Histochemistry (1990) 94:441–447

Histochemistry

Light microscopic histochemistry of the postnatal development and localization of carbonic anhydrase activity in glial and neuronal cell types of the rat central nervous system

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Received March 7, 1990; Accepted May 12, 1990

Summary. The postnatal expression of carbonic anhydrase (CA) activity in glia and neurons was investigated by a modified light microscopical method of Hansson. Strong CA activity was observed during the first postnatal week in ameboid microglia, clustering in the cingulum, the periaqueductal region, the roof of the lateral ventricles and the white matter of the cerebellum. The intensity of staining gradually decreased during the second week and finally disappeared. From the 9th postnatal day on, cerebellar Purkinje cells expressed strong CA activity, which completely disappeared by the end of the investigation period. CA staining of the oligodendrocytes and pericytes could be observed from the first postnatal day on. The present results raised the possibility that carbonic anhydrase activity may play a role in the regulation of the development and proliferation of some cell types, perhaps via intracellular pH changes.

Introduction

The enzyme carbonic anhydrase (CA; E.C. 4.2.1.1.) is widely distributed in different cells of animals, plants and certain bacteria (Giacobini 1961; Hansson 1967; Korhonen and Hyyppa 1967; Maren 1967; Nógrádi and Mihály 1988). It plays an important role in acid secretion by the kidneys, removal and transport of carbon dioxide produced by glycolysis, but its function is still unknown in some biochemical processes (Deutsch 1987).

Since Van Goor (1948) first reported carbonic anhydrase in brain tissue, it has been localized histochemically in glial and choroid plexus cells (Giacobini 1961; Korhonen et al. 1964; Nógrádi and Mihály 1988), in pericytes (Kazimierczak et al. 1986), in sensory ganglion cells (Riley et al. 1984; Kazimierczak et al. 1986), in the wall of capillaries and in some axons (Riley et al. 1984; Nógrádi and Mihály 1988). Although the neurons in the mesencephalic nucleus of the trigeminal nerve contain the enzyme (Aldskogius et al. 1988) these nerve cells are of peripheral origin, migrating into the brainstem during the embryonic life (Lieberman 1976). The only central neurons that display detectable CA activity are the granule cells of the islets of Calleja in the adult rat (Nógrádi et al. 1989).

Biochemical studies on isolated oligodendroglia and brain homogenates suggested the increase of CA activity during postnatal development (Nair and Bau 1969; Delaunoy et al. 1980; Sapirstein et al. 1980; Snyder et al. 1983; Davis et al. 1987) supporting the functional role of the enzyme in the myelination process (Sapirstein et al. 1978). The rapid postnatal increase in the number of astrocytes and oligodendrocytes also indicates the importance of CA in developmental processes (Ling and Leblond 1973; Hirano and Goldmann 1988), because CA was believed to be a specific marker for oligodendrocytes (Ghandour et al. 1979; Roussel et al. 1979) and recently CA has been observed in protoplasmic astrocytes (Cammer and Tansey 1988). The presence of the enzyme was also indicated in the radial glial cells during the prenatal ontogenesis (Hirano and Goldmann 1988).

Considering the postnatal development of histochemically detectable CA activity in the rat CNS, no systematic attempts have been made so far. The aim of the present study is to reveal some information about glial cell ontogenesis and to elucidate the possible role of the enzyme during the neural maturation.

Materials and methods

Wistar rats pups ($n=30$) were perfused via the left ventricle of the heart with ice-cold 0.1 *M* phosphate-buffered 2.5% glutaraldehyde solution (pH 7.4) at the ages 1, 3, 4, 5, 7, 9, 11, 14, 17, 22, 24, 26, 28, 30, 32 days postnatally. The cerebrum, the brainstem, the cerebellum and the spinal cord were removed and postfixed for 8–10 h in the same fixative. Then the tissue was sliced and rinsed in 0.05 *M* phosphate buffer containing 0.2 *M* sucrose (pH 7.4). 15-μm-thin consecutive sections were cut on a freezing

