Histol Histopathol (1999) 14: 103-117

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Microglial cells during the lesion-regeneration of the lizard medial cortex

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Summary. The lizard medial cortex (lizard fascia dentata) is capable of neural regeneration after being lesioned by the anti-metabolite 3-acetylpyridine (3AP). This study was aimed at detecting microglial behaviour during the medial cortex lesion-regeneration process using tomato lectin histochemistry to label microglia (both with light and electron microscopy) and proliferating cell nuclear antigen (PCNA) immunocytochemistry to label proliferating cells. As expected, 1-2 days post-injection lectin-labelled microglia cells could not be observed in the medial cortex plexiform layers, but later (7 days post-injection) abundant lectin-labelled microglia cells re-populated the regenerating medial cortex. Abundant PCNA-immunolabelled nuclei were detected both in the subjacent ependymal neuroepithelium (neuroblasts, maximum at 2 days post-injection) as well as in some parenchymal cells which were also lectin-labelled (microglia, maximum at 7-15 days post-injection). Re-invasive microglia were also detected in the vicinity of ventricular ependymal lining, blood vessels and meninges. The electron microscope demonstrated that these microglial cells participate in cell debris removal, especially of neural granular cell somata. Other cell types related to microglia (mast cells, peri-vascular cells and meningeal cells) were also present during the scavenging process. Significant numbers of microglial cells remained in close relationship with the ependymal proliferative areas, even in control non-lesioned animals. This is indirect evidence for the working hypothesis that microglia are not only implicated in cell debris removal, but also in the regulation of newly generated neuroblast incorporation onto the cortical areas. Whether they phagocytose immature neuroblasts or induce cell death in them or even prevent their migration onto the principal layer areas are likely possibilities that remain to be proven.

Key words: Phagocytosis, Tomato lectin histochemistry, Ultrastructure, Hippocampus, 3-acetylpyrydine

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Introduction

Microglial cells populate the cerebral cortex of control lizards, showing a layered-pattern distribution (Berbel et al., 1981; Castellano et al., 1991). In the medial cortex they are located juxtasomatically in both plexiform layers and also in a stratum adjacent to the ependyma; usually no microglial cells are seen in the outermost limit of the medial cortex outer plexiform layer near the meninges. In the dorsomedial cortex microglial cells are observed near the cerebral surface, in a stratum adjacent to the granular layer and also near the ependyma (Castellano et al., 1991). Surprisingly, when the medial cortex principal cells and their zinc-enriched axonal projection are lesioned with the intraperitoneal injection of antimetabolite 3AP, microglia transitorily disappear from the lesioned area (López-García et al., 1994). This microglial behaviour in the lesioned lizard medial cortex contrasts with that shown by microglia in the lesioned central nervous system of mammals (Davis et al., 1994; Mallat and Chamak, 1994) and non-mammalian vertebrates (Battisti et al., 1995; Lázár and Pál, 1996) where microglia cells participate actively in the debris removal. Obviously, debris removal seems to be a prerequisite for neuronal regeneration.

In order to investigate the role of microglial cells, as well as radial glia-ependymocytes (Nacher et al., 1999), in the brain of lesioned-regenerating lizards, electron microscopy and tomato lectin histochemistry as a microglia cell marker (Acarin et al., 1994) have been used. As microglial reaction to mammalian CNS lesion usually implies the proliferation of microglial cells (Giulian and Baker, 1986; Graeber et al., 1988; Glenn et al., 1992) whether a similar phenomenon occurs in the lizard cerebral cortex was also an objective of this study. Proliferating cells have been labelled with an antibody against "proliferating cell nuclear antigen" (PCNA). Double PCNA immunocytochemistry was performed in the same sections as lectin histochemistry to asses microglia cell proliferation. PCNA immunocytochemistry will also be useful to follow the "reactive neurogenesis" period subsequent to lizard medial cortex lesion (Molowny et al., 1995) and to distinguish it from the hypothetical microglial proliferation period. The

hypothesis that the transitory disappearance of microglia could be an important permissive factor for reactive neurogenesis and posterior regenerative events (López-García et al., 1994) has been examined by assessing the relationship between microglia cells and the proliferating ependymal areas.

Materials and methods

37 healthy adult lizards (4-5.5 cm snout-vent) of the species *Podarcis hispanica*, captured in the surroundings of Burjassot, Valencia (Spain) were used in this study. After their capture, lizards were maintained in terraria simulating their environmental conditions. Experimental protocols were carried out according to institutional guidelines concerning animal care and protection.

An intraperitoneal injection of the neurotoxin 3-acetylpyridine (3AP) (dose: 150 mg Kg^{-1} b/w) was performed in 32 lizards. Non-injected lizards (n=5) were used as negative controls. At various days post-lesion (1, 2, 4, 7, 15, 30, 42 and 90 days), animals under ether anaesthesia were transcardially perfused with either 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2-7.4 (n=19) for light microscopy, or with 2% paraformaldehyde, 2% glutaraldehyde in 0.1M phosphate buffer (n=18) for electron microscopy. Then the brains were removed and postfixed in the same fixative for 6 hours at 4 °C. After dehydrating, the brains were either embedded in Polywax (BDH, Poole) and serially sectioned (parallel series of 10 μm-thick transverse sections) or transversely sliced (75-100 μm-thick slices) using a vibratome. Glutaraldehyde-fixed slices were postfixed in 1% osmium tetroxide and embedded in epoxy resin (TAAB, Aldermarston). Semithin sections were toluidine blue-stained and observed under the light microscope to asses the lesion extent of the neurotoxin. Selected sections were re-embedded; ultrathin sections obtained from them were lead-stained and observed under the electron microscope.

Lectin histochemistry/PCNA immunocytochemistry

Polywax, vibratome and semithin resin-embedded sections were histochemically stained with biotinylated tomato lectin (L-9389; Sigma, St. Louis, MO). Polywax sections were dewaxed and processed for tomato lectin histochemistry as described by Acarin et al. (1994) with slight modifications. Endogenous peroxidase activity was blocked with 3% H_2O_2, 10% methanol in 0.1M Tris-buffered saline (TBS). The sections were incubated with the biotinylated tomato lectin (1:50) for 1 h at room temperature, then with an avidin biotin complex (Dakopatts; Golstrupp, Denmark) for 1 h at room temperature and finally with 3,3'-diaminobenzidine 4HCl (Sigma, St. Louis) plus nickel ammonium sulphate and H_2O_2 for colour development. Vibratome sections were processed free floating following the protocol described for Polywax sections. Semithin sections, after being treated with sodium etoxide for 2 min at 60 °C, were processed as described for Polywax sections, reducing the incubation times. Controls without biotinylated lectin were done for assessment of correct staining.

