Acta Neuropathol (1999) 97 : 359–368

REGULAR PAPER

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Progressive parenchymal deposition of β-amyloid precursor protein in rat brain following global cerebral ischemia

Received: 3 July 1998 / Revised, accepted: 6 October 1998

Abstract In addition to producing acute neuronal necrosis within selectively vulnerable brain regions, our recent studies have shown that global cerebral ischemia may also be followed by protracted degenerative changes occurring over the course of 10 weeks. Chronic brain pathology may be associated with the abnormal deposition of β-amyloid precursor protein (βAPP). In the present study, we used a monoclonal antibody to the N-terminal portion of βAPP to characterize the brains of rats surviving 1–10 weeks following 10 min of global brain ischemia produced by bilateral carotid artery occlusions plus systemic hypotension. After ischemia, increased βAPP immunolabeling emerged in several brain regions. In the hippocampus, granular deposits appeared in the damaged CA1 area by 2 weeks, and by 4–10 weeks the remnants of necrotic CA1 neurons were also immunolabeled. In striatum and thalamus, regions with necrotic cell death also revealed granular βAPP deposits. The neocortex was devoid of overt ischemic neuronal damage but revealed prominent βAPP immunoreactivity. Large ovoid deposits of low-density βAPP immunostaining occurred in cortical neurons at 1–2 weeks. At 4–10 weeks, large round or oval deposits immunoreactive for βAPP appeared in several cortical regions. The highest density of deposits was seen in the temporal and piriform cortices. Our results indicate that abnormal βAPP deposition may result from ischemic as well as chronic neurodegenerative processes.

Key words Amyloid · Immunochemistry · Chronic pathology · Neurodegeneration

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Introduction

Global brain ischemia typically gives rise to selective neuronal changes in vulnerable areas within the first few days after ischemia [8, 34]. More recently, however, it has become recognized that neuropathological processes may continue well beyond the acute stage. We recently studied a rat model of 10-min forebrain ischemia followed by survival periods ranging from 1 to 10 weeks, and we documented a maturation of cellular damage in the striatum occurring over several weeks, which correlated with persistent astrocytic and microglial activation [24]. Although damage to hippocampal pyramidal neurons was evident within 1 week, glial reactions were long lasting. In that study, we detected eosinophilic deposits in some brain structures, suggesting that additional pathological processes were set into motion by ischemia at long survival periods [24].

APP is a transmembrane glycoprotein which is up-regulated in response to neuronal injury and plays a role in cell adhesion, mediating inflammatory responses as well as the development of plasticity [25, 27]. An abnormal accumulation of βAPP is known to be stimulated by metabolic or excitotoxic insults [17, 27], including brain ischemia [4, 29, 30, 33, 35, 39, 40] and trauma [7, 12, 23], and may contribute to intracellular calcium-ion dyshomeostasis and free radical formation [25]. The present study was designed to determine the extent and topography of βAPP accumulation over the chronic interval of 1–10 weeks following a 10-min global ischemic insult. We employed the immunocytochemical visualization of βAPP in sections adjacent to those in which we have previously characterized chronic neuronal, astrocytic and microglial alterations by light-microscopic and immunohistochemical methods [24]. Changes in βAPP were analyzed with a monoclonal antibody to its N-terminal region. This approach allowed us to identify abnormal sites of intra- or extracellular deposition of βAPP or its N-terminal splice variants. By following changes of βAPP across closely spaced postischemic survival time points, we hoped to gain insight into the sequence of βAPP cellular changes.

Materials and methods

Animal preparation

Male Wistar rats weighing 265–370 g were used in this study following an overnight fast. All protocols were approved by the University of Miami's Animal Care and Use Committee. Animals were initially anesthetized with 3% halothane and 70% nitrous oxide. The femoral arteries were cannulated with polyethylene tubing to permit blood pressure measurements and blood sampling. The common carotid arteries were exposed bilaterally in the neck, and close-fitting loops of polyethylene tubing contained within another dual-bore Silastic tubing were placed around each carotid artery. Rats were endotracheally intubated, immobilized with pancuronium bromide (0.75 mg/kg i.v.), and ventilated mechanically with 0.5% halothane, 70% nitrous oxide, and a balance of oxygen so as to maintain the arterial PO_2 and PCO_2 in the normal range. Plasma glucose was measured before ischemia and was normal. Brain temperature was measured with a sterile 33-gauge thermocouple implanted stereotaxically in the left frontal cortex through a small burr hole drilled in the skull. Brain and rectal temperatures were held within the normal range (36.5–37.0 °C and 37.0–37.5 °C, respectively) throughout the experiment by separate warming lamps above the body and head.