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microtome, collected in the same buffer and mounted onto the outer surface of a tubular dialysis membrane (pore size approx. 2.5 nm, Union Carbide), as previously described (Mihály et al. 1988). The dialysis tube was filled with the incubation medium (1.75 m*M* $CoSO_4$ + 11.7 m*M* KH_2PO_4 + 157 m*M* $NaHCO_3$ + 53 m*M* H_2SO_4; pH 6.9, after Hansson (1967) and Brown (1980)). Since the sections were on the outer surface of the membrane, the continuous aeration was secured (Mihály et al. 1988). The specificity of the histochemical reaction has been controlled by adding 10^{-4} *M* acetazolamide to the incubation medium. Incubation time was 25 min at room temperature. Then the sections were rinsed in phosphate buffered saline (0.67 m*M* NaCl in 0.1 *M* phosphate buffer, pH 5.9, after Brown (1980)) and immersed in 0.5% Na_2S in distilled water, dehydrated and mounted on gelatinized slides. Every 5th section was stained with cresyl-violet and the brain structures showing CA activity were identified with the help of a stereotactic atlas (Sherwood and Timiras 1970).

In separate experiments, non-specific esterase (NSE) has been localized and its activity compared to that of CA. Rat pups ($n=10$) were perfused transcardially with Baker's formol-calcium at the ages 1, 2, 3, 5, 7 days postnatally. 20-μm-thin frozen sections were cut from the cerebrum, cerebellum, brainstem and spinal cord and incubated in a medium containing 1-naphthyl-acetate and freshly prepared hexazotized pararosaniline as a coupler (Davis and Ornstein 1959). Incubation time was 15 min at room temperature. After incubation the sections were rinsed in distilled water, dehydrated and coverslipped. Some of them were counterstained with 2% methyl green.

The different macroglial cell types were distinguished by using the classical light microscopical (Golgi 1894; Schroeder 1929) and more detailed fine structural descriptions (Ling et al. 1973). The basis of classification of these cells was the size of their cell body, the form and distribution of their processes and the intensity of CA staining (Giacobini 1961; Lönnerholm 1974).

Results

On the 1st postnatal day the neuronal cell bodies were nonreactive in contrast to the glial cells which exhibited different degrees of CA activity. Apart from oligodendroglia and astrocyte-like forms (Figs. 1, 2), a great number of intensely stained cells bearing short, simple processes were present (Fig. 3). Characteristic microglia with finely branching processes could never be seen, therefore they were considered as the ameboid form of microglia (Ling 1976; Ivy and Killackey 1978; Ling et al. 1982; Innocenti et al. 1983). These cells were clustered in discrete regions of the brain, i.e. in the medial periventricular zone of the wall of the lateral ventricles and in the cingulum (Figs. 4, 5). They were scattered in the white matter, too. The ameboid cell clusters showed intense NSE-staining (Fig. 6). In the wall of blood vessels pericyte-like cells showed CA-positivity. Occasionally, astrocyte-like cells were anchored to the vessels. No axonal staining was observed at this stage.

Reactive glial cells with fine processes could be observed in the pons and the medulla surrounding the unstained axons and neurons. The CA-positive ameboid microglia cells were present only around the 4th ventricle and the central canal (Fig. 7). None of them possessed long, fine processes.

Only a few CA-reactive microglial cells were seen around the central canal in the spinal cord. Ependymal cells displayed no CA-staining. In the cerebellar white matter a few oligodendroglial cells and clusters of heavily stained ameboid microglial cells were found, while in the grey matter blood vessels exhibited CA-staining.

On the 3rd day the overall pattern of CA-staining was similar. However, the number of CA-positive ameboid microglia cells considerably decreased in the periventricular regions. On the other hand, the raphe in the brainstem contained branching, strongly stained cells with different morphology. Most of them had simple processes resembling the ameboid microglia, but some of them gave rise to long, fine branches. No stained microglial cells were present around the central canal of the spinal cord, although the white and the gray matter displayed glial- and blood vessel CA activity. The distribution of NSE-containing cells was similar to that of the CA-stained microglia.