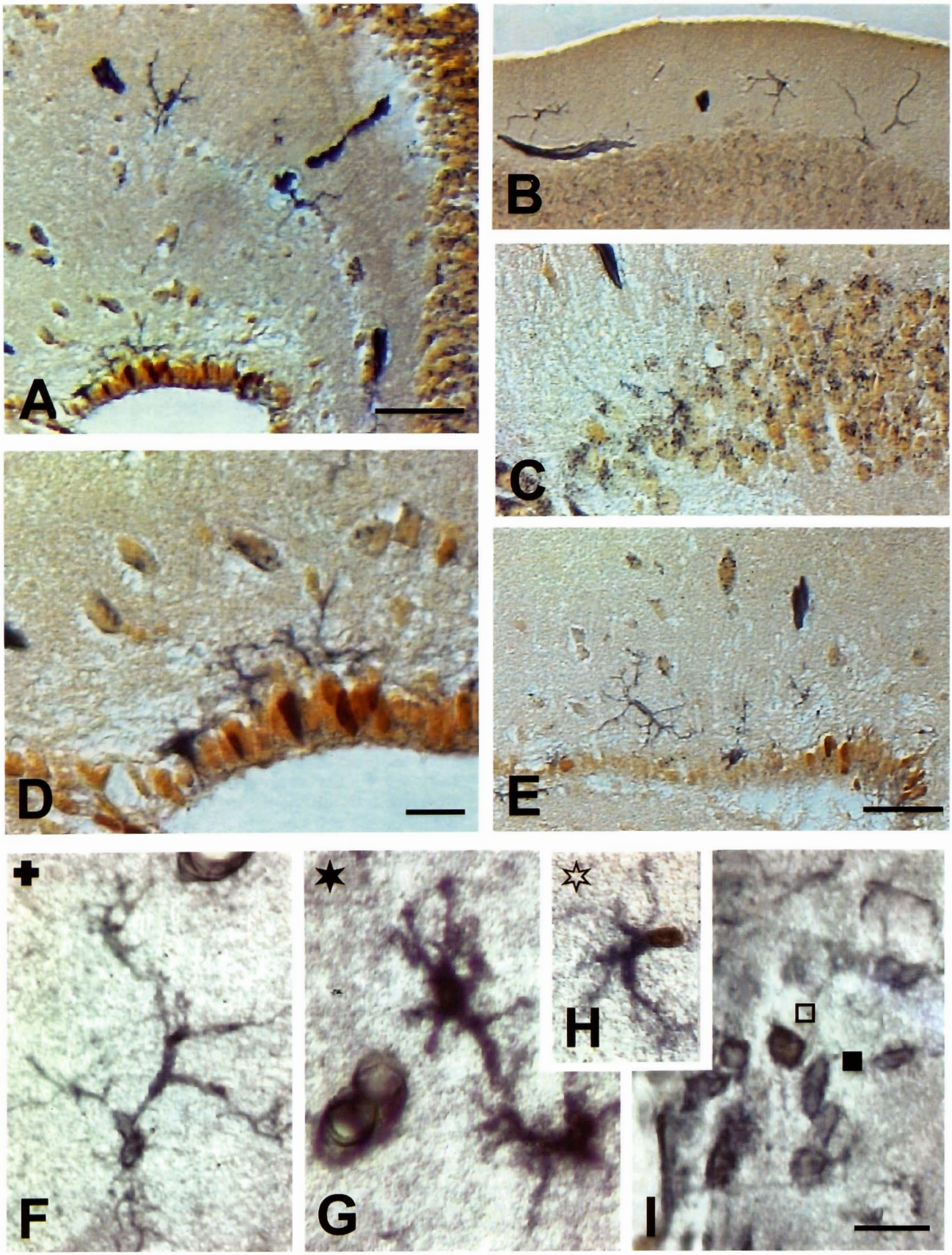
After blocking the peroxidase activity as above, Polywax and vibratome sections were immunostained with an anti-PCNA antibody. Briefly, the sections were incubated in mouse anti-PCNA PC10 Clone monoclonal antibody (Boehringer, Mannheim) 1:50 dilution. The second layer was goat anti-mouse IgG (Sternberger-Meyer, Baltimore) (1:100), followed by the third layer: mouse peroxidase-antiperoxidase (PAP) complex (Sternberger-Meyer, Baltimore), 1:100. Controls without primary antibody were done for assessment of correct immunostaining. Development of the immunostaining was carried out using diaminobenzidine and H_2O_2.

Some glutaraldehyde-fixed vibratome sections were processed for lectin histochemistry as above, then they were osmium postfixed and processed for electron microscopy.

Results

The histological appearance of the cortex of control and lesioned-regenerating lizards matched that described in previous experiments (López-García et al., 1992a, 1994; Nacher et al., 1999). Lectin-labelled microglial cells appeared under different morphological aspects (Fig. 1). In order to distinguish between this continuum of morphologies we have classified lizard microglial cells following the work of Davis et al. (1994) who distinguished between ameboid microglia, resting (ramified) microglia, activated microglia, reactive microglia and giant multinucleated cells. Although true ameboid cells are usually restricted to developmental stages, the term ameboid has been used when reactive microglial cells displayed this morphology. PCNA immunocytochemistry was also performed in order to detect proliferating cells

Fig. 1. Double immunostaining for PCNA (brown nuclei) and tomato lectin (black); control non-lesioned animal; sections from Polywax-embedded material. **A.** Medial cortex inner plexiform layer; observe the presence of lectin-labelled restiform microglia and blood vessels; some PCNA-immunoreactive nuclei are visible in the ependyma (sulcus septomedialis). **B.** Medial cortex outer plexiform layer. **C.** Medial cortex cell layer; most neuronal somata appear loaded with dark lectin granules. **D.** The ependymal sulcus septomedialis; lectin-labelled restiform microglia cells appear close to and intermingled within ependymal cells, some of them with PCNA immunoreactive nuclei. **E.** The inner plexiform layer of the dorsomedial cortex; observe the presence of conspicuous restiform microglia cells in the vicinity of the ependyma. **F-I.** Distinct microglial cellular morphologies in the lizard cerebral cortex and the symbol used to represent them in figure 3. **F.** Restiform microglia (dark cross). **G.** Activated microglia (dark star). **H.** Activated microglia with PCNA-immunoreactive nucleus (white star). **I.** Reactive ameboid microglia with PCNA-immunoreactive nucleus (white square) and without (dark square). Scale bars: 10 μm in A; 10 μm in E, B and C; 10 μm in D; 10 μm in I and F to H.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Lectin histochemistry/PCNA immunocytochemistry

The morphology and ultrastructure of the tomato lectin-labelled cells confirmed that they were mainly microglial cells. Nevertheless, neuronal somata also showed lectin-positive granules which could be identified as lysosome-like granules (Fig. 2A). In addition, as occurs in the rat brain, other cells were also labelled with the lectin: endothelial cells (Fig 2B) as well as some ependymocytes which showed a weak staining in their apical region, although their cilia were negative.

In control non-lesioned lizards, the distribution pattern of lectin-labelled microglia was similar to that previously described for this and other lizard species (Berbel et al., 1981; Castellano et al., 1991). In the medial cortex, microglial cells are mainly distributed in a thick stratum of the inner and outer plexiform layers, surrounding the granular layer; occasionally they could be seen near the ependyma (Fig. 2C). In the dorsomedial cortex, microglial cells were mainly found in the inner plexiform layer, near the cerebral surface, in the surroundings of the granular layer and near the ependyma.

The medial cortex

In animals sacrificed 2 to 4 days after the lesion, microglial cells were virtually absent from the medial cortex plexiform layers (Figs. 3, 4). However, some lectin-labelled cells could be detected, close to the ependyma and the granular layer in the inner plexiform layer of these animals. These cells had an ameboid morphology, were radially oriented and abounded particularly in the ventral region of the 4-day post-injection medial cortex (Figs. 4, 6B). Electron microscope examination confirmed that only rare microglial cells could be seen in the plexiform layers (Fig. 5). Nevertheless, other cell types related to microglia, such as mast cells, could be distinguished in the inner plexiform layer.

At 7 days post-injection restiform and activated labelled cells repopulated the plexiform layers, especially at their caudal levels (Figs. 3C,D). In these animals most lectin-labelled cells displayed PCNA immunoreactivity (Figs. 4, 1H). The number of labelled cells was greater than that of controls and labelling was observed even in the zinc-positive stratum of the inner plexiform layer (Fig. 4). Ultrastructural examination of the plexiform layers revealed microglia-like and perivascular cells filled with cellular debris similar to that of degenerated synapses (Fig. 5A). In the animals that survived more than 15 days, restiform microglial cells populated the plexiform layers (Fig. 5B). Their number was still higher than in controls and thus were especially abundant in the dorsomedial cortex; very few of them were PCNA immunoreactive. Meningeal cells with many residual structures could be seen next to the pial end-feet of the radial glia ependymocytes.