Fig. 2a–i βAPP immunoreactivity in hippocampus after 10-min▶ global ischemia. **a** Absence of βAPP immunoreactivity in CA1 sector at 7 days. **b** Extensive granular βAPP deposition in strata radiatum and oriens of CA1, sparing the pyramidal cell layer, at 2 weeks. **c**, **d** Extensive deposition of enlarged immunoreactive granules throughout CA1 at 4 weeks. **e**, **f** At 6 weeks, positive staining for βAPP is seen only within the pyramidal cell layer of CA1; the granular deposits visible in strata radiatum and oriens at 2–4 weeks have now disappeared. A few ovoid structures are also visible. **g**, **h** At 8 weeks, βAPP immunoreactivity persists in cells of CA1 pyramidal layer (comprising injured neurons as well as glia). In addition, numerous ovoid structures are visible in strata radiatum and oriens and within the dentate hilus. **i** βAPP immunoreactivity persists in cells of CA1 pyramidal layer at 10 weeks. **a**, **b**, **f**, **i** × 1000, **c**, **e**, **g** × 100, **d**, **f** × 250

Production of cerebral ischemia

Transient forebrain ischemia was induced by the method of bilateral carotid artery occlusions plus systemic hypotension. Blood was gradually withdrawn into a heparinized syringe to reduce mean arterial blood pressure (MABP) to 45–50 mm Hg, and the

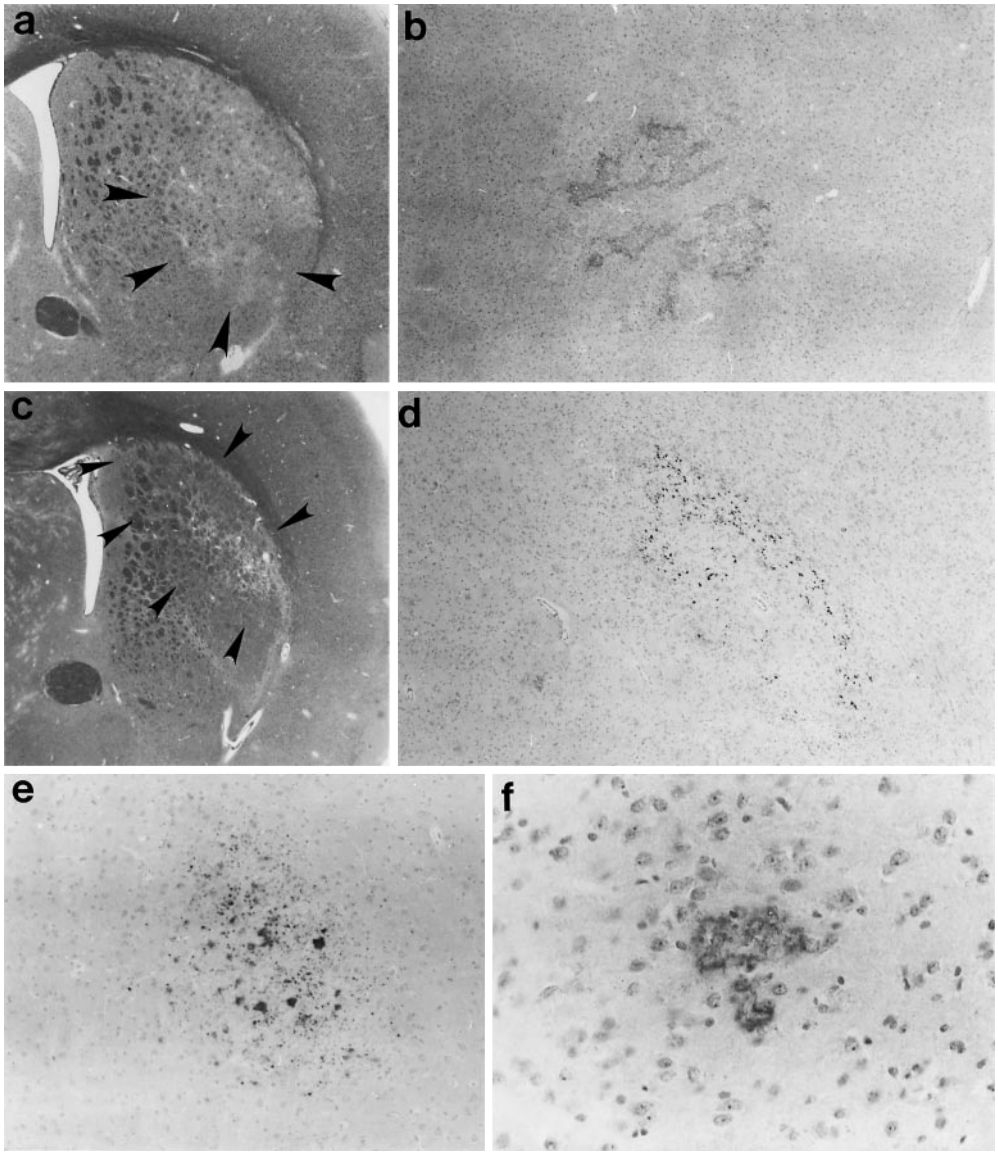
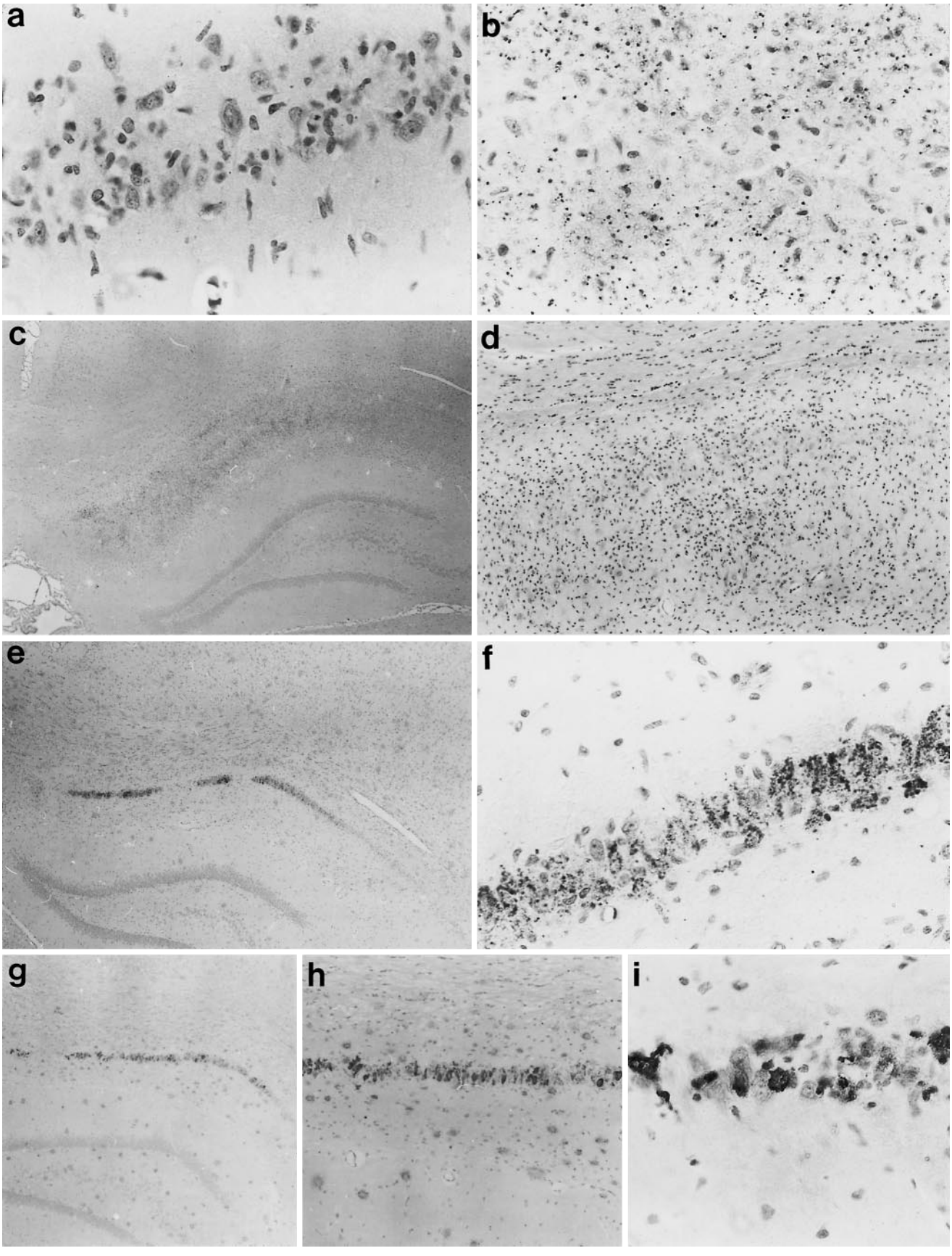


