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# Radial glial cells, proliferating periventricular cells, and microglia might contribute to successful structural repair in the cerebral cortex of the lizard *Gallotia galloti*

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**Abstract**

Reptiles are the only amniotic vertebrates known to be capable of spontaneous regeneration of the central nervous system (CNS). In this study, we analyzed the reactive changes of glial cells in response to a unilateral physical lesion in the cerebral cortex of the lizard *Gallotia galloti*, at 1, 3, 15, 30, 120, and 240 days postlesion. The glial cell markers glial fibrillary acidic protein (GFAP), glutamine synthetase (GS), S100 protein, and tomato lectin, as well as proliferating cell nuclear antigen (PCNA) were used to evaluate glial changes occurring because of cortical lesions. A transitory and unilateral upregulation of GFAP and GS in reactive radial glial cells were observed from 15 to 120 days postlesion. In addition, reactive lectin-positive macrophage/microglia were observed from 1 to 120 days postlesion, whereas the expression of S100 protein remained unchanged throughout the examined postlesion period. The matricial zones closest to the lesion site, the sulcus lateralis (SL) and the sulcus septomedialis (SSM), showed significantly increased numbers of dividing cells at 30 days postlesion. At 240 days postlesion, the staining pattern for PCNA, GFAP, GS, and tomato lectin in the lesion site became similar to that observed in unlesioned controls. In addition, ultrastructural data of the lesioned cortex at 240 days postlesion indicated a structural repair process. We conclude that restoration of the glial framework and generation of new neurons and glial cells in the ventricular wall play a key role in the successful structural repair of the cerebral cortex of the adult lizard.


*Keywords:* Cerebral cortex; Injury; Regeneration; Gliosis; Immunohistochemistry; Cell proliferation; Radial glial cells; Sulci; Reptile

## Introduction

The reactive gliosis that occurs after injury in the central nervous system (CNS) of vertebrates directly influences the degree of success of the spontaneous regeneration process in these tissues. The nonpermissive microenvironment formed by glial cells and extracellular matrix is partially responsible for the limited regenerative capacity of the injured CNS in mammals and birds (Blaugrund et al., 1992; Ramón y Cajal, 1914; Schwab and Caroni, 1988; Schwartz et al., 1990). In mammals, reactive astrocytes not only form a physical barrier that stop axonal regrowth but also express several inhibitors of axonal growth (Qiu et al., 2000, review). In contrast, transected axons in fishes, amphibians, and lizards regrow in the presence of reactive astrocytes that have a similar appearance to those in mammals (Anderson et al., 1984; Lang et al., 2002; Stafford et al., 1990). It has been proposed that oligodendrocytes and myelin sheaths in fishes express very low levels of growth inhibitors (Bastmeyer et al., 1991; Sivron and Schwartz, 1994) and upregulate growth-promoting molecules after CNS injury (Ankerhold et al., 1998; Bastmeyer et al., 1991) that support the restoration of neuronal connections and functions (Attardi and Sperry, 1963; Gaze, 1970; Maturana et al., 1959; Stuermer et al., 1992). On the other hand, it has been shown that oligodendrocytes and myelin sheaths in mammals,

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reptiles, and amphibians express inhibitors of the axonal growth (Lang et al., 1995, 1998; Schnell and Schawb, 1990) in the intact and lesioned CNS.

Regenerative success in the CNS of lizards has been demonstrated in the transected caudal spinal cord of *Anolis carolinensis* (Simpson and Duffy, 1994, review), in the transected optic nerve of *Ctenophorus ornatus* (Beazley et al., 1997) and *G. galloti* (Lang et al., 1998, 2002), and after a neurotoxin-induced degeneration in the cerebral cortex of *Podarcis hispanica* (Font et al., 1995; López-García et al., 1992). Taking into account the importance of the glial response in the regenerative process of the CNS of vertebrates and the few studies on this topic in reptiles, we have focussed this study on the progress of reactive gliosis after a physical lesion in the dorsal cerebral cortex of the lizard, corresponding to the medial pallium of mammals (Lanuza et al., 2002).