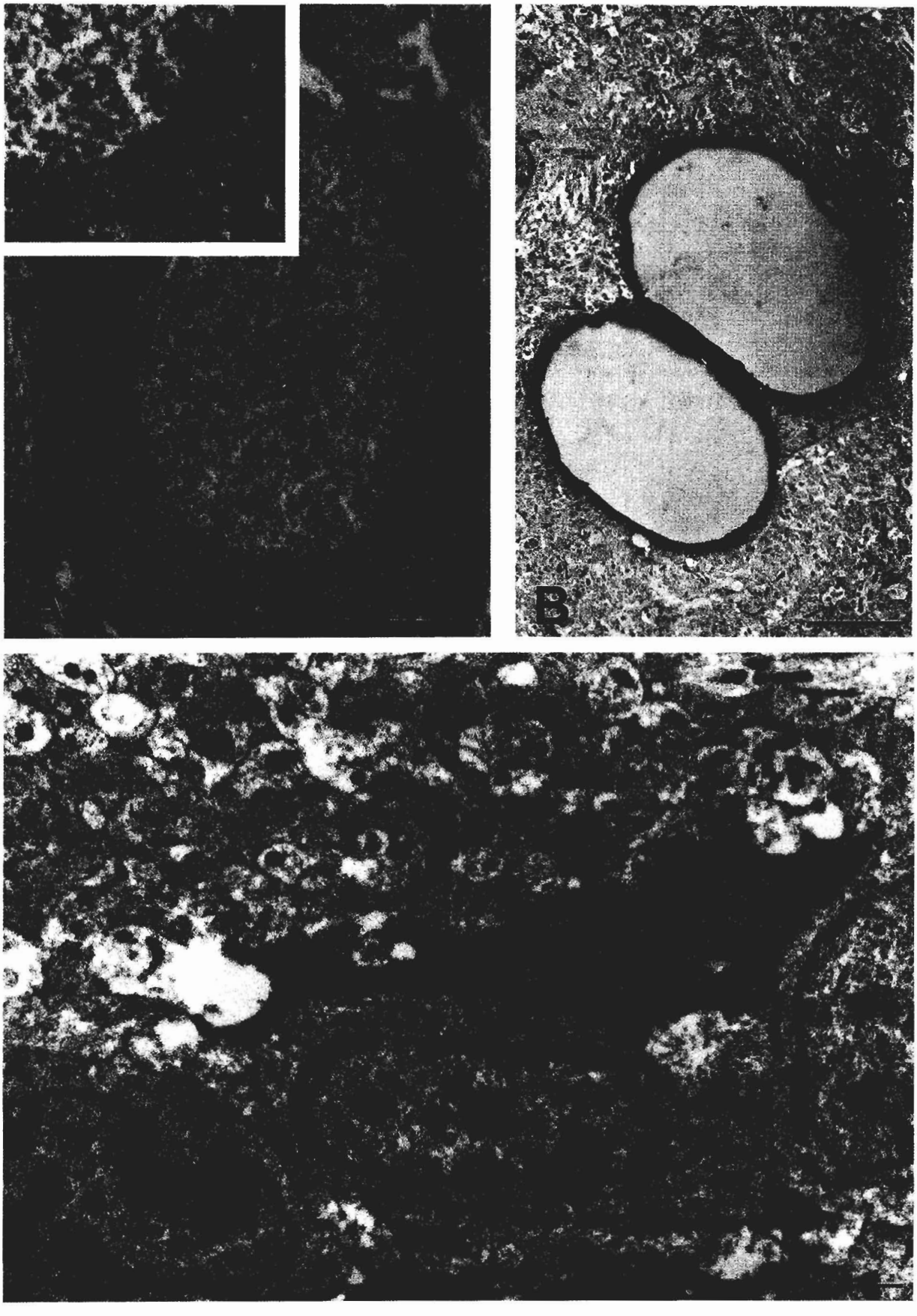
The cell layer started to show labelled cells in the 1-2-day post-injection animals (Fig. 6A), although they were very scarce and accumulated in its most ventral/caudal extremity. These cells displayed a reactive (ameboid) or activated morphology and were usually close to the cell layer rather than intermingled within granular somata. At 4 days post-injection (Fig. 6B) round ameboid cells could be found throughout the cell layer and adjacent to it. Beyond the 7-day postlesion, the number of reactive labelled cells inside the cell layer was progressively reduced; the adjacent cells became more abundant and displayed restiform morphology (Fig. 6C). Some of these cells appeared as active phagocytic when observed under the electron microscope (Fig. 6D,E).

Other cortical and telencephalic areas

In the first days after the neurotoxin injection (Fig. 7A) the dorsomedial and dorsal cortices presented few labelled cells, especially in the zinc-enriched areas of its plexiform layers. These cells were mainly restiform microglia, although some reactive cells could be observed in the medialmost region of the dorsal cortex. 4 days after the lesion the number of labelled cells in the plexiform layers of the dorsomedial and medial cortices increased, especially in the outer plexiform layer. This increase persisted in the 7- and 15-day postlesion animals (Fig. 7B) in which PCNA immunoreactivity was activated and reactive labelled cells could be observed, some of them associated with the meninges, although they were very scarce in the zinc positive fields. Reactive cells still populated the medial extremity of the dorsal cortex cell layer. In the animals sacrificed 30 and 42 days after the lesion almost every labelled cell could be classified as restiform, although some gliotic foci with reactive PCNA immunoreactive cells could be detected in the animals which survived 42 days.

Microglial reaction was not only confined to the medial, dorsal and dorsomedial cortex, other telencephalic and extra-telencephalic areas seemed to be involved. As soon as 1 day after the lesion, the cell layer of the ventral part of the lateral cortex and the dorsolateral ADVR (two regions characterised by the presence of zinc-enriched neuronal somata) showed many reactive and activated cells. These features continued in the 15 day postlesion animals, but were absent in the lizards with longer survival times. The

Fig. 2. Electron microscopy of lectin-labelled structures. **A.** Section of a neuronal somata showing two lectin-containing granules; they appear near the nuclear envelope (insert). **B.** Heavy staining of the endothelial lining of the typical paired capillaries of the lizard cortex; perivascular cells appear unlabelled; inner plexiform layer. **C.** Lectin-labelled microglia-like cell juxtaposed to the ependyma; the staining delineates the plasma membrane. Scale bars: 2 μm in A; 10 μm in B; 1 μm in C.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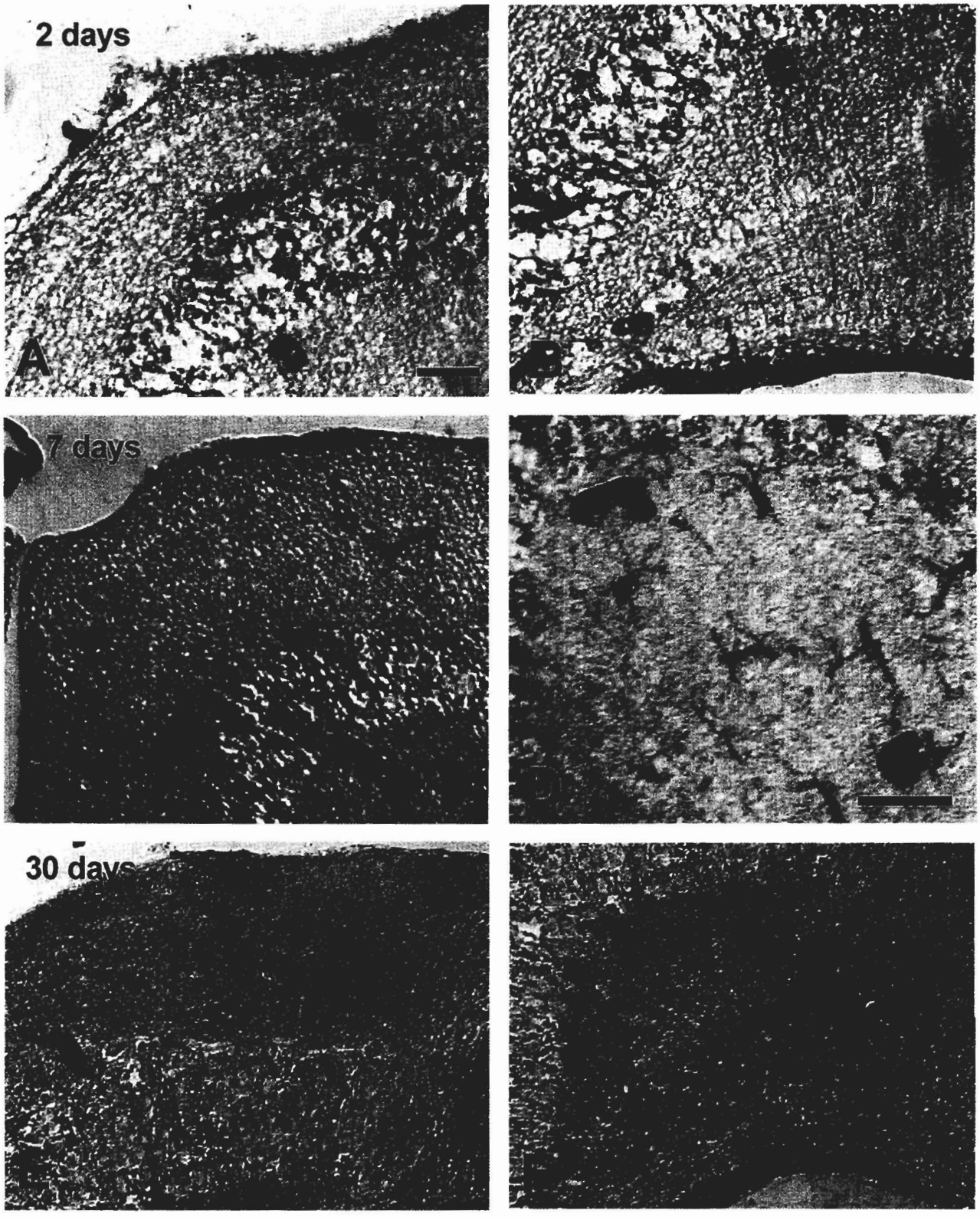