Fig. 1 Paraffin-embedded sections of brains studied 4 weeks (**a**, **b**, **e**) and 8 weeks (**c**, **d**, **f**) following 10 min of forebrain ischemia. Adjacent sections were stained with H&E (**a**, **c**) and βAPP (**b**, **d**–**f**). Discrete lesions of variable size, characterized by pallor on H&E stains, involve the striatum (**a**, **c**). Adjacent sections (**b** and **d**, corresponding to the areas marked by *arrows* in **a** and **c**, respectively) contain widespread deposits of round, punctate immunoreactive material. At 8 weeks, neuronal somata at the border of the striatal lesion are immunoreactive for βAPP (**d**). The ventrolateral thalamus at 4 weeks (**e**) contains intraneuronal as well as extracellular granular βAPP deposits; on H&E stain (not shown), gliosis is present. **f** Extensive βAPP deposition in the hypothalamus at 8 weeks, associated with neuronal clumping (*H&E* hematoxylin and eosin, *βAPP* β amyloid protein precursor). **a**, **c** × 35, **b**, **d** × 87, **e** × 218, **f** × 545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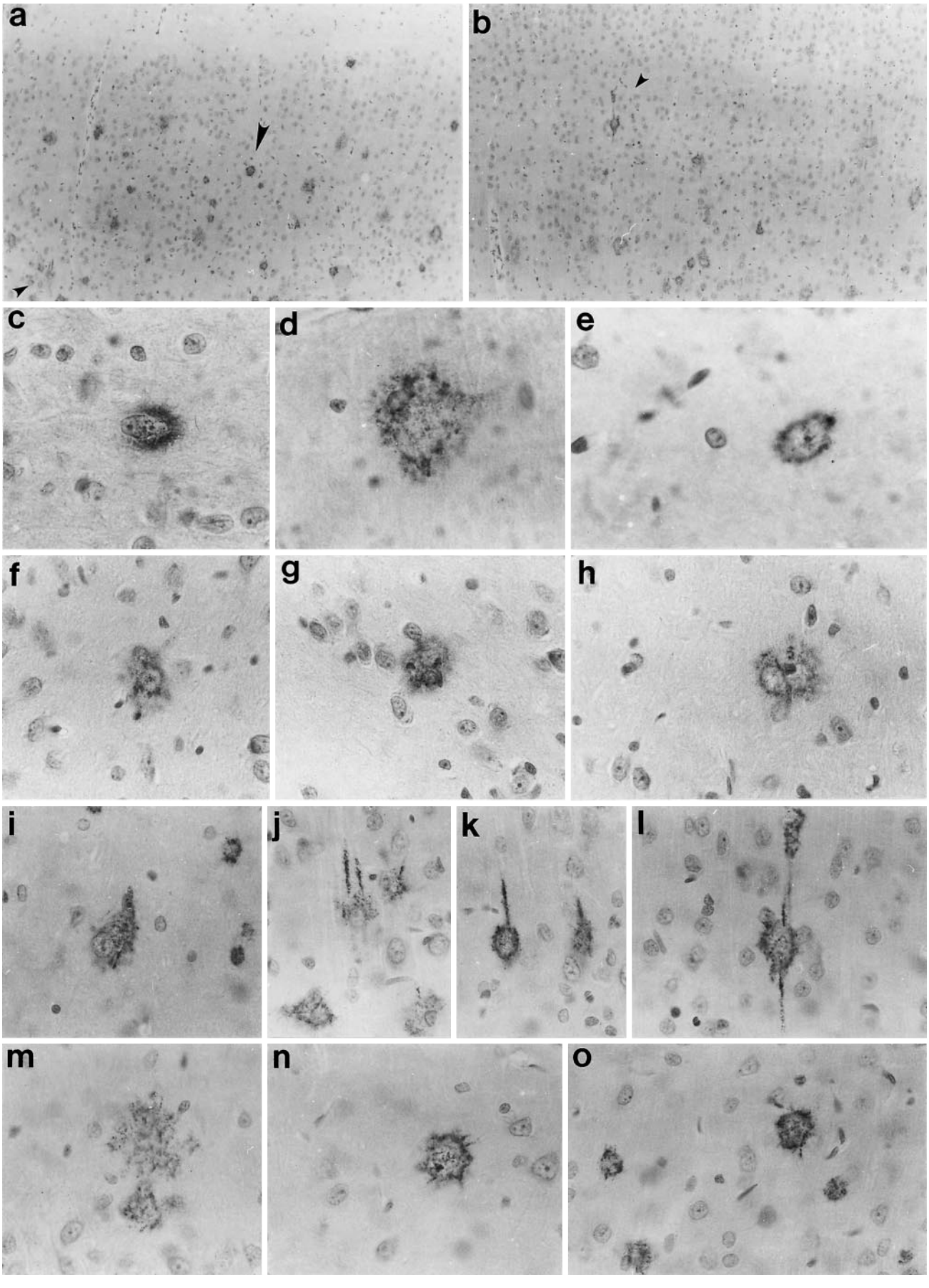
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◀ **Fig. 3a–o** βAPP immunoreactivity in temporal and piriform cortex at 8 weeks. **a** Numerous ovoid structures are apparent in cortical layers II–V. **b** These structures involve cortical layers V and VI. **c** Cortical pyramidal neuron with both extra- and intracellular βAPP deposition. Karyorrhexis is evident. **d** Large, diffuse ovoid structure. **e** Neuronal perikarya is immunolabeled for βAPP. Note enlarged nucleus and abnormal immunopositive nucleolus. **f–h** Clusters of βAPP-immunoreactive neurons. Some neurons exhibit karyolysis. **i** Neuronal inclusions in a structure that resembles a neurofibrillary tangle. **j–l** βAPP is contained within dendrites and axons as well as in the cytoplasm of pyramidal neurons; cytomegaly and karyolysis are present (**j** and **l** are derived from region of *small arrowheads* in **a** and **b**, respectively). Note entrapment of apparently normal neuron by βAPP in **j**. **m** Diffuse ovoid structure. **n**, **o** More dense structures (**o** is derived from region of *large arrowhead* in **a**). **a**, **b** × 250, **c–e** × 1500, **f–o** × 1000