In 5 to 7-day-old animals the number of ameboid microglia cells around the lateral ventricles further decreased. On the contrary, no decrease was seen in the cingulum of the corpus callosum, where the microglia cells persisted, changed their shape and developed long, fine processes (Fig. 9). NSE could be localized in these cells. In the brainstem small decrease in the number of heavily stained periaqueductal microglia cells could be observed. Some of the ependymal cells lining the central canal in the medulla oblongata started to exhibit CA reactivity on the 5th postnatal day. The cells lining the middle part of the lateral walls of the central canal exhibited stronger activity compared to the cells covering the anterior and posterior poles of the lumen (Fig. 8). The first appearance of CA-stained epithelial cells of the choroid plexus was seen on the 4th postnatal day.

On the 9th day a peculiar staining pattern appeared in the cerebellar cortex: the developing Purkinje cells began to show CA-staining, strongest in the perikarya, but also detectable in their dendrites and axons. Apart from the Purkinje neurons, oligodendroglia cells were also stained (Fig. 10). The first signs of axon staining were seen on this day in the cerebrum, and later this staining pattern continuously strengthened.

In 11-day-old animals the CA activity of microglia disappeared, but the number of other CA-positive glial cells increased considerably. Strongly stained oligodendroglia cells were present in the white matter and faintly stained astrocytes were found in the grey matter. The CA activity of the Purkinje cells became stronger; they formed a contrasted line between the weakly stained molecular layer and the unstained granule cells (Fig. 11). At this age all the ependymal cells of lateral ventricles showed temporarily increased activity (Fig. 12).

From the 14th day on the features of CA activity in the investigated brain areas were very similar to that

Fig. 1. Strongly stained oligodendroglial cell in the subcortical white matter of an 1-day-old pup. Not only the cell body but also the fine processes were CA reactive. *Bar* = 10 μm

Fig. 2. Pale staining showing astrocyte-like cell in the developing neocortex. The fine, branching processes (*arrows*) also showed CA-staining. *Bar* = 10 μm