Fig. 3. Light microscope aspects of the medial cortex of lesioned animals sacrificed 2 days (A, B), 7 days (C, D) and 30 days after the 3AP-neurotoxin injection; Polywax sections double-labelled for PCNA and lectin. **A.** only some scarce lectin-positive activated cells can be seen close to the granular cell layer. **B.** inner plexiform layer; few ameboid-fusiform lectin-labelled cells appear radially oriented near the ependyma where many PCNA-immunoreactive nuclei appear. **C and D.** 7 days postlesion; activated and restiform microglia cells populate the outer (C) and the inner (D) plexiform layers. **E and F.** 30 days postlesion; restiform lectin-labelled cells populate the outer (E) and the inner (F) plexiform layers. Scale bars: 25 μm in A, B, C, E, F; 25 μm in D.

mural layer of the nucleus sphericus also presented reactive and activated cells in the first days after the lesion. The hypothalamic area also presented many activated and reactive microglia coinciding with the highest expression of PCNA in the third ventricle ependyma (7-15 days post-injection) (Fig. 7C). The microglial reaction also affected the anterior telencephalic commissure, especially in the 7-day postlesion animals; in this case, many microglial cells were also PCNA immunoreactive (Fig. 7D). The cerebellum also presented PCNA immunoreactive nuclei and associated activated and reactive microglia.

PCNA immunocytochemistry

The sulcus septomedialis was the ependymal zone in which most PCNA immunoreactive nuclei were detected, specially in its medialmost and ventral-caudal

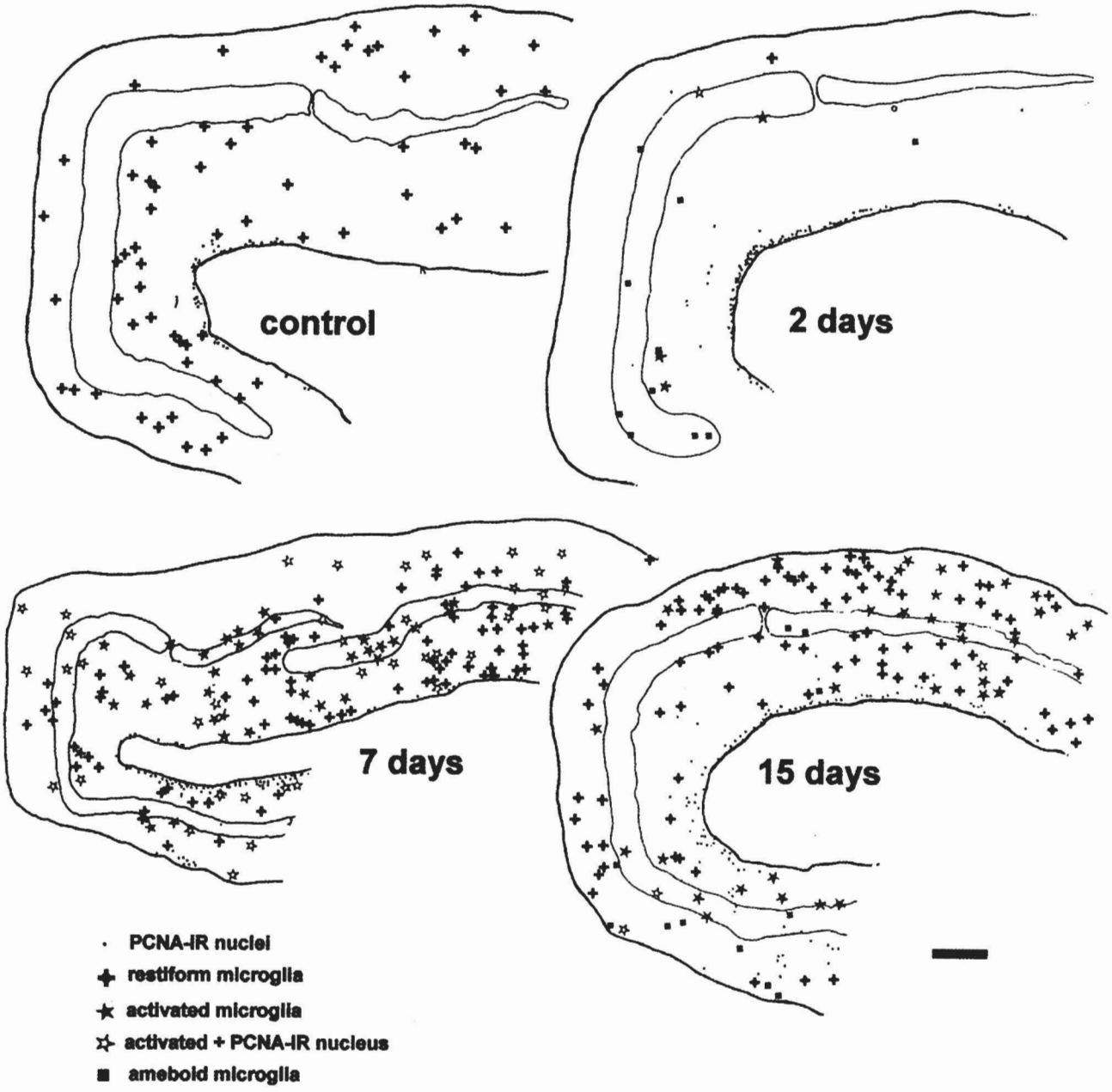


Fig. 4. Camera lucida drawings of the medial and dorsomedial cortices to show the presence and position of PCNA-immunoreactive nuclei (dots) and the different forms of microglia cells (crosses for restiform microglia, dark stars for activated microglia, white stars for activated microglia with PCNA-labelled nucleus, dark squares for ameboid microglia and white squares for ameboid microglia with PCNA-labelled nucleus); from Polywax commissural-to-slightly-post-commissural transverse sections of animals sacrificed at different times after the 3AP-neurotoxin injection. Scale bar: 100 μm.

extremities. Only scarce isolated labelled nuclei or occasional 2-3 cell nests were found in the dorsomedial and dorsal cortex ependyma.