## Materials and methods

### *Animal maintenance and surgical procedures*

We used 60 adult lizards *G. galloti* (Bischoff, 1982), indigenous to the island of Tenerife. They were collected in their natural environment under license and in compliance with local legislation, and maintained and treated in the laboratory according to the Spanish law (Real Decreto 223/1988) and European animal welfare legislation. The animals were kept in large holding tanks fitted with heaters and overhead lighting (daily cycles of 12-h light/dark; lights on at 7:30 AM), and free access to water and to a mixed diet of commercially available cat food as well as a variety of live insects and fruit. The temperature in the holding tanks was maintained around 20 °C according to the average temperature in their natural environment.

The lizards were anesthetized on ice before lesioning. Under a surgical microscope (Zeiss), a small hole was carefully made in the right frontoparietal osteodermal plaque, using a dental drill, to expose the surface of the telencephalic cortex. The meninges were cut and a unilateral incision was made in the dorsal telencephalic cortex using iridectomy scissors. Finally, the surface of the injury was carefully covered with sheets of fibrin (spongostan) and dentistry cement. Recovery was rapid, and the animals behaved normally after the unilateral incision. The animals were divided into six groups that were allowed to recover over periods of 1, 3, 15, 30, 120, and 240 days postlesion, respectively, and were processed in the same way as unlesioned (control) lizards.

### *Immunohistochemistry*

The lizards were anesthetized with ether vapor and perfused transcardially with Bouin's fixative. The brains were dissected out, cleaned of meninges, and immersed in the same fixative for 7 h. Then, the brains were dehydrated in ethanol, embedded in paraffin, and sectioned at 10 μm in the transverse plane.

Double immunoperoxidase labeling for the detection of the proliferating cell nuclear antigen (PCNA) and glial markers such as glial fibrillary acidic protein (GFAP), glutamine synthetase (GS), tomato lectin, and S100 protein (S100) was performed using sequential detection of the antigens according to established protocols. Sections were hydrated and immersed in a solution of 10% $H_2O_2$ in TBS (0.05 M Trizma base containing 150 mM of NaCl, pH 7.4) to block endogenous peroxidase and then preincubated in TBS containing 1% bovine serum albumen for 3 h. Afterwards, the sections were incubated with monoclonal anti-PCNA antibody (Novocastra; 1:60) overnight at 4 °C, followed by incubation with biotinylated anti-mouse IgG antibody (Amersham; 1:200) for 1 h, and avidin–peroxidase (Sigma; 1:400) for 1 h. The peroxidase activity was revealed with a solution of diaminobenzidine (DAB) containing 1% ammonium nickel sulfate to obtain a black staining. After the anti-PCNA staining, the endogenous peroxidase was blocked again, as described above, and the detection of the corresponding second antigen (GFAP, GS, Lectin or S100) carried out.

The staining of GFAP performed according to Yanes et al. (1990). Sections were incubated with the monoclonal anti-GFAP antibody (Sigma; 1:300) for 48 h at 4 °C, followed by treatment with goat anti-mouse IgG antibody (Sternberger; 1:30) for 30 min, and with the PAP-Complex (Sternberger; 1:100) for 1 h. Finally, the peroxidase activity was revealed with DAB.

The GS was detected according to Monzón-Mayor et al. (1990a). The sections were incubated with monoclonal anti-GS antibody (Sigma; 1:500) overnight at 4 °C, followed by incubation with biotinylated goat anti-mouse IgG antibody (Vector; 1:200) for 1 h and avidin–peroxidase (Sigma; 1:400) for 1 h. Finally, sections were immersed in DAB to reveal the peroxidase activity.

Tomato lectin histochemistry was performed according to Plaza-Pérez (2002). The sections were incubated in biotinylated tomato lectin (Sigma; 1:60) overnight at 4 °C, followed by incubation with avidin–peroxidase (Sigma; 1:400) for 1 h. Finally sections were immersed in DAB to reveal the peroxidase activity.

Immunostaining of S100 was performed according to Romero-Alemán et al. (2003). The sections were incubated with rabbit anti-cow S100 (Dako; 1:1000) for 72 h at 4 °C, followed by incubation in biotinylated goat anti-rabbit IgG (Vector; 1:200) for 1 h, and avidin–biotin complex kit (Vector). Finally, labeling was visualized with DAB.

After each of the steps described above, sections were washed three times in TBS. Double staining was clearly distinguishable under the microscope since anti-PCNA labeling was black whereas anti-GFAP, anti-GS, anti-lectin, and anti-S100 stainings were brown. In negative controls, the relevant primary antibodies were omitted.