carotid ligatures were then tightened bilaterally for 10 min. MABP was held at 45–50 mm Hg throughout this interval by controlled exsanguination. After 10 min of ischemia, the carotid ligatures were removed, and the warmed shed blood was reinfused to restore normotension. Respiratory adjustments were made to normalize arterial blood gases, and full physiological monitoring was continued for 3 h into the postischemic period. Arterial and venous catheters were then removed with appropriate caution; all skin incisions were closed and incisions infiltrated with 1% lidocaine. Rats were placed in cages at room temperature with free access to water and food pellets.

Sham-operated rats received similar operative preparation but were not subjected to carotid occlusions or blood withdrawal.

Tissue preparation and immunohistochemistry

Animals were killed at the end of 1 (n = 5), 2 (n = 4), 4 (n = 4), 6 (n = 5), 8 (n = 5), and 10 (n = 6) weeks after the ischemic insult. Under deep halothane anesthesia, rats were perfused transcardially with FAM (a mixture of 40% formaldehyde, glacial acetic acid, and methanol, 1:1:8 by volume) for 20 min following a 1-min initial perfusion with physiological saline delivered into the ascending aorta at a pressure of 110 mm Hg. The heads were immersed in FAM at 4 °C for 1 day. Brains were then removed, placed in FAM for another day, and blocked. Coronal tissue blocks were embedded in paraffin for sectioning. Brain sections (10 μm thick) were prepared at 250-μm intervals and were stained for the light microscopic immunocytochemical visualization of the N-terminal portion of βAPP (22C11, 0.1 μg/ml, Boehringer Mannheim).

For βAPP immunohistochemistry [10], sections were rehydrated and placed in 1.5% H_2O_2 in methanol to block endogenous peroxidase activity. They were then rinsed, placed in a solution of citrate buffer, and microwaved for 15 min. Sections were dipped in 0.05 M PBS and incubated with normal horse serum. The primary antibody was applied overnight at 4 °C. To test for nonspecific staining, negative controls were conducted in which the primary antibody was omitted and, instead, mouse IgG (1:500) was used during tissue processing. Sections were then rinsed with PBS and the secondary antibody applied. The avidin-biotin complex (Vector, Burlingame, Calif.) was used for antibody detection followed by peroxidase reaction with diaminobenzidine (DAB) and H_2O_2. Slides were washed in 0.5% Triton X-100 and counterstained with hematoxylin. These procedures were similar to those recently reported from our laboratory [10].

Identification of βAPP accumulation

Representative areas of neocortex, striatum (~ 0.2 mm posterior to bregma) and the hippocampal CA1 sector (~ 3.6 mm posterior to bregma) were examined by an observer (B.L.) blinded to the experimental groups. Specific βAPP staining was identified by its dark-brown appearance. Quantitation of βAPP deposits was carried out in a standardized region of inferomedial temporal and piriform/entorhinal cortices (bregma ~ –3.6 mm). Statistical differences over time were assessed by one-way analysis of variance (ANOVA) with post-hoc Bonferroni comparisons.

Results

Pre-ischemic physiological variables in all groups were within the normal range: MABP, 123 ± 8 mm Hg; PaCO_2, 39 ± 2 mm Hg; PaO_2, 123 ± 12 mm Hg; pH 7.42 ± 0.03; and plasma glucose, 122 ± 20 mg/100 ml. There were no inter-group differences (see [24] for a complete description).

Light microscopic histopathology

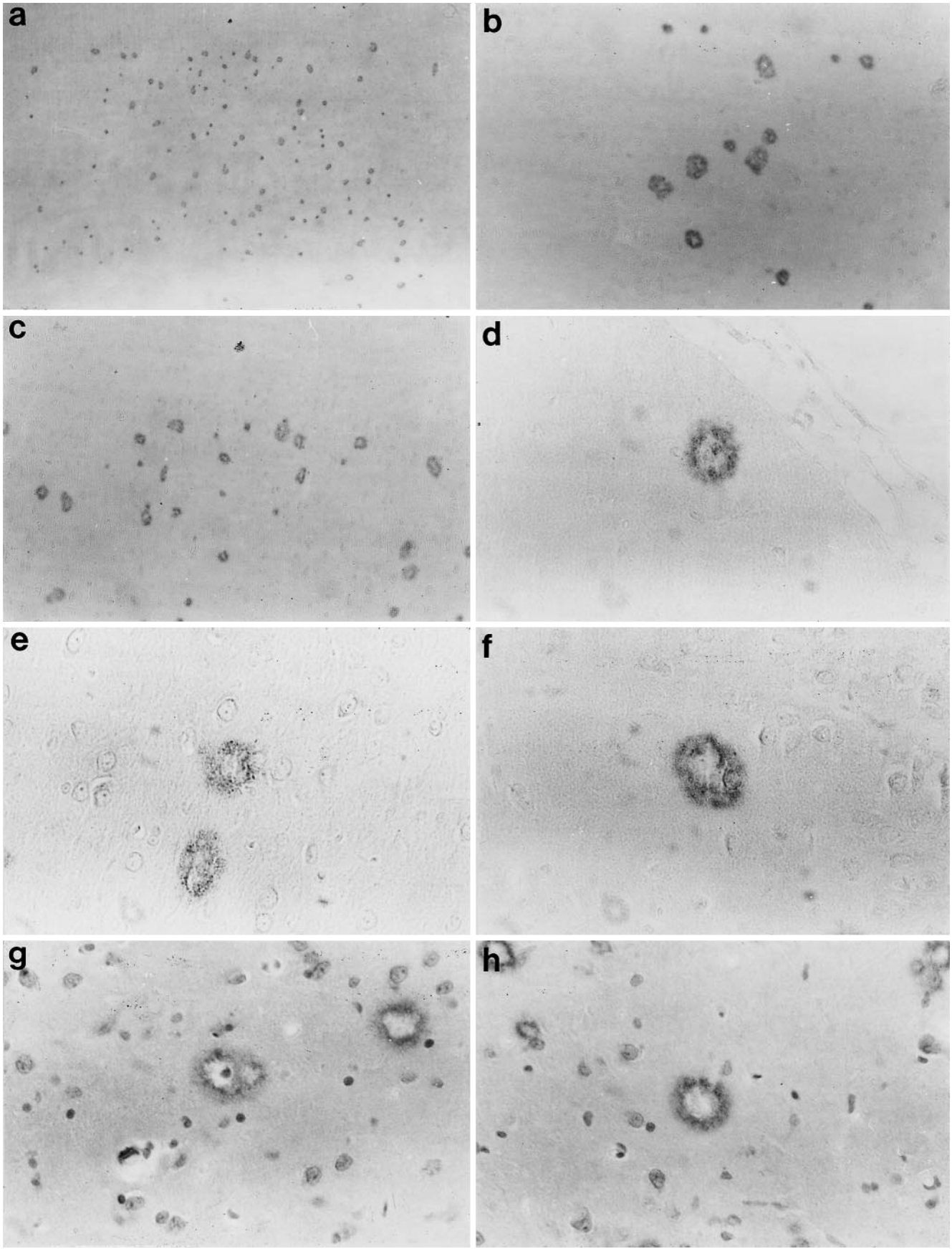
Our previous report [24] provides a comprehensive description of neuropathological alterations in rats with 1- to 10-week survival following this 10-min ischemic insult. The CA1 sector of hippocampus showed consistent high-grade loss of pyramidal neurons from 1 week onwards, associated with glial fibrillary acidic protein (GFAP)-reactive astrocytes and isolectin-positive activated microglia. Significant loss of small striatal neurons first became evident only at 4 weeks. Regions of delayed striatal infarction were evident in approximately one-quarter of the brains, and foci of hippocampal necrosis in approximately one-half of the cases. In addition, eosinophilic material suggestive of amyloid protein was noted within necrotic areas of CA1 and striatum at 6–10 weeks. The neocortex showed neuronal rarefaction and spongy changes, and rare necrotic cortical neurons were present at 1 and 4 weeks. There was no laminar necrosis, however, and frank infarction was rare. A mild cortical microglial reaction was observed at 1–10 weeks, as were a few GFAP-positive reactive astrocytes; no clusters of microglia or astrocytes were noted.