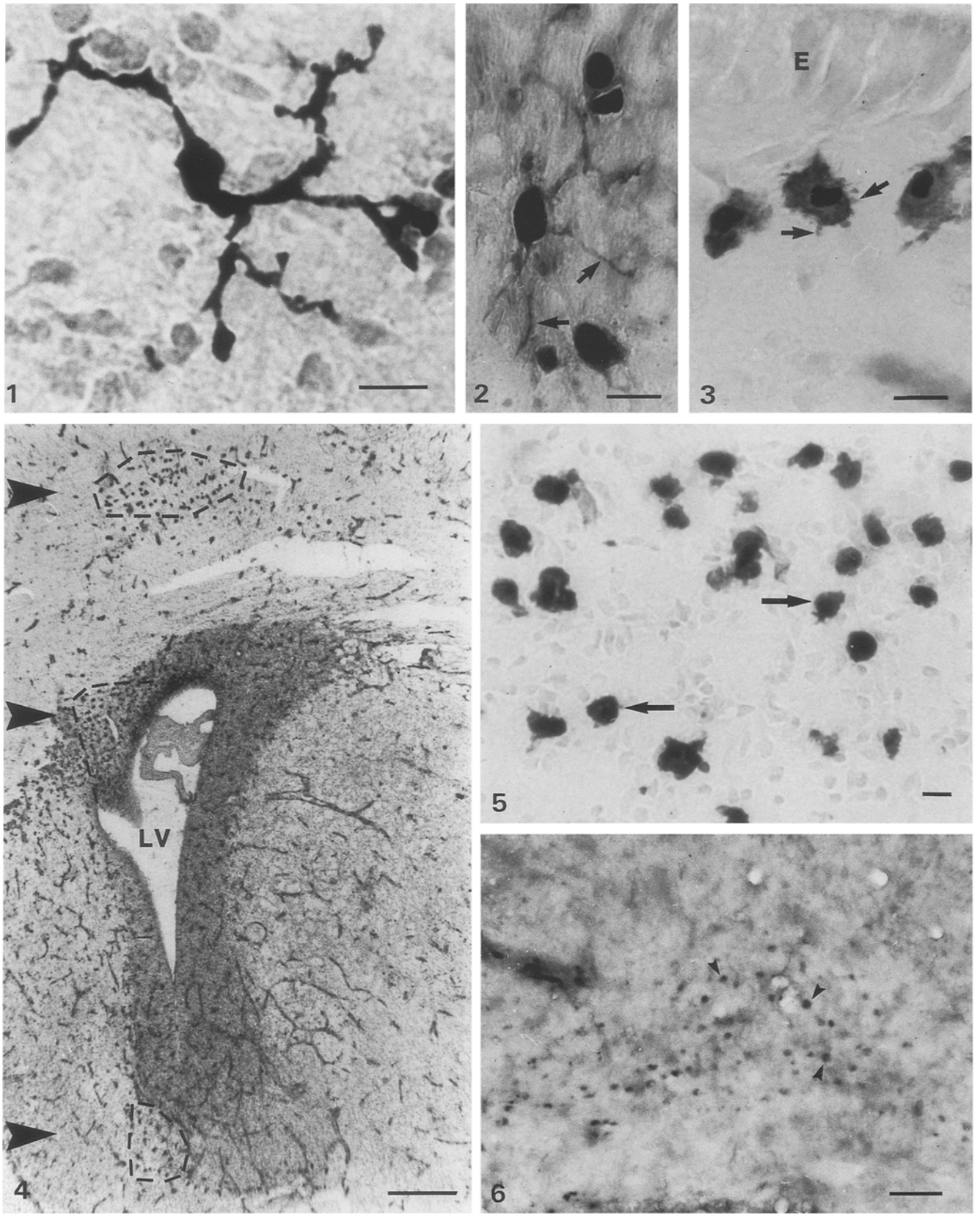
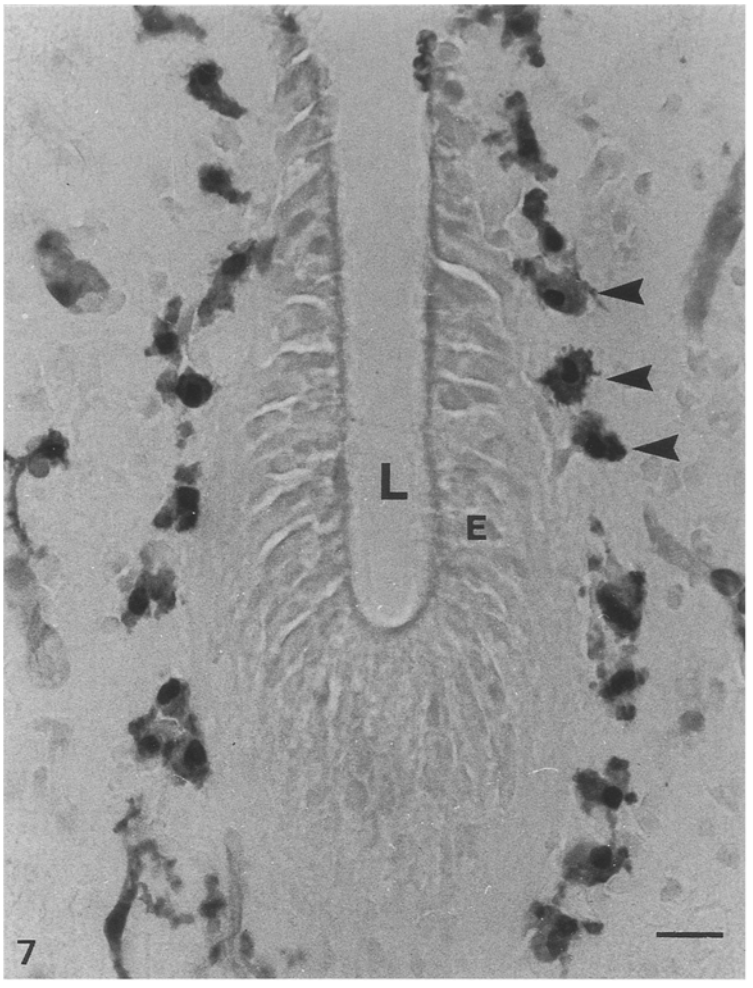