*Golgi–Hortega technique*

Lesioned and control lizards were processed for the Golgi–Hortega technique according to Romero-Alemán et al. (1995). Lizards were anesthetized with ether vapor and perfused with a fixative containing 40% formaldehyde, 3% potassium dichromate, and 3% chloral hydrate in distilled water. Brains were dissected out, postfixed in the same fixative for 58 h, and transferred to 1% silver nitrate solution for 60 h. Transverse sections of 60- to 80-μm thickness were obtained with a vibratome and then preserved with the Lavilla stabilization method (Lavilla, 1943). Finally, the sections were dehydrated in a graded ethanol series and xylene and mounted in Araldite.

*Electron microscopy*

Electron microscopy was performed according to Monzón-Mayor et al. (1990b). Briefly, five lizards at 240 days postlesion were perfused with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Afterwards, the brains were dissected out and kept in the same fixative for 4 h at 4 °C. After several washes in PB, the brains were immersed in PB containing 1% osmium tetroxide for 2 h at 4 °C and then in a solution of 2% uranyl acetate in distilled water for 1 h. After several washes in PB, the brains were dehydrated in ethanol and propylene oxide and embedded in Epon. Ultrathin sections were cut on an Ultracut-E ultramicrotome (Leica) and mounted on copper grids. After air-drying and counterstaining in lead citrate, the sections were analyzed with a Zeiss 910 electron microscope.

*Quantitative analyses*

To detect the variation in the numbers of PCNA-positive cells during the regenerative process, we counted the labeled cells under a 40× lens in 55 transverse sections of the telencephalon, out of three controls and three lesioned lizards for each postlesion stage evaluated. We counted the stained cells in the lesion site, in the four ependymal zones, and four sulci of the ventricular wall ipsilateral and contralateral to the lesion. The optical dissector method was used to avoid double counting. These data were processed using the SPSS statistics software for Windows 95 (version 6.1.2.). The mean values and standard errors were represented graphically. The Kruskal–Wallis test was used to compare mean data from more than two groups whereas the Wilcoxon test was used to compare mean values between both hemispheres in the same group. Differences were considered statistically significant at $P \leq 0.05$.

*Image acquisition and processing*

Photomicrographs were taken using a Leitz DMRB light microscope and a Zeiss 910 electron microscope. The negatives and slides were scanned and digitally processed using Adobe Photoshop 5.0 software. Only general contrast adaptations were made and figures were not otherwise manipulated.

## Results

The telencephalic cortex consists of three layers: the inner plexiform layer (ipl), the cellular layer (cl), and the outer plexiform layer (opl), and is divided into the medial cortex (MC), dorsomedial cortex (DMC), dorsal cortex (DC), and lateral cortex (LC). According to Yanes-Méndez et al. (1988a,b), the telencephalic ventricular wall is divided into four ependymal zones: (a), (b), (e), and (f) and four sulci or matricial zones: the sulcus septomedialis (SSM), the sulcus lateralis (SL), the sulcus terminalis (ST), and the sulcus ventralis (SV) (Fig. 1). The lesion was made in the dorsal cortex (Fig. 1, asterisk).

*Double labeling for PCNA/GFAP and PCNA/GS in the lesioned cerebral cortex*

In the control telencephalon, PCNA labeling was observed in the lateral ventricular wall, mainly in the sulcus ventralis, lateralis, and terminalis, but scarcely in the four ependymal zones (not shown). In the cerebral cortex, glial