In the sulcus septomedialis of the animals sacrificed one day postlesion, there was a transient decrease in the number of PCNA-immunolabelled nuclei (Fig. 8A), but from the second to seventh day postlesion (Fig. 8B,C) large numbers of PCNA-immunostained nuclei were detected. Fifteen days postlesion, although the distribution of the labelled nuclei in the medial cortex ependyma was still the same, their number was greatly reduced. At 30 days postlesion (Fig. 8D) both the distribution and the number of PCNA immunostained nuclei in the medial cortex ependyma resembled that of a non-injected lizard.

In the medial cortex parenchyma, between the second and the fifteenth days postlesion (especially in 7 days post injection survived lizards), great numbers of PCNA-labelled cells populated the inner plexiform layer, the cell layer and even, to a lesser extent, the outer plexiform layer; most of these PCNA immunoreactive cells were also lectin immunoreactive (Figs. 1H, 4).

Relationships between microglia cells and the ependyma

At 1 day postlesion, labelled cells and their processes appeared intermingled within the ependymocytes in the ependymal sulcus septomedialis (Fig. 8A). However, 2 days post lesion, at the time of massive bulk PCNA labelling of ependymal cells (neuroblasts), the lectin-labelled structures were almost absent from the ependyma (Fig. 8B). 7 days after the lesion these lectin-labelled cells and processes could be observed again in the medial cortex ependyma, but were absent in regions with many PCNA immunoreactive ependymocytes, such as the ventral region of the sulcus septomedialis (Fig. 8C). From 15 days postlesion and onwards, many lectin-labelled cells extending their processes above and between the ependymocytes appeared in the sulcus septomedialis independently of the presence of PCNA immunoreactive nuclei in ependymocytes (Fig. 8D,E). Supraependymal (Kolmer) cells (Fig. 8F) were particularly abundant in animals sacrificed a long time after the lesion (7-90 days post-injection), especially in the vicinity of the choroid plexus; some of these cells were even observed in the 1 day postlesion animals.

In other ependymal areas (e.g., the dorsomedial and dorsal cortices) some lectin-labelled cells were seen above the spots of PCNA immunoreactive ependymocytes, at 1 day postlesion (Fig. 7A). Later, 7-15 days post-injection, many activated cells and processes were present in the ependymal regions that displayed high PCNA immunoreactivity (Fig. 7B). The PCNA immunoreactive ependymocytes of the nucleus sphericus, lateral cortex, ADVR, striatum and some regions of the third ventricle in the hypothalamus also presented abundant associated lectin-labelled cells and processes, especially in the animals sacrificed from 2 to 15 days post lesion. These cells were usually reactive or activated and appeared as phagocytosing PCNA-labelled ependymocytes.

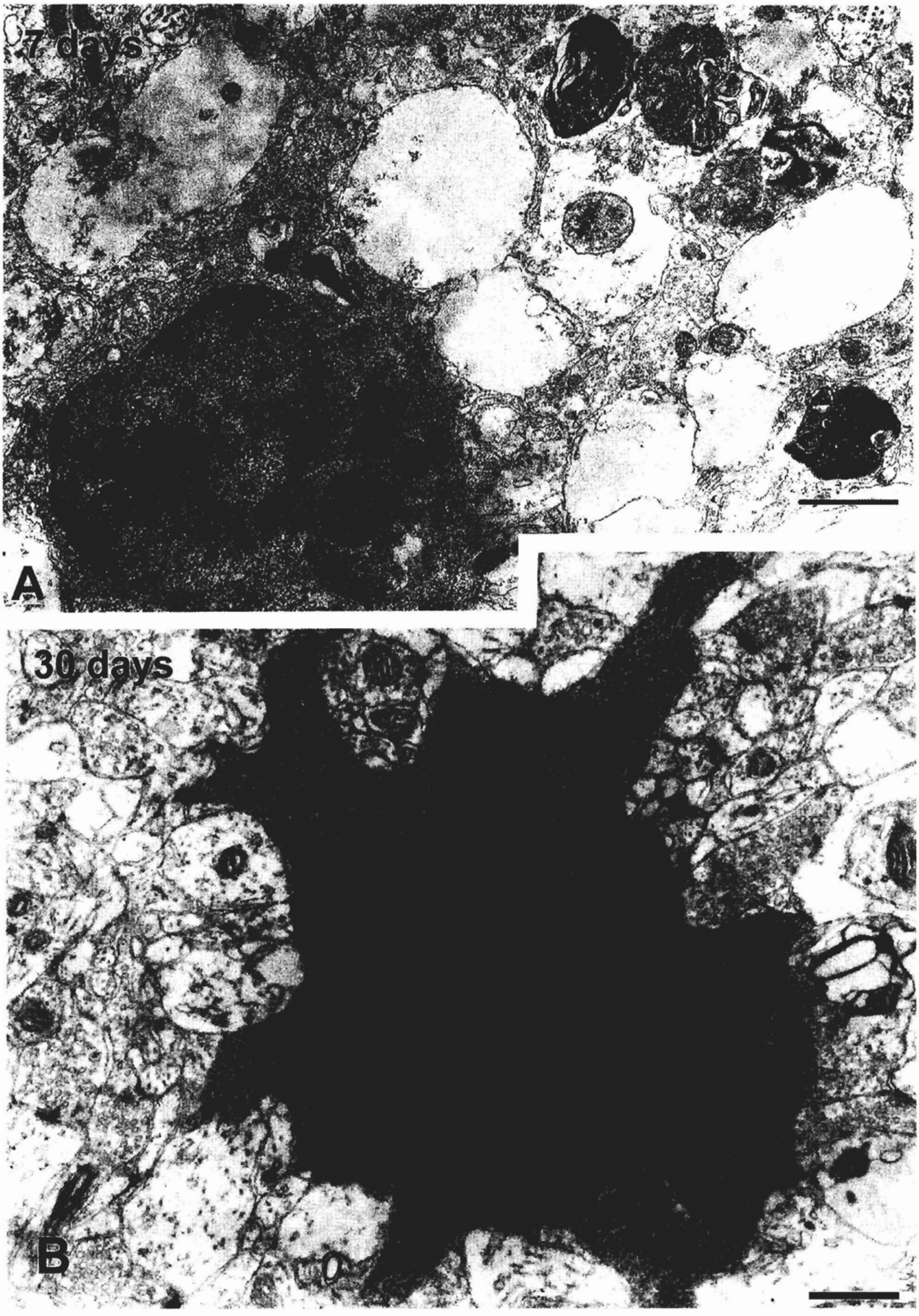
Discussion

In previous studies, either silver impregnation or nucleoside di-phosphatase (NDPase) histochemistry have been the main tools used to identify microglia cells in the lizard brain (Berbel et al., 1981; Castellano et al., 1991; López-García et al., 1994). In this study, histochemistry of tomato lectin which recognises poly-N-acetyl lactosamine residues in the cell surface (Acarin et al., 1994) has been used to identify microglia cells. As with NDPase histochemistry, endothelial cells and the ventricular surface of some ependymocytes were also labelled with the lectin. In neuronal somata, NDPase-labelled dictyosome-associated endo-membrane cisterns (probably Golgi-endoplasmic-reticulum-lysosome membranes) whereas tomato lectin-labelled the final compartments, i.e., the lysosome-like granules. In any case, both NDPase and lectin histochemistry label the same cells in the lizard brain and both are useful tools, the later being more reliable as lectin histochemistry may be performed either in pre- or post-embedded material and easily combined with other immunostaining.