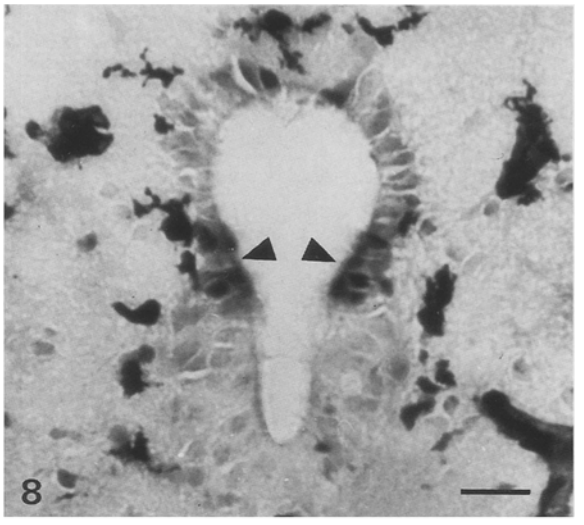
Fig. 3. Two CA-reactive ameboid microglial cells near the ependymal cell lining (E) of the IVth ventricle of an 1-day-old pup. Note the fine, simple processes (*arrows*) of the microglial cells. *Bar* = 10 μm

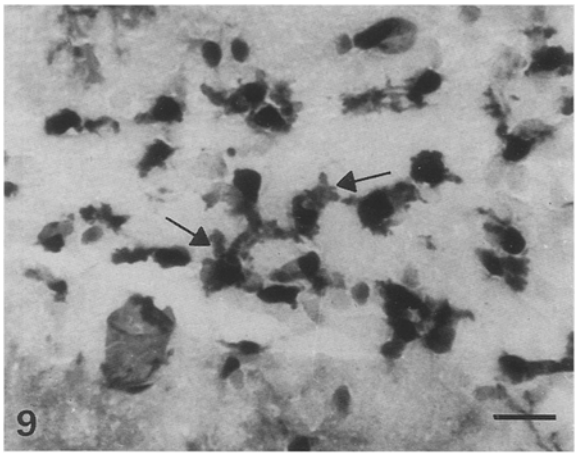
Fig. 4. Low magnification picture of the periventricular region of the lateral ventricle (LV) (1-day-old animal). *Scattered lines* represent the margins of ameboid cell clusters in the cingulum of the corpus callosum and in the medial periventricular zone (*arrowheads*). *Bar* = 250 μm

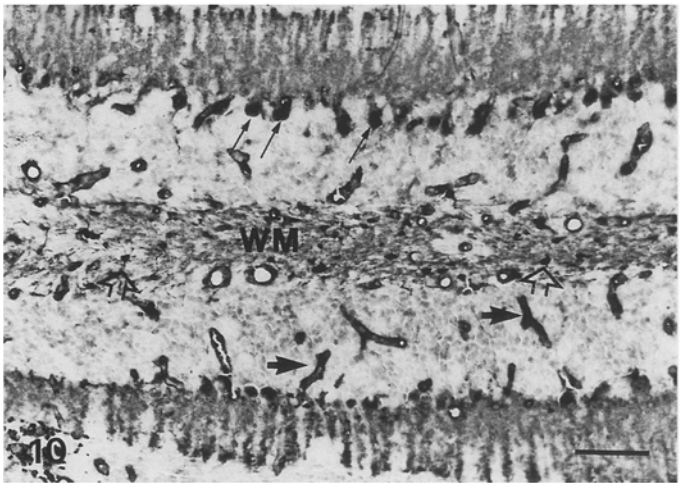
Fig. 5. CA-stained ameboid microglia cells clustering in the cingulum of a 3-day-old animal (*arrows*). *Bar* = 10 μm