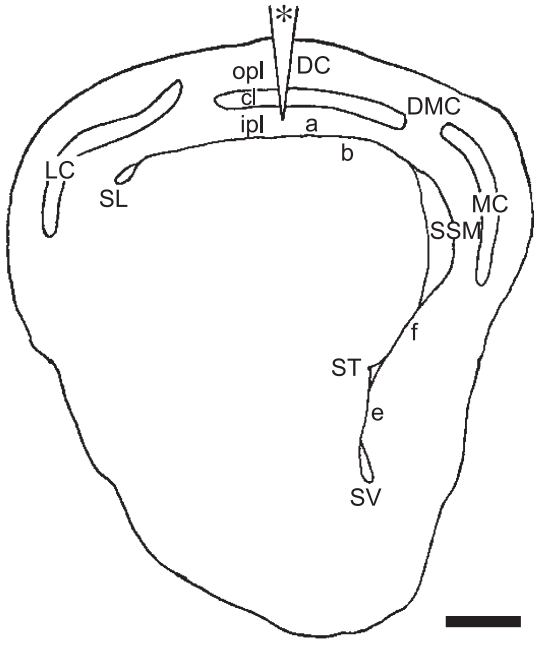


Fig. 1. Camera lucida drawing of a transverse hemisection of the telencephalon in the lizard *G. galloti*. Dorsal telencephalon is up and medial is to the right. The cerebral cortex presents three strata: the outer plexiform layer (opl), the cell layer (cl), and the inner plexiform layer (ipl) and it is divided into the medial cortex (MC), dorsomedial cortex (DMC), dorsal cortex (DC), and lateral cortex (LC). The injury was made in the DC (asterisk). The lateral ventricle is divided into four ependymal zones: (a), (b), (f), and (e) and four sulci: sulcus lateralis (SL), sulcus septomedialis (SSM), sulcus terminalis (ST), and sulcus ventralis (SV). Scale bar = 1000 μm.

processes, including subpial and perivascular endfeet, as well as the ependymal zone (a) were GFAP- and GS-positive (Figs. 2A, B). In addition, scattered GS-positive glial cells were observed in all cortical layers (Fig. 2B).

At 1–3 days postlesion, PCNA-positive cells were observed along the incision line, from the pial surface to the underlying ependymal zone (a) (Fig. 2C). These proliferating cells were GFAP- and GS-negative and absent from the contralateral cerebral cortex. Both GFAP and GS immunostaining were absent from glial processes located along the incision line and in the underlying ependymal zone (a) (Fig. 2D, trapezoid), whereas they persisted similar to control levels in the ependymal zone (b), in the cortex around the incision (Fig. 2D), and in the contralateral telencephalon.

At 15–30 days postlesion, a marked increase in the number of PCNA-positive cells was observed in the lesion site at the pial surface, in all cortical layers, as well as in the underlying ependymal zone (a) (Fig. 2E). In addition, GFAP and GS immunoreactivity were increased in the lesioned dorsal cortex (Figs. 2E, F) as well as in the adjacent dorsomedial and lateral cortex. By contrast, labeling of PCNA, GS, and GFAP in the contralateral hemisphere appeared similar to controls. The underlying ependymal zone (a) changed into a hypertrophic and pseudostratified epithelium showing a peak-shape (Figs. 2E, F, asterisks), whereas the contralateral ependymal zone (a) persisted unmodified (not shown). Cell bodies in the underlying ependymal zone (a) were GFAP-, GS-, PCNA-positive, and showed double labeling for PCNA/GFAP and PCNA/GS (Figs. 2E, F). In the lesioned cortex, scarce PCNA-positive cells displayed GFAP-positive processes (not shown). Scattered GS-positive glial cells in the lesion site remained PCNA-negative and appeared as numerous as in controls (Fig. 2F, arrow-heads).

At 120 days postlesion, PCNA staining appeared similar to that in controls, whereas GFAP and GS immunoreactivity were still increased (Figs. 3A, B). The underlying ependymal zone (a) reverted into a non-hypertrophic simple epithelium, but it retained its peak-shape (Fig. 3A, asterisk). Scattered GS-positive glial cells in the lesion site persisted unchanged (Fig. 3B, arrow-heads).

At 240 days postlesion, double labeling for PCNA/GFAP and for PCNA/GS appeared similar in both telencephalic hemispheres and resembled that in controls (Figs. 3C, D). The only persisting difference between both hemispheres was, in individual specimens, the peak-shape of the ependymal zone (a) in the lesioned cortex (Fig. 3C, asterisk).

*Double staining for PCNA/lectin in the lesioned cerebral cortex*

In the unlesioned telencephalon, microglia, blood vessels, and the ependymal epithelium displayed lectin labeling (not shown). The different morphologies of lectin-positive microglial cells observed in the unlesioned lizard matched the previous classification of Plaza-Pérez (2002) who distinguished between ameboid, pseudopodic, primitive rami-