Microglial reaction to the lesion

Transient accumulation of microglia/brain macrophages occur when physiological (Ferrer et al., 1990; Pearson et al., 1993; Moujahid et al., 1996) or induced cell death (Banati et al., 1993; Landis, 1994) occurs in the brain. In both mammals and non-mammalian vertebrates the response to lesion is characterised by increased lectin binding and transformation of the resting ramified microglia into two different morphological stages in a progressive fashion: activated and reactive microglia respectively (Streit et al., 1988). Both stages also occur during the lizard medial cortex lesion. Activated and reactive microglial cells are active macrophages as they express complement receptors and major histocompatibility complex. Although the functional role of these cells is not fully understood, they appear to participate in immune responses and are capable of migration and phagocytosis (Streit et al., 1988; Gehrmann et al., 1995). In the lesioned lizard

Fig. 5. Electron microscope aspects of microglia in the plexiform layers of the medial cortex of lesioned animals. **A.** Microglia-like cell in the inner plexiform layer of the medial which appears phagocytosing electron dense debris resembling degenerating synapses; seven days after 3AP injection. **B.** Restiform microglia-like cell in the inner plexiform layer of a lizard sacrificed 30 days after 3AP injection; observe the healthy appearance of the surrounding neuropile. Scale bars: 0.5 μm.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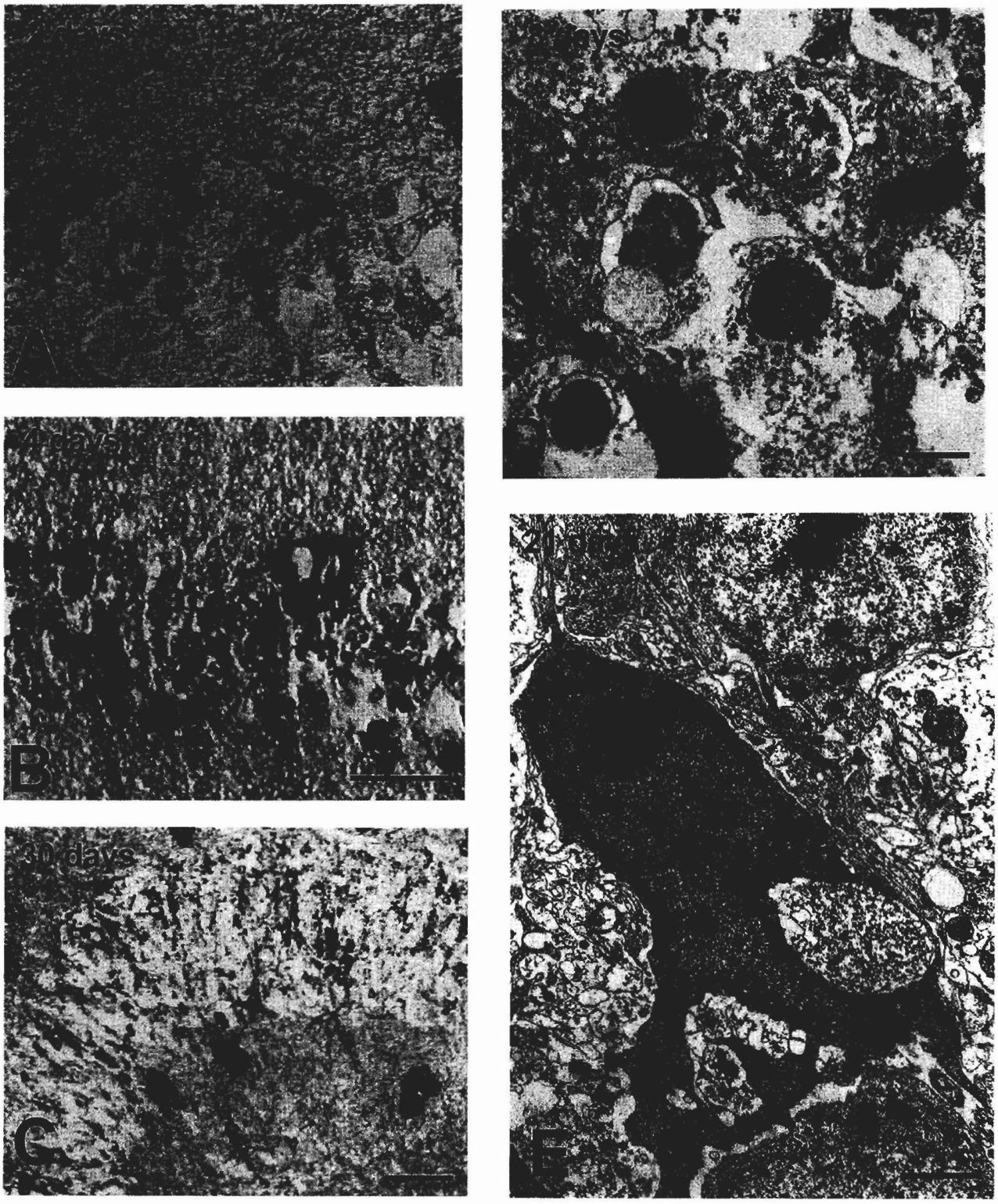


Fig. 6. Light and electron microscope aspects of the medial cortex cell layer at different survival times after the 3AP-neurotoxin injection. A, B, C. Polywax sections double labelled for PCNA and lectin. **A**. 2 days; scarce ameboid activated microglia appear in the borderline of the cell layer. **B**. 4 days; activated microglia cells can be seen within the cell layer. **C**. 30 days; the cell layer appears almost free of lectin-labelled cells. **D**. 8 days; electron microscope aspect of the cell layer showing microglia cells and pyknotic nuclei of lesioned neurons. **E**. 21 days; electron microscopic aspect of a microglial cell surrounding a cell process which seems to be phagocytosed. Scale bars: 25 μm in A, B, C; 5 μm in D; 1 μm in E.

medial cortex, reactive and activated microglia also phagocytose degenerating neural structures during the lesion regeneration process. Furthermore, mammalian microglia are capable of significant proliferation (Giulian and Baker, 1986; Graeber et al., 1988; Glenn et al., 1992) like that we have demonstrated in the lesioned lizard cerebral cortex. All these features indicate that microglial response after 3AP injection in lizards is very similar to that described for the lesioned mammalian CNS, with the only difference, in the lizard medial cortex, that it is delayed as a consequence of the early transitory disappearance after the lesion (López-García et al., 1994).

In the lizard brain, 3-AP not only lesions the medial cortex granule neurons, but also other zinc-enriched neurons (Molowny et al., in preparation). The presence of abundant activated and reactive microglia next to all of the cortical and extra-cortical areas containing zinc-enriched neurons confirms the role of microglia in removing cell debris.

Apart from microglial cells other related cells, mast cells and perivascular cells appeared in the lesioned medial cortex. Like microglia, mast cells enter the brain during development via penetrating blood vessels; they are strongly regulated by hormones especially by glucocorticoids, and may influence synaptic transmission in the CNS and coordinate some aspect of the immune response (Silver et al., 1996). Perivascular cells act as neuronophages during the lesion of the rat facial nucleus (Angelov et al., 1996). The presence of cellular debris in these microglial-related cells suggest that they are involved at least partially in the scavenging processes in