βAPP immunohistochemistry in brains with sham procedure

Three of four rats studied at 1 week, and three of four rats studied at 10 weeks following sham procedures showed an absence of specific immunostaining; one brain of each group contained infrequent small, low-density spots of cortical βAPP immunopositivity.

βAPP immunoreactivity in ischemic brains

In marked contrast to the findings in sham-operated animals, striking βAPP immunoreactivity was observed in animals recovering from forebrain ischemia. In general, two distinct patterns of βAPP immunoreactivity were noted: (1) a diffuse punctate pattern of tiny round βAPP-labeled granules distributed in cells of those areas of the hippocampal CA1 sector, striatum, and thalamus identified in our previous study as ischemically lesioned [24]; and (2) a pattern of non-diffuse, larger, non-punctate ex-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◀ **Fig. 4** βAPP-immunohistochemical staining in temporal and piriform cortex at 10 weeks, without (**a–f**) and with (**g**, **h**) hematoxylin counterstain. Ovoid structures are larger and more dense than at 8 weeks (Fig. 3). Their cores are relatively empty compared to 8 weeks (**g**, **h**). **a** × 100, **b**, **c** × 250, **d–h** × 1000

tra- and intraneuronal foci of βAPP deposition, most common in ventral cortical regions – zones not affected by necrosis in our previous study [24].

Areas of ischemic damage

Striatum and thalamus

No βAPP accumulation was present in brains of rats surviving 1 or 2 weeks after ischemia. With 4- to 10-week survival, numerous tiny granular deposits specifically stained by βAPP antibodies were observed within those regions of striatum and thalamus that exhibited well-demarcated foci of necrosis when stained by H&E or by GFAP or isolectin immunochemistry (Fig. 1) [24]. At 8 weeks, dense intracellular βAPP accumulation was present at the borders of large striatal lesions, while the lesion centers contained no immunolabeling (Fig. 1). Most of the affected cell bodies were not enlarged.

Hippocampus

In area CA1, specific βAPP immunostaining was absent at the end of 1 week but was present in all brains by 2 weeks postischemia (Fig. 2). Numerous tiny granular deposits were observed throughout the strata radiatum and oriens but not in the pyramidal cell layer. By 4 weeks postischemia, in three-quarters of the rats, these granules enlarged, became rounded, and occupied the pyramidal cell layer as well (Fig. 2). At 6 weeks, the punctate immunostaining became more intense but was confined only to cells of the pyramidal layer. At 8 and 10 weeks, affected cells were densely immunostained. This staining affected only the subgroup of rats which exhibited a more severe pattern of ischemic injury by H&E [24]. In some brains, neurons of hippocampal CA3 had very mild βAPP immunostaining at 1 week.