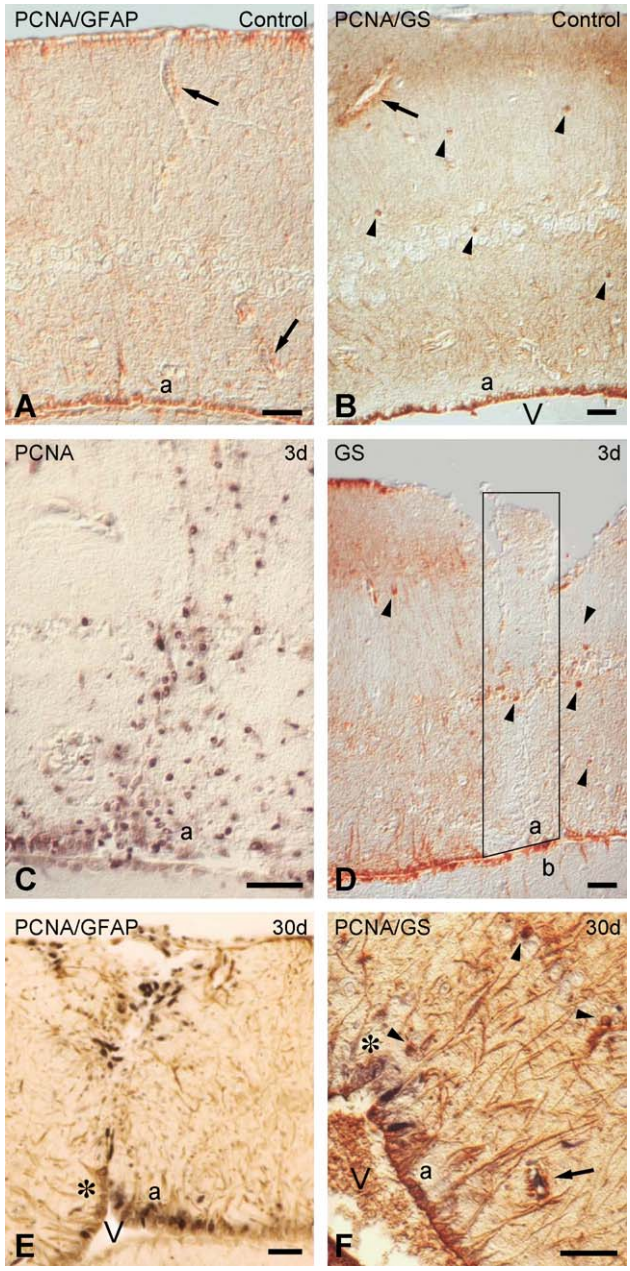


Fig. 2. Labeling for the PCNA, GFAP, and GS in the control and lesioned cerebral cortex. (A, B) Nomarski optics. Double staining for PCNA/GFAP (A) and PCNA/GS (B) in the control cerebral cortex. Glial processes, including subpial and perivascular endfeet (arrows), and the ependymal zone (a) are GFAP- and GS-positive. Some scattered glial cells in all cortical layers are GS-positive (arrow-heads). The PCNA labeling is not observed in these sections. (C) Nomarski optics. PCNA-positive cells are shown in all cortical layers at 3 days postlesion. (D) The GS staining in glial processes is decreased along the incision line (in the trapezoid) at 3 days postlesion. The underlying ependymal zone (a) is GS-negative, whereas the nearby areas and the ependymal zone (b) are stained normally. Scattered GS-positive glial cells (arrow-heads) are stained as in controls. (E, F) Double labeling for PCNA/GFAP (E) and for PCNA/GS (F) at 30 days postlesion. Upregulation of the GFAP and GS and PCNA is shown. The darkest staining correspond to the PCNA labeling. The ependymal zone (a) presents a pseudostratified epithelium showing a peak-shape (asterisks). Note scattered PCNA-negative/GS-positive glial cells (arrow-heads in F) and PCNA-positive nuclei into a blood vessel (arrow in F). a: ependymal zone (a); V: ventricle. Scale bars = 50 μm.