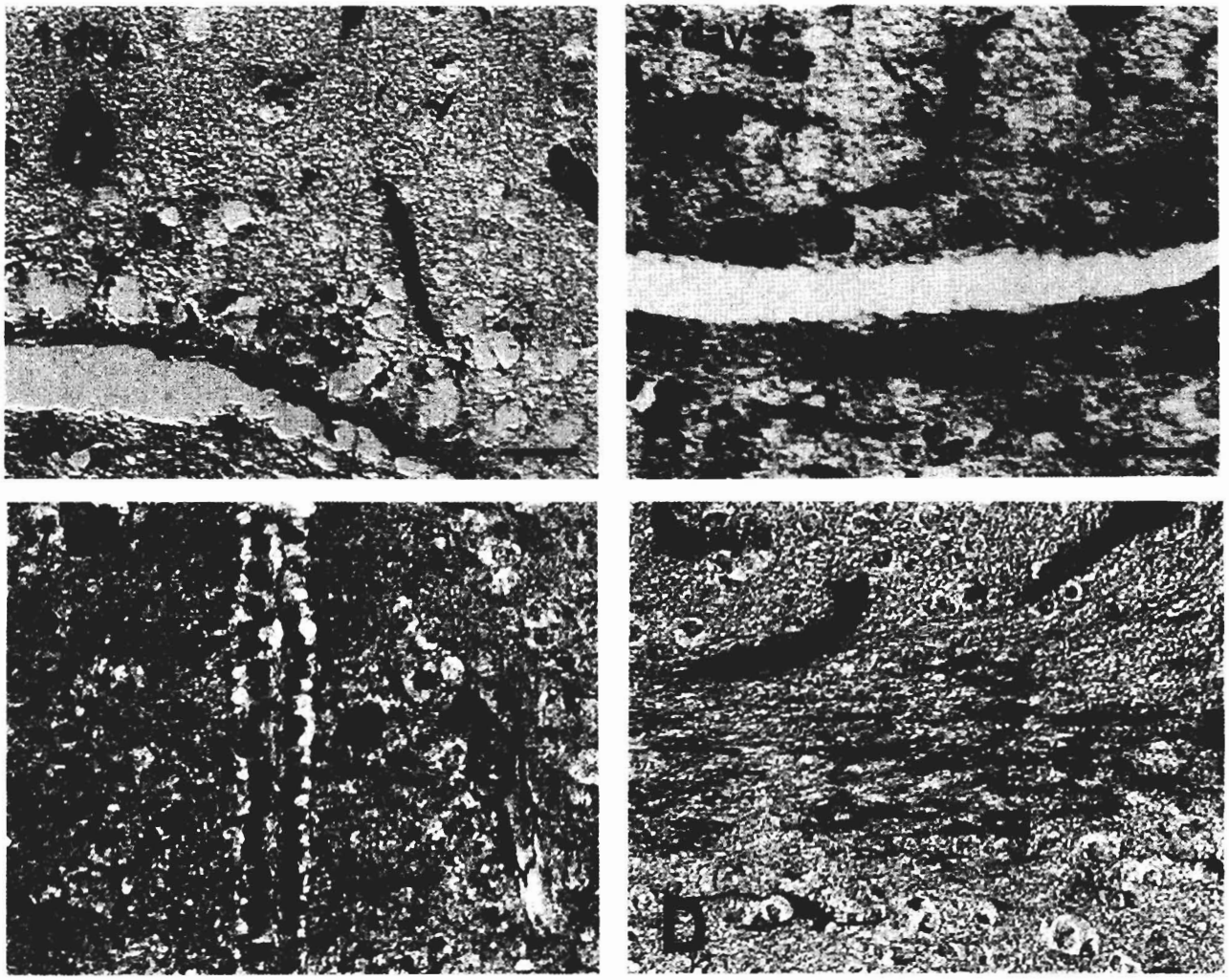


Fig. 7. Light microscope aspects of other telencephalic structures containing lectin-labelled cells and PCNA-immunoreactive nuclei. **A and B.** Dorsomedial cortex inner plexiform layer and ependyma; note the presence of lectin-labelled cells close to "hot spots" of PCNA-labelled nuclei; A: 1 day post-lesion; B: 7 days postlesion. **C.** Lectin-labelled cells surrounding the third ventricle ependyma PCNA-immunoreactive nuclei; 7 days postlesion. **D.** Aspect of the anterior commissure at 7 days postlesion; note the presence of abundant lectin-labelled cells resembling restiform-reactive microglia, some of them have PCNA-immunoreactive nuclei. Scale bars: 25 μm in A, C, D; 25 μm in E.

the lesioned medial cortex. The appearance of meningeal cells with debris in their cytoplasm in close relation with the radial glia end-feet suggests that these cells also participate in the scavenging process, probably getting the debris from the radial glia-ependymocytes, as occurs with perineurial glia and macrophages in the insect embryonic nervous system (Sonnenfeld and Jacobs, 1995).

Transitory disappearance of microglia

Using NDPase histochemistry, a transitory disappearance of microglia from the lizard lesioned medial cortex in the first days after the lesion has been described (López-García et al., 1994). Lectin histochemistry confirms that this disappearance is almost complete in the plexiform layers but not in the cell layer where some scarce reactive and activated microglial cells can be found. Migrations from the plexiform layers are the likely explanation for their absence in these strata; nevertheless, the reduced number of cells in the granular layer indicates that only a minimal fraction of microglia might migrate to the granular layer. The fate of these missing microglia is uncertain, but the simultaneous increase in microglial cells in adjacent cortical areas i.e. dorsomedial and medial cortices, suggests that at least part of them could migrate there. The presence of supraependymal Kolmer cells shortly after lesion may indicate another escape route through the ependymal layer. The presence of debris-full macrophage-like cells in the meninges could signal another way of escape of microglia cells which have passed the glial-limiting membrane.

The period of microglial absence is highly variable among individuals and depends on the environmental photoperiod and temperature variables (unpublished observations) as well as the neurogenetical activity in the ependyma (Ramirez et al., 1997). In the frog brain, the removal of cobalt-labelled neurons by microglia-like cells is twice as fast during the summer as during the winter (Lázár and Pál, 1996). Thus, seasonal-induced differences may be the likely explanation for faster/slower timetable sequences of "microglia early disappearance" (López-García et al., 1994) and the "reactive neurogenesis" (Molowny et al., 1995) periods observed in other experimental lesion-regeneration series performed in our own laboratory but in different seasons. In any case, as shown in this study, the overlapping of these two events occurs in any experimental series and suggests some degree of causality between them, i.e. it is likely that the transitory absence of microglia permits the burst of "reactive neurogenesis".