Areas devoid of ischemic damage

Cortex and hypothalamus

Large oval deposits of low-density βAPP immunostaining were noted at 1 and 2 weeks postischemia in scattered cortical neurons of some brains. A few deposits were larger and more dense. Unexpectedly, this pattern of βAPP deposition again emerged in the neocortex at 6–10 weeks postischemia, where enlarged foci of βAPP were located predominantly in the basal cortical regions. These deposits were scattered symmetrically within the cortex and involved layers II–VI. By 8 weeks postischemia, the number of these deposits had increased, their immunostaining density was more pronounced, and the deposits were associated with neuronal enlargement or karyolysis (Fig. 3). Extracellular βAPP deposits appeared to entrap neurons so as to form large, diffuse ovoid foci. No glial clusters were observed.

At 10 weeks postischemia, the density and size of these ovoid βAPP structures increased, and in many instances their cores disappeared (Fig. 4). The size of these structures in cortical layers II–V was larger than that in layer

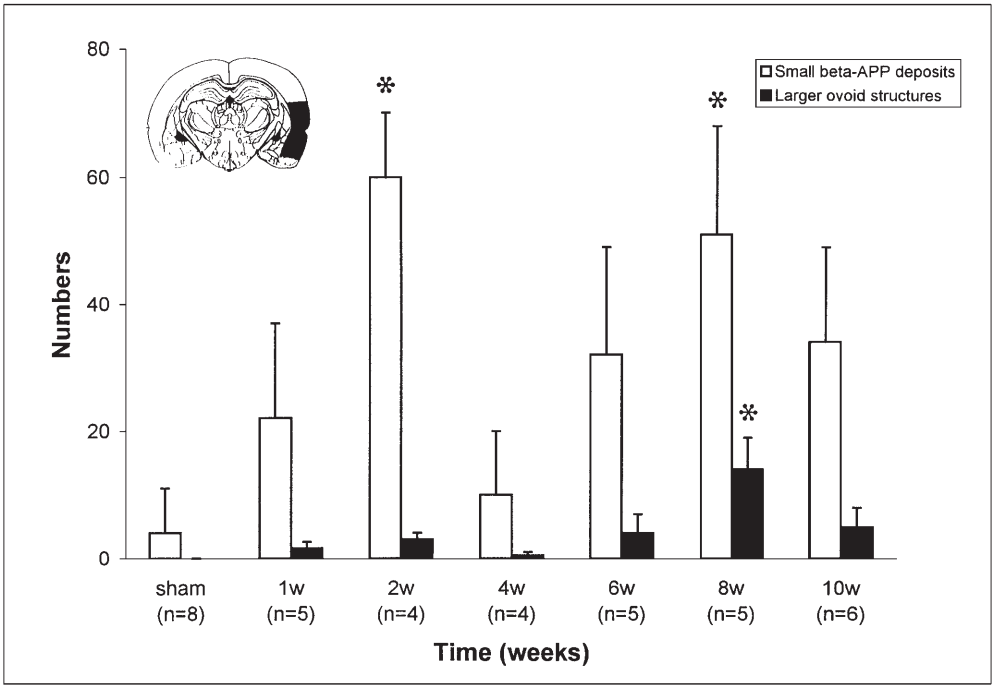

Fig. 5 Cortical βAPP deposition in brains of rats subjected to 10-min global ischemia followed by 1–10 weeks of survival. Numbers of small (< 15 μm) βAPP deposits and of larger ovoid structures were quantitated in a standardized region of inferomedial cortex (inferior auditory, entorhinal, and dorsal piriform areas at bregma level ~ –3.6 mm) and are shown as means ± SEM. *Asterisks* denote significant difference from pooled shams ($P < 0.05$, one-way analysis of variance followed by Dunnett's test)

VI; however, these foci were more numerous in the deeper cortical layers. By 8 weeks postischemia, these structures were present in dendritic areas of hippocampus in most rats.

Figure 5 shows the time course of βAPP deposits and ovoid structures in this study. Two peaks of βAPP deposition were noted: the first at 2 weeks, the second at 6–10 weeks.

Ovoid structures were also observed in the hypothalamus (Fig. 1) but not in striatum or thalamus. Occasional small intraneuronal βAPP deposits were observed in the thalamus, peaking at 2 weeks postischemia. A few small oval immunoreactive profiles were present in the corpus callosum and external capsule at 1 week in a few cases, but these changes had disappeared by 2 weeks.

Glial cells and endothelia showed no βAPP immunostaining. There was no relationship between blood vessels and sites of βAPP deposition.

Discussion

The present study reports, for the first time, the progressive emergence of immunoreactivity to the N-terminal portion of βAPP in the brains of rats followed over a 10-week period after a brief (10-min) period of global forebrain ischemia. Immunohistochemical labeling was adjusted so that sham-ischemic animals failed to show baseline labeling of the neuronal cytoplasm. Under these conditions, our immunohistochemical study revealed a complex pattern of βAPP deposition over time in different structures. In regions most affected by ischemia – the CA1 sector of hippocampus and dorsolateral striatum – diffuse punctate, granular βAPP deposition increased progressively over the 8–10 week period (Fig. 2). At longer survival periods, severely damaged CA1 neurons were intensely labeled. A second pattern of βAPP deposition was noted in the cortex at longer survival times, when prominent extracellular and intracellular ovoid plaque-like βAPP deposits were present and were particularly frequent in the basal aspects of the forebrain, despite the fact that these areas were, in other respects, not damaged by ischemia (Figs. 3, 4). These ovoid structures were not identified in the striatum but occasionally occurred in the dendritic layers of CA1.