Fig. 6. Ameboid microglial cells showing strong NSE activity in the cingulum of a 3-day-old pup. *Bar* = 100 μm









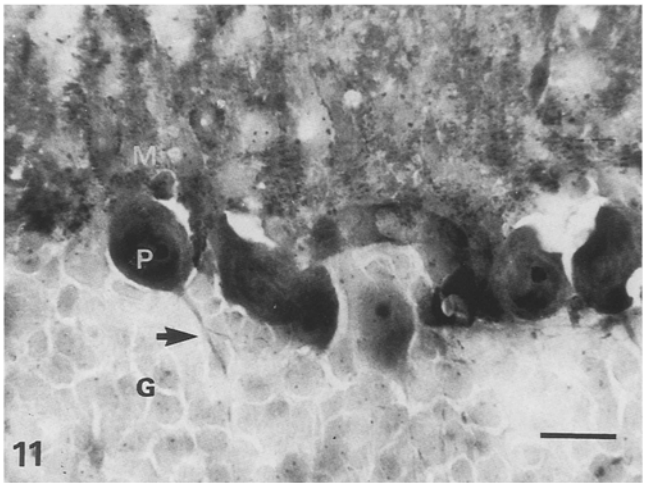


Fig. 7. CA-reactive ameboid microglial cells (*arrowheads*) surrounding the lumen of the IVth ventricle (*L*). The microglial cells were closely related to the ependymal cell (*E*) lining of the ventricle. *Bar* = 20 μm

Fig. 8. Staining pattern of the ependymal cells of the central canal in oblongated medulla (6-day-old pup). The ependymal cells lining the middle part of the lateral wall of the central canal showed strong CA reactivity (*arrowheads*). *Bar* = 20 μm

Fig. 9. Transforming ameboid microglial cells in the cingulum of a 5-day-old pup (*arrows*). Note the fine, branching processes of CA-reactive cells. *Bar* = 15 μm

Fig. 10. Low power micrograph from the cerebellum of a 14-day-old animal. The Purkinje cells (*thin arrows*) and the molecular layer contain the reaction product, in the latter presumably the dendrites of Purkinje cells were stained. In the white matter (*wm*) oligodendrocytes (*outlined arrows*) and capillaries (*thick arrows*) could be seen. *Bar* = 100 μm

Fig. 11. Higher magnification of the developing cerebellar cortex. Note the diffuse cytoplasmic staining of the Purkinje cells (*P*) between the slightly reactive molecular layer (*M*) and the non-reactive granule cells (*G*). The axons of the Purkinje cells (*arrow*) were stained, too. *Bar* = 20 μm

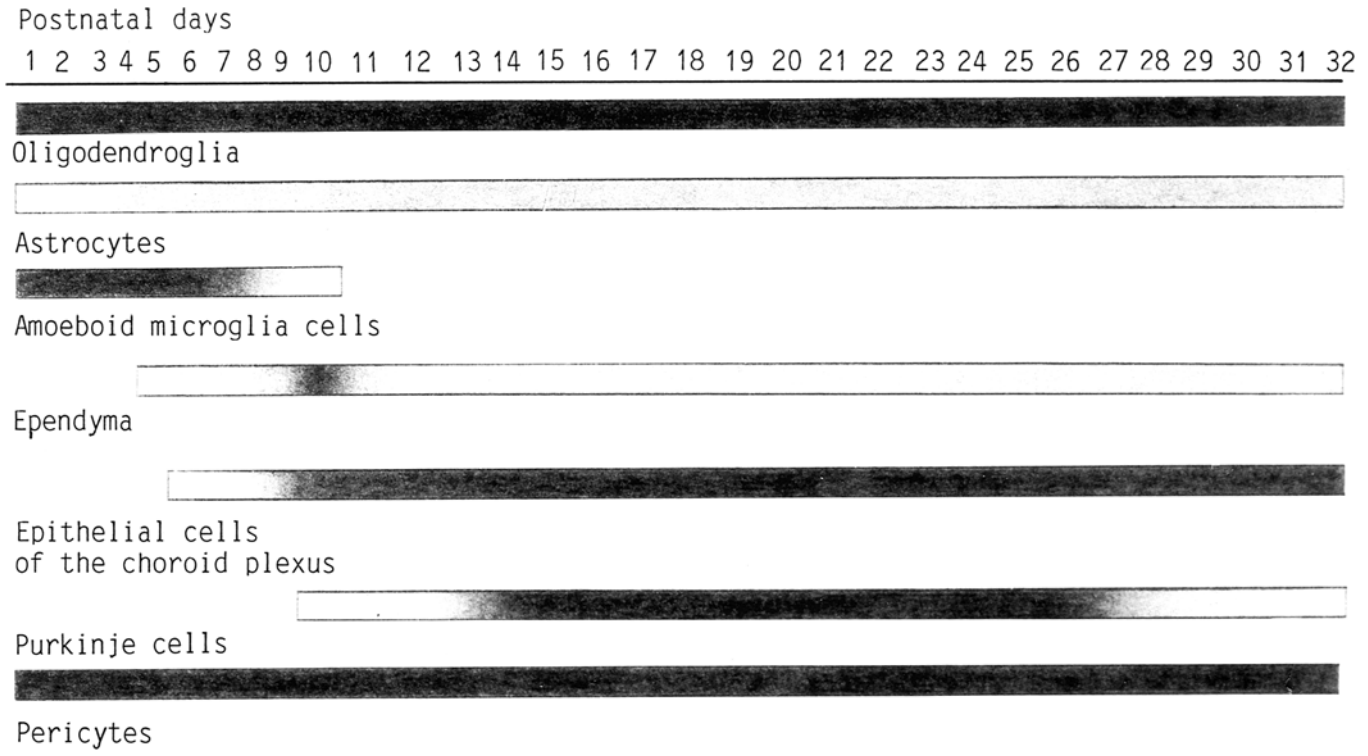


Fig. 12. Schematic diagram of temporal changes in CA reactivity of different cell types in the developing CNS. Note the strong reactivity of oligodendrocytes and pericytes already in newborn rats

of the adult (Korhonen et al. 1964, Nógrádi and Mihály 1988). The only exception was the CA staining of the Purkinje neurons, which was detected continuously till the 26th day. From this time it started to decrease and completely disappeared by the 32nd day.

10^{-4} *M* acetazolamide completely inhibited the enzyme reaction: none of the above described staining patterns were seen. Only the nucleoli of neurons and glia showed faint, greyish staining.

Discussion

Our histochemical results confirmed the biochemical data on the early appearance of CA activity during the postnatal development in the CNS (Sapirstein et al. 1978; Snyder et al. 1983). We have already shown in the 1-day-old CA containing glial cells, pericytes and ameboid microglia cells in the developing rat brain. One may expect therefore that at least some of these cells were present prenatally. Indeed, CA containing radial glial cells were reported in the spinal cord at the 18th embryonic day (Hirano and Goldman 1988), and it was suggested that these cells may be the origin for the oligodendroglia (Skoff 1980; Hirano and Goldman 1988). Contrary to this, Davis et al. (1987) have biochemically detected CA activity in oligodendrocytes of the optic nerve only from the 5th postnatal day, but this may be characteristic only for the optic nerve. Though astrocytes increase in number in the late prenatal and early postnatal period, oligodendrocytes seem to proliferate mainly in the postnatal period and myelinogenesis follows the onset of oligodendrogliosis (Tennekoon et al. 1977). This fact would serve the explanation for the absence of axonal staining till the 9th postnatal day. On the other hand, CA-IV, the membrane-associated carbonic anhydrase was reported to be fully expressed by the 16th postnatal day (Sapirstein et al. 1978), though the myelin membrane is still maturing at this age. Thus the histochemically observed axon-staining is in accordance with biochemically detected CA-IV levels. We have observed an increase in the intensity of the histochemical reaction and in the number of CA reactive glial cells, mainly oligodendrocytes during the postnatal development which is in agreement with the literature data regarding the postnatal increase in number of glial cells in young animals (Ling and Leblond 1973).