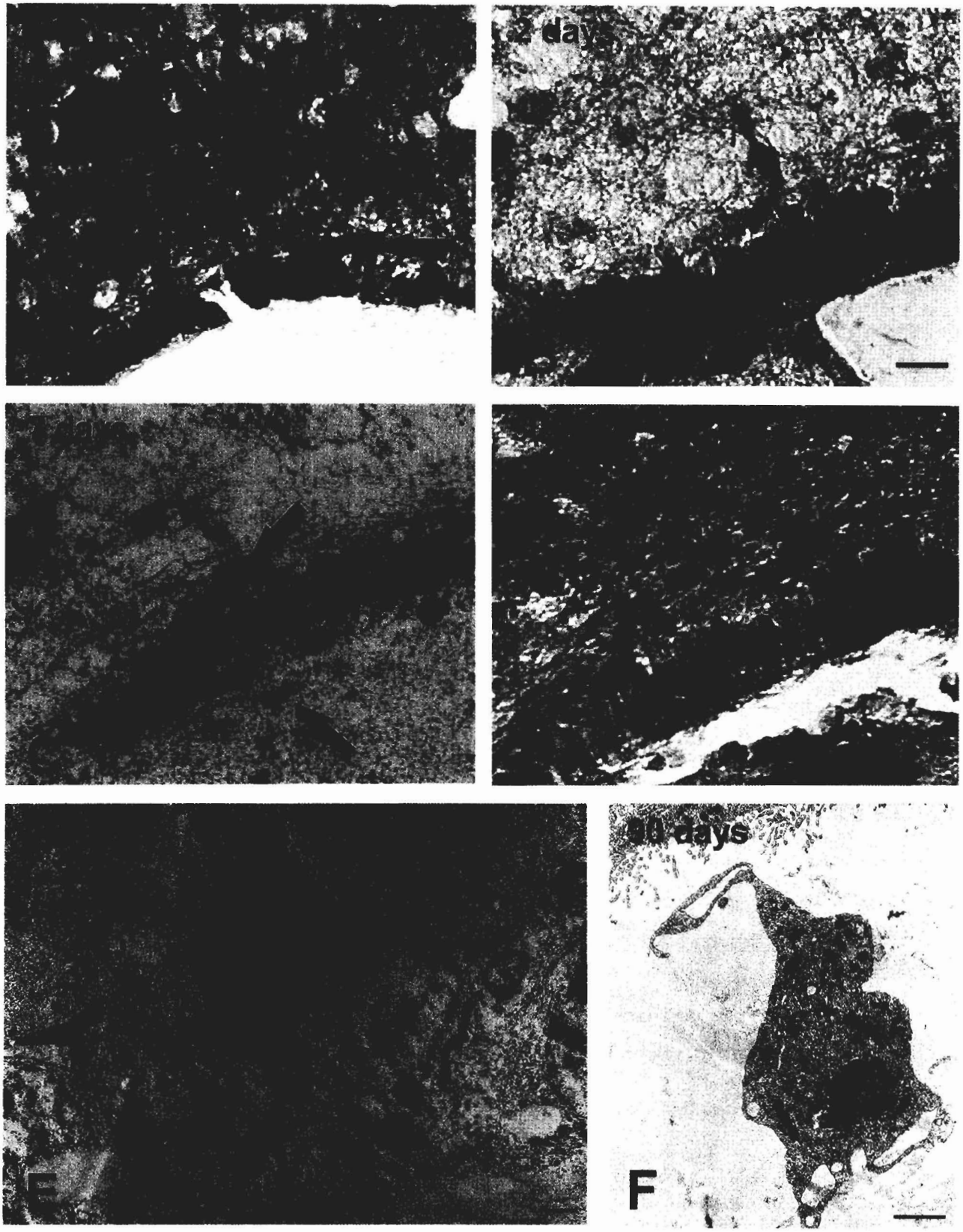
Microglia and neurogenesis

Microglial cells constitute a permanent population in the lizard CNS ependyma (Castellano et al., 1991), although their distribution may differ between different regions. The presence of proliferating cells in the ependyma is not only confined to the sulcus septomedialis of the medial cortex; other areas of the lateral and third ventricle ependyma also display this property (unpublished results). Nevertheless, the fate of the newly generated cells in the ependyma appears clearly different among different ependymal areas: radial migration and recruitment in the principal cell layer is only limited to the medial cortex (García Verdugo et al., 1986; López-García et al., 1988; Molowny et al., 1995). Our results show that microglial cells are absent from ependymal areas in which neuroblasts experiment radial migration, e.g., the medial cortex. This phenomenon is especially evident after lesioning the medial cortex; the "transitory disappearance of microglia" and the peak of "reactive neurogenesis" overlap in time and the former seems to be a permissive factor for the latter. Moreover, the absence of microglia cells may also favour migration and recruitment of the newly generated cells. In salamanders microglia must be kept away from the denervated retina by resident Muller cells to allow successful regeneration (Naujoks-Manteuffel and Nieman, 1994).

During the ontogenesis of the lizard cortex, microglial cells appear in the lizard cerebral cortex after the burst of cortical neurogenesis (Nacher, 1996), as occurs during the rat fascia dentata histogenesis in which microglial cells are absent from the neuronal proliferating areas and then appear after birth (Dalmau et al., 1997). Supporting our hypothesis of a microglial control of neurogenesis, it has been suggested that microglia play a role in the rejection of cells and tissue transplanted into the brain (Finsen et al., 1990) and that microglial cells may induce cytotoxic effects on proliferating cancer cells (Murata et al., 1997).

The present results raise new questions about the participation of microglial cells in the control of postnatal neurogenetical activity. To our knowledge there are no studies about the role of these cells in adult mammalian hippocampus neurogenesis. Future observations of microglial cell participation after adrenalectomy, which implies an increase in adult hippocampal neurogenesis (Cameron and Gould, 1994) could elucidate the role of the relationship of microglia

Fig. 8. Aspects of the sulcus septomedialis at different survival times after the 3AP-neurotoxin injection. A-D. Polywax sections double-labelled for PCNA and tomato lectin. **A.** 1 day; there are scarce PCNA immunoreactive nuclei and lectin-labelled microglial cells (arrows). **B.** 2 days; highly proliferating sulcus with abundant PCNA-labelled nuclei and almost devoid of lectin-labelled cells. **C.** 7 days; there are fewer PCNA-immunoreactive nuclei and scarce lectin-labelled processes appear intermingled with the PCNA-immunoreactive ependymocytes (arrow). **D.** 30 days; labelled restiform microglia cells appear in the vicinity of the sulcus with some PCNA-immunoreactive nuclei. **E.** 21 days; electron microscope aspect of a juxtaependymal microglia cell. **F.** 90 days; electron microscope aspect of a supraependymal "Kolmer cell" free in the ventricle but in the proximity of the apical surface of the ependyma. Scale bars: 10 μm in A to D; 10 μm in E, F.


2 days
F

and neurogenesis.

Re-invasion of the medial cortex by microglial cells

The dead and dying neurons provide stimuli for monocyte macrophage invasion of the mammalian CNS after a variety of lesions (see Davis et al., 1994; Barron, 1995, for reviews), and could be the unique source of brain macrophages in some circumstances (Streit et al., 1988). These invasive blood-derived microglia enter the CNS in a similar way to during development (Leong and Ling, 1992). In the mammalian hippocampus a dual pial hematogenous origin of microglia has been suggested (Dalmau et al., 1997). This observation partially coincides with our study of the microglial cells during lizard cortex development (Nacher, 1996) and the present results in the lesioned medial cortex. However, we also suggest a third ventricular origin, both after lesion and during development, coinciding with previous studies in the quail CNS (Cuadros et al., 1994). In the regenerating medial cortex microglial cells are in close relationship to radial glial cells, as occurs during hippocampal development (Dalmau et al., 1997). Microglial cells probably use the radial glial "guides" to migrate to the granular layer in a similar way to newly generated neurons (Rakic, 1972; Garcia-Verdugo et al., 1986).

Microglial participation in neuronal debris removal

Activated and reactive microglia remove cell debris generated after Wallerian degeneration in the adult mammalian CNS, although this process is very slow (Milligan et al., 1991). In contrast, the microglial reaction in fish (Velasco et al., 1995) and amphibian (Wilson et al., 1992) CNS is stronger and faster, allowing successful nerve regeneration. Thus tissue remodelling may be a function of microglial cells in CNS repair, probably removing inhibitors of axon growth. A recent study indicates that after Wallerian degeneration, lizards are capable of successful structural, although non-functional, regeneration (Beazley et al., 1997).

Although the bulk of the zinc-enriched axonal projection emitted by the lesioned medial cortex is also the subject of lesion-regeneration phenomena (López-García et al., 1992a,b), the synaptic fields occupied by this prominent zinc-positive projection were devoid of microglia. We have shown (Nacher et al., 1999) that in the lesioned lizard cerebral cortex degenerated axons and presynaptic boutons might be mainly eliminated by radial glia-ependymocytes rather than by microglia. This agrees with the fact that reactive microglia do not phagocytose presynaptic terminals after axotomy (Svensson and Aldskogius, 1993). Nevertheless, the participation of lizard microglia in the elimination of neurite debris cannot be absolutely excluded; in fact, some exchange of debris could exist between radial glia and microglial cells, similar to what happens with perineurial glia and macrophages in the insect embryonic nervous system (Sonnenfeld and Jacobs, 1995).

Microglia are responsible for the synaptic stripping phenomena, displacing the afferent synaptic terminals from the dying neurons (Blinzinger and Kreutzberg, 1968). Axonal projections to the lesioned lizard medial cortex appear to be maintained, especially those coming from the olfactory lateral cortex (or "lizard perforant path") (unpublished). It is likely that microglial cells in the lesioned medial cortex could perform this synaptic stripping in the dying medial cortex neural somata.

Acknowledgements. The authors are grateful to Ms. Sabina Wolsky for excellent technical assistance and Mr. Peter Kanis for improving the English. This study was supported by DGESeIC PM97-0104 and GV-D-VS-20-126-96 grants.

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Accepted June 2, 1998