In previous studies, prominent immunoreactivity of the N-terminal portion of βAPP has been described within the 1st week after a cerebral ischemic insult, and up to 30 days after trauma; however, βAPP immunoreactivity was absent in postischemic hippocampal CA1 neurons [7, 23, 27, 29, 30, 39, 40, 42, 43]. βAPP deposition has been observed in the border zone of an involving infarct, accumulating in dystrophic axons and neuronal perikarya at 4–7 days after focal ischemia [39, 43]. Plaque-like cortical βAPP deposits have also been described at 6–12 months after ischemia, but N-terminal immunoreactivity was inapparent at that time [33].

A combination of events could explain our findings. Since βAPP is produced within neurons [6, 9, 20, 36, 38], there is normally some protein in the cytoplasm and in axons [9], but these physiological protein concentrations lie below the level of detection by the present method. Previous studies have described the appearance of diffuse punctate APP immunoreactivity in axonal swellings and, less often, within neuronal cell bodies following brain trauma [7, 23] and ischemia [39]. These changes have been attributed to alterations of axonal transport of βAPP [7, 20, 23, 44, 45]. Focal ischemia is known to disrupt the fast axonal transport of βAPP due to cytoskeletal breakdown [44].

Since some βAPP deposits of this study bore a resemblance to abnormal neurons (Fig. 3c–i), the question arises whether a delayed form of neuronal degeneration occurs in the neocortex after a brief period of global brain ischemia. Studies in the gerbil have reported central chromatolysis of cortical neurons after ischemia [28, 40, 43]. Chromatolysis is a typical response to axonal damage, and the initial changes of βAPP localization are compatible with an abnormality of axonal transport. The increased cytoplasmic staining of neurons after ischemia might also reflect either the decreased removal of βAPP from neurons, or alternatively, an increase in βAPP synthesis. In other studies, however, the total amount of βAPP mRNA in brain did not change over a 21-day period after global or focal ischemia [1–3, 18, 19], suggesting that neuronal βAPP synthesis did not increase during this period. Abnormal metabolism of βAPP following ischemia may also have contributed to its accumulation in the brain. βAPP is processed via an endosomal/lysosomal pathway [21, 26, 46]. βAPP is also internalized from distal axons or terminals and retrogradely transported back to perikarya in organelles [45]. It is possible that ischemia, even if insufficient to induce neuronal necrosis, might impair the lysosomal system and Golgi apparatus, slowing the neuronal degradation of βAPP and promoting its accumulation. Finally, the cerebral deposits of immunoreactive βAPP in this study may also have derived from the blood [30], facilitated by ischemia-induced blood-brain barrier damage [31], which would permit penetration of circulating APP [22, 31, 32]. In our companion study, we documented chronic endothelial degenerative changes after global ischemia [24], which may have contributed to βAPP entry into brain. However, the fact that perivascular βAPP deposits were absent in the present study makes it unlikely that APP-immunoreactivity was derived from the plasma.

The punctate granular pattern of βAPP deposition in hippocampus and striatum in this study may constitute a reaction to chronic inflammation. Interleukins (IL) are known to be released in response to cerebral ischemia and injury. In particular, IL-1 appears to play a key role in the initiation of neuronal βAPP synthesis [5, 11, 15, 37]. Ischemia triggers IL-1 release, which then mediates βAPP accumulation [16]. Activated microglia containing IL-1 increase threefold in number in the acute period following neural injury, and this correlates with a sevenfold increase in numbers of neurons with elevated βAPP levels [14]. In the present study, a striking microglial reaction was ob-

served starting at 1 week after global ischemia, that persisted for 8 weeks [24], so that it is likely that IL-1 and -6 were released over a prolonged postischemic period and thus influenced βAPP formation. In this context, it is of interest that the distribution of punctate granular βAPP deposits throughout the strata oriens and radiatum of CA1 exactly matched the zone of activated microglia in our companion study [24]. Astrocytes are also involved in cytokine cycles [13] and may have participated in the process of βAPP accumulation [41]; this is suggested by our observation of granular βAPP deposition around injured astrocytes at 8 weeks postischemia [24].

In summary, the present study provides evidence suggesting that βAPP is a reactive protein involved in the processes of ischemic brain damage and repair. Two types of βAPP reactivity were observed in the subacute and chronic period after brief transient global ischemia. On the one hand, βAPP was retained within damaged neurons. In addition, large ovoid accumulations of βAPP evolved in non-necrotic areas. Our findings raise the possibility of a relationship between ischemic and chronic neurodegenerative processes. Thus, an important question to be addressed in future studies is whether ischemia-induced βAPP deposition is accompanied or followed by the deposition of amyloidogenic peptide fragments in the rat brain.

Acknowledgements This study was supported by US Public Health Service grant NS 05820. Ms. Lin Li provided expert assistance with immunocytochemical procedures. Ms. Helen Valkowitz helped to prepare the typescript.

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