CA has been believed to be an exclusive marker for oligodendrocytes, but in the case of astrocytes there was some controversy. The CA-staining of astrocytes was denied by several authors (Ghandour et al. 1979; Roussel et al. 1979), while recently CA containing protoplasmic astrocytes were found in double-immunostained specimens (Cammer and Tansey 1988). Similarly to this observation we found astrocyte-like cells in the developing CNS; sometimes their end-feet were seen surrounding blood vessel, too.

The ameboid microglial cells appear in the late prenatal age in the white matter of the cerebrum and cerebellum, in the gray matter of the brainstem and spinal cord and seem to disappear on the second postnatal week (Ling 1976; Matsumoto and Ikuta 1985). They were reported to show non-specific esterase enzymatic activity, and according to this they are considered to

take origin from blood macrophages (Ling et al. 1982; Perry and Gordon 1988; Streit et al. 1988). Since the cell clusters showing NSE activity were highly reactive for CA, too, we considered that ameboid microglial cells contain CA. Their typical shape (Ling 1976) and distribution in the CNS (Ling 1976; Matsumoto and Ikuta 1985; Perry and Gordon 1988) provided further possibility to distinguish them from the other glial cell types. Their functional role in the developing nervous system is thought to remove debris and degenerated axons (Jordan and Thomas 1988) which is in accordance with their disappearance at the end of myelinogenesis. On the other hand, pericytes are also thought to be a special form of brain macrophages, possessing strong CA activity (Kazimierczak et al. 1986; Nógrádi and Mihály 1988). The fact, that transforming ameboid microglia cells showed no longer CA activity, whilst pericytes may preserve that, requires further investigations in order to ascertain the real function of CA in CNS macrophages.

The maturation of the Purkinje cells can be divided into several phases (Altman 1972). According to the first phase the Purkinje cells are distributed in the developing cerebellar cortex between the molecular layer and the white matter. At the fourth day after birth the majority of the Purkinje cells become dispersed in a monolayer. Later, at the 8-day-old animal these cells have well-developed apical cones, and at this stage appears the first sign of the glial covering of the Purkinje cells (Altman 1972). Although the migration of these cells terminates for this age, the maturation process lasts till the 21st postnatal day. Recent studies raised the possibility that the initiation of cell growth may depend on intracellular pH, too (Thomas 1989). This involves the Cl-HCO_3 exchange mechanism, because bicarbonate is the best buffer for intracellular pH regulation. This fast buffering needs carbonic anhydrase. Thus, supposing a strong metabolic activity during maturation, the temporary activation of CA is not so surprising. However, intracellular pH seems to be too fundamental to regulate growth processes directly, whilst growth factors may stimulate the pH-regulation of the cell (Boron 1984) in order to satisfy the increased metabolic requirements during development. Though we possess no considerable information about the expression and supression mechanism of CA genes (Deutsch 1987), it is not inconceivable that growth factors can activate CA genes through one or more messengers.

The nucleolar CA-staining did not disappear after 10^{-4} M acetazolamide treatment, though acetazolamide proved to be a specific inhibitor of the enzyme (Lönnerholm 1974). The nucleolar appearance of the cobalt precipitate may be due to the unspecific binding of the cobalt-phosphate complex to the proteins of the nucleolus (Häusler 1988).

Our results provided evidence for the functional role of CA in the developing CNS. The early postnatal CA reactivity of macroglial cells and the transient CA-staining exhibited by brain macrophages suggest the importance of the enzyme in the regulation of developmental processes. The appearance of Purkinje cell CA activity is thought to be put in action by genome mechanisms, which requires further molecular investigations. The verification of CA activity of different types of microglia is just under way in our laboratory.

Acknowledgements. The authors are indebted to Prof. B. Csillik for criticism of the manuscript. The technical assistance of Mrs. K. Lakatos, Miss I. Bódi and Mr. István Faragó are greatly appreciated.

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