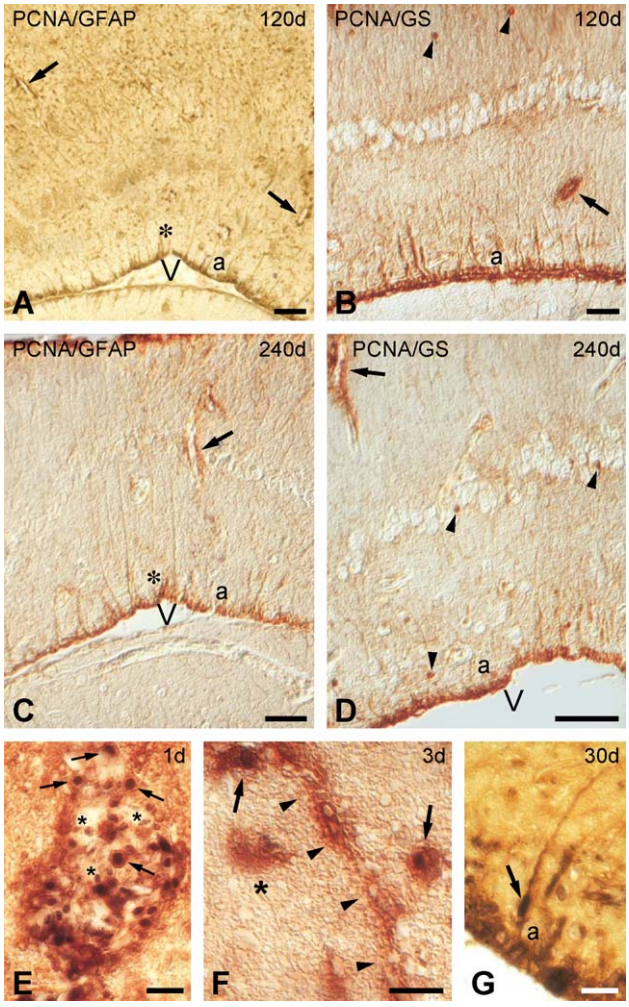


Fig. 3. Staining for the PCNA, GFAP, GS, and lectin in the lesioned cerebral cortex. (A, B) Double labeling for the PCNA/GFAP (A) and PCNA/GS (B) at 120 days postlesion. The upregulation of the GFAP and GS is observed in processes, including perivascular endfeet (arrows). The ependymal zone (a) returns into a simple epithelium still showing a peak-shape (asterisk in A), and the PCNA staining is absent. Scattered GS-positive glial cells (arrow-heads in B) persist unmodified. (C, D) Nomarski optics. Double labeling for PCNA/GFAP (C) and PCNA/GS (D) at 240 days postlesion. Note a staining pattern similar to that in controls (see also Figs. 2A, B). Note GS-negative neurons (D) next to scattered GS-positive glial cells (arrow-heads in D). (E–G) Nomarski optics. Double labeling for PCNA/lectin at 1, 3, and 30 days postlesion (E, F, and G, respectively). (E) PCNA+/Lectin+ cells (arrows) as well as PCNA+/Lectin− cells (asterisks) are shown. (F) Note PCNA+/Lectin+ (arrows) and PCNA−/Lectin+ cell bodies (asterisk) at both sides of the Lectin-positive incision line (arrow-heads). (G) A PCNA+/Lectin+ cell displaying a radial process (arrow) is observed closed to the ependymal zone (a). a: ependymal zone (a); V: ventricle. Scale bars (A to D) = 50 μm. Scale bars (E to G) = 20 μm.

fied, and ramified microglia in the cerebral cortex of adult *G. galloti*.

At 1–3 days postlesion, lectin-positive cells increased in the lesion site. Most of them had an ameboid appearance and were PCNA-positive (Figs. 3E, F, arrows) whereas others were PCNA-negative (Fig. 3F, asterisk). The incision line was also labeled by lectin (Fig. 3F, arrow-heads).

At 15–30 days postlesion, the increase in lectin labeling of macrophages/microglial cells was more evident in the earlier stages. In addition, some PCNA-positive glial cells adjacent to the ependymal zone (a) displayed lectin-positive radial processes (Fig. 3G, arrow).

At 120 days postlesion, lectin labeling remained increased above control levels, whereas the PCNA staining in the lesioned cortex disappeared. However, at 240 days postlesion, the number and morphology of microglial cells in the lesioned cortex became similar to that in control animals.

### *Double labeling for PCNA/S100 in the lesioned cerebral cortex*

S100-positive glial cells in the unlesioned cortex were scarce and PCNA-negative (Romero-Alemán et al., 2003). No differences were observed between the S100 staining in the control and lesioned cortex at any time postlesion.

### *Summary of the changes in PCNA, GFAP, GS, and lectin labeling patterns in the lesioned cortex*

In control lizards, PCNA-positive cells were only observed in the ventricular wall. GFAP and GS labeling were observed in glial processes and in the ependymal epithelium (Fig. 4A). Scattered GS-positive glial cells and lectin-positive macrophages/microglial cells were also observed in all cortical layers (Fig. 4A).

At 1–3 days postlesion, PCNA staining appeared in the lesion site whereas it persisted similar to that in controls in the underlying ependymal zone (a). Upregulation of lectin labeling was observed in the lesion site: PCNA+/lectin−, PCNA+/lectin+, and PCNA−/lectin+ cells were observed along the incision line. By contrast, GFAP- and GS-positive radial glia was absent along the incision line whereas scattered GS-positive glial cells persisted unmodified (Fig. 4B).

At 15–30 days postlesion labeling for all markers described above increased in the lesion site. Numbers of PCNA-positive cells were markedly increased in all cortical layers, as well as in the underlying ependymal zone (a), which changed into a hypertrophic and pseudostratified epithelium showing a prominent peak-shape. GFAP and GS reactivity increased in radial glial cells, while scattered GS-positive glial cells persisted as in unlesioned controls. Lectin-positive macrophages/microglia increased in numbers in all cortical layers, some of them were also PCNA-positive (Fig. 4C). It is noteworthy that at 30 days postlesion, numbers of PCNA-positive cells decreased in the cortical layers whereas they increased in the ependymal zone (a) (Fig. 4D).

At 120 days postlesion, GFAP, GS, and lectin labeling still appeared increased in the lesion site, whereas PCNA staining returned to levels similar to those in controls (Fig. 4E). At 240 days postlesion, all markers considered here had returned to control levels (Fig. 4F).

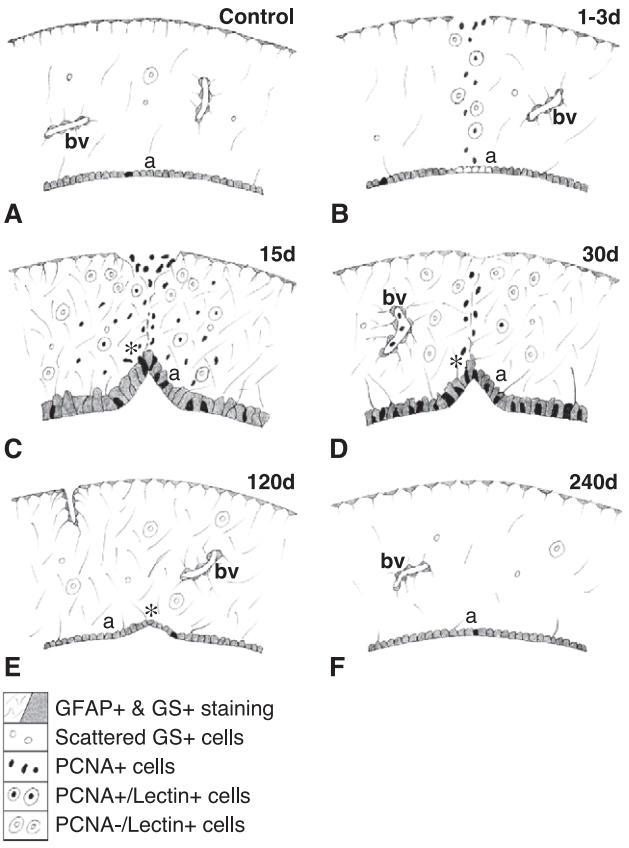


Fig. 4. Diagram illustrating the main changes in the staining for the PCNA, GFAP, GS, and lectin in the control and lesioned cerebral cortex. Both, GFAP and GS labelings are represented in the gray-colored ependyma, glial processes, and subpial endfeet. (A) In the control, cerebral cortex, the ependymal zone (a) and radial glial processes are GFAP- and GS-positive. In addition, some scattered glial cells are GS-positive whereas microglial cells are lectin-positive. PCNA staining is absent from the cortical parenchyma and scarce in the underlying ependymal zone (a). (B) At 1–3 days postlesion, PCNA-positive cells are observed in all cortical layers. The number of lectin-positive cells is increased in the lesion site and some of them are PCNA-positive. The GS and GFAP stainings are decreased along the incision line. (C) At 15 days postlesion, the GFAP, GS, and lectin are upregulated. An important increase in the number of PCNA-positive cells is observed in the lesion site, some of them are also lectin-positive. The ependymal zone (a) changes from a simple to a pseudostratified epithelium showing peak-shape, hypertrophic cell bodies and upregulation of the PCNA staining. (D) At 30 days postlesion, