many cells possess both long, radial glia-like processes oriented parallel to the white matter fibers as well as shorter astrocitic processes close to the cell body. D, (P7) - stellate cells with short, stocky processes. E, (P14) - elongated cells with slender processes. F, (P21) fibrillary astrocytes in the ventral part of the external capsule. Scale bars equal to 250 μm (A) and 50 μm (B, C, D, E, F).

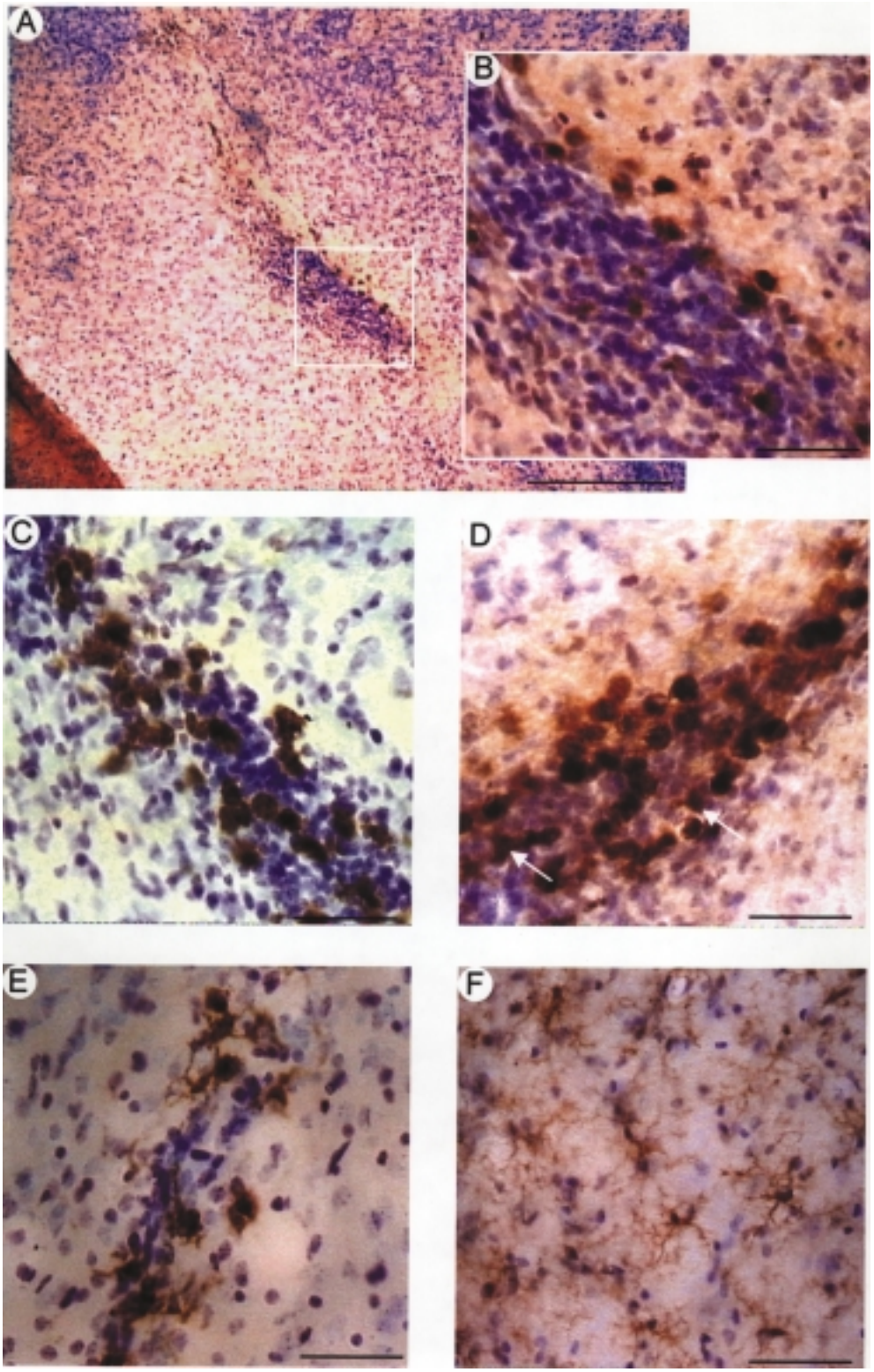

Fig. 2. Microglial cells of the paraclaustral reservoir during postnatal development of the rat brain. A, B (day of the birth; P0) and C, (P4) – ED1/OX-42-positive ameboid cells, oval or round in shape with filopodia or occasional pseudopodia. D, (P7) - ED1/OX-42-positive ameboid cells - many emit short pseudopodial processes (arrows). E, (P14) - ED1/OX-42-positive ramified ameboid microglia with variable number of short, thick processes. F, (P21) - OX-42-positive cells in the ventral part of the external capsule possess numerous bushy branching processes. Scale bars equal to 250 μm (A) and 50 μm (B, C, D, E, F).

still clearly differed from the surrounding white and gray matter.

During the second week of postnatal life the number of ameboid ED1/OX-42-immunopositive cells in the paraclaustral reservoir decreased gradually. On P14 microglial cells (referred to as ramified ameboid microglia) were oval or multiform and possessed a variable number of short, thick processes (Fig. 2E).

At P21 the ameboid ED1/OX-42-immunopositive cells did not occur in the ventral part of the external capsule; starting from this stage only OX-42-immunopositive ramified microglia were observed. These cells possessed numerous bushy branching processes (Fig. 2F). Their distribution and density did not differ from the other parts of external capsule.

DISCUSSION

Our results show that during postnatal development, besides neuronal cells, both microglial and astroglial populations coexist in the paraclaustral reservoir.

Astroglial cells of the paraclaustral reservoir

In our study, from birth (P0) astroglial cells of the paraclaustral reservoir are GFAP-positive. From the day of birth to P4 most of GFAP-immunopositive cells in the paraclaustral reservoir possessed both long, radial glia-like processes oriented parallel to the white matter fibers of the external capsule as well as shorter astrocytic processes close to the cell body. This astroglia phenotype is reminiscent of that classically described by Cameron and Rakic (1991) as transitional astroglia. Starting on P7, GFAP-positive cells of the paraclaustral reservoir showed the morphology of astrocytes present in the adult. The pattern of the morphological changes of astroglia present in the paraclaustral reservoir was similar to that observed by Hunter-Schaedle (1997) and Bignami (1991) in other regions of the brain. Similarly, fully mature astrocytes did not start appearing in developing cerebral cortex until P6 (Hunter-Schaedle 1997).

The earliest form of astroglia that appears during embriogenesis is radial (Hunter-Schaedle 1997). Because it is widely accepted that functions of radial glia cells are exclusively developmental, they undergo dramatic changes of phenotype and function, transforming into mature astrocytes (Hunter-Schaedle 1997). The transformation of radial glia into the paraclaustral reservoir probably begins before birth, because on P0 many cells show the phenotype of transitional astroglia. On P7 many of paraclaustral reservoir astrocytes resemble protoplasmatic astroglia (multipolar, stellate cells) – they are localized mainly near boundaries and some of them probably migrate to the surrounding gray matter structures. At P21 astrocytes observed in the ventral part of the external capsule possess a morphology typical of white matter astrocytic cells classically called fibrous. They can originate either from radial glia of the paraclaustral reservoir or directly from glial precursors generated in the ventricular zone and traverse the paraclaustral reservoir mainly after P4 (Maciejewska et al. 1999).

As suggested previously by Ramon y Cajal (1911) and confirmed by other authors (Angevine and Sidman 1961, Rakic 1972, O'Rourke et al. 1995) radial glia are strictly related to neuronal migration. The radial network coincides with neuronal histogenesis suggesting that this serves as a scaffolding for nerve cell migrating from their germinal zones towards the developing brain structures (Compston et al. 1997).

As we have described previously (Maciejewska et al. 1999), during the postnatal period, the majority of neurons reach the paraclaustral reservoir between P0 and P4; the last neurons leave this structure before P7. This is coincident with the appearance of GFAP-positive cells showing features of radial glia - after P7 these were not observed in the paraclaustral reservoir. Besides structural function, radial glia provide a cellular substrate which supports and directs the migration of young neurons (Hunter-Schaedle 1997). Astroglia are implicated in neurite outgrowth and synaptic plasticity (Muller et al. 1995) as well as provide energy, nutrient and growth factor support for neurons (Tsacopoulos and Magistretti 1996, Compston et al. 1997).

Astroglia are probably also involved in microglia development. Sievers et al. (1996) put forward the hypothesis that astrocytes are able to regulate microglial morphology and functional state. Their experimental study shows that *in vitro* astrocytes are necessary for transformation of microglia from the ameboid to the ramified form. Taking this into consideration, we suggest that the astroglial population of the paraclaustral reservoir may play a significant role in the morphological transformation of microglial cells.

Microglial cells of the paraclaustral reservoir

Microglia present at the first stage of postnatal maturation constitute a population of ameboid cells which

have morphological, histochemical, and immunological features similar to those of macrophages outside the brain; for that reason many authors call them brain macrophages (Ling and Wong 1993, Wang et al. 1996, Dalmau et al. 1997). In animal studies, macrophages have been described as a heterogeneous population, consisting of two main classes: primitive/fetal macrophages and monocyte-derived macrophages. It was later postulated that primitive fetal macrophages, which originate from the yolk sac, colonize the CNS early in development and remain thereafter as resident macrophages/ microglia (Dalmau et al. 1997). The other group of macrophages, which derive from circulating monocytes, with advancing age undergo the transitory stage - ameboid microglial cells residing mainly in loosely organized subcortical white matter and circumventricular regions (Ling 1976, Xu and Ling 1994, Earle and Mitrofanis 1997) . According to Milligan et al. (1991), Wang et al. (1996), as well as Ling and Wong (1993) these cells show vigorous expression of complement receptor type 3 (CR3) and proteins of unknown function (ED1).

Microglial cells present in the paraclaustral reservoir during the first two weeks of postnatal life exhibit immunoreactivity to complement receptor type 3 (marker: antibody OX-42) and macrophage proteins of unknown function (marker: antibody ED1). Until P7, they are small in size, oval or round in shape, usually without processes. According to the nomenclature of the Dalmau et al. (1997) they represent ameboid microglia types 2 and 3. During the second postnatal week ED1/OX-42-positive microglial cells change their morphology and began to emit short processes – Dalmau et al. (1997) described them as primitive ramified microglia. After P21 only OX-42-immunopositive, ramified cells were observed in the ventral part of the external capsule. This is in accordance with the observation of Chugani et al. (1991) who described that in other brain regions this transformation of ameboid into ramified microglia takes place between postnatal days 4 and 14.

During the first two postnatal weeks, the paraclaustral reservoir is a place of accumulation of the ameboid microglial cells – their density is clearly higher than in the neighboring part of the external capsule and surrounding structures. Hurley and Streit (1996), using lectin histochemistry, identified eight ameboid microglial cell clusters in the rat postnatal forebrain - each of these is first apparent on P0 and disappears by P13. Ling (1976) described concentrations of ameboid microglia in the corpus callosum of the neonatal rat. In his opinion, this specific location may possibly serve as a resting-place before the cells migrate to their adult positions. Alternatively, these cells may be involved in local development. Similarly, the paraclaustral reservoir could serve as a "waiting room" for microglial cells migrating to the other brain structures, or ameboid microglial cells accumulated in the paraclaustral reservoir could perform local developmental functions.

Ameboid microglia are also implicated in neuronal migration (Perry and Gordon 1988, Nakajima and Kohsaka 1993) . Moreover in culture these cells can synthesize some molecules with inherent neurotrophic and neuronal differentiating activities (Mazzoni and Kenigsberg 1997) such as nerve growth factor, basic fibroblast growth factor and interleukin-3 (Mallat et al. 1989, Shimojo et al. 1991, Gebicke-Haerter et al. 1994) . Consequently, in the paraclaustral reservoir, ameboid microglial cells can potentially influence the survival and migration of neuronal cells that migrate to the piriform cortex and probably into the intercalated masses of the amygdaloid body (Bayer 1980), as well as into areas of the basal telencephalon (Bayer 1993).

Although association of microglia with developmental cell death was observed in some brain regions (Ashwell and Bobryshev 1996), it seems that concentration of amebiod microglial cells in the paraclaustral reservoir is not closely connected with apoptosis, because our previous investigation showed a rather low number of apoptotic cells in this structure (Maciejewska et al. 1999). According to Milligan et al. (1991) and Cuadros et al. (1997), the distribution of microglia during development frequently does not correlate with physiological neuronal death, but rather with the phagocytic activities in the course of cell differentiation and with remodeling of their connections (Wang et al. 1996). Although some neurons in the paraclaustral reservoir can probably serve as transient targets for reciprocal cortico-subcortical connections and eventually die after they fulfill their developmental function (Maciejewska et al. 1999), it is possible that ameboid microglia of the paraclaustral reservoir can act to scavenge redundant cells.

The localization of the paraclaustral reservoir in the external capsule implies an influence of its microglial population on developing white matter fibers. According to Hamilton and Rome (1994) white matter microglia may play a direct role in either generation of oligodendrocytes or in the early stage of myelinogenesis. *In vitro*, microglia can stimulate the synthesis of my-

elin-specific protein (Ling et al. 1991). Initial contact between microglia and this protein could be established in early prenatal life and may play a role in development of autoimmune diseases later in life (Andjelkovic et al. 1998). The other possible role of microglia from the paraclaustral reservoir is the elongation of external capsule tract axons, as such a role is observed in the developing human spinal cord (Hutchins et al. 1992).

Ameboid microglia of the paraclaustral reservoir may play a role in the development of the blood-brain barrier as was postulated by Earle and Mitrofanis (1998). According to these authors, a transient population of ameboid microglial cells present in the internal capsule during the first three weeks of postnatal life is closely associated with the vasculature and together with astrocytes and radial glia may play a role in the development of the blood-brain barrier in this white matter region.

After P21 only OX-42-immunopositive, ramified cells have been observed in the ventral part of the external capsule. Their density did not differ from neighboring parts of external capsule. Thus, the question arises what happens to ameboid microglial cells of the paraclaustral reservoir? According to our previous observations with the use of the TUNEL method in which we did not observe much apoptotic cell death, we suspect that most of the ameboid microglia transform into ramified microglia during development (Heiskari et al. 1986). On the other hand, we cannot exclude the possibility of death of some ameboid microglial cells of the paraclaustral reservoir as was described in other region of the brain by Imamoto and Leblond (1978).

To sum up our previous (Maciejewska et al. 1999) and present investigations, we suggest that the paraclaustral reservoir is a place of transient accumulation for both migrating neurons as well as glia (microglia and astrocytes). Neuronal population of this structure disappears about P7, whereas the glial population disappears over a week later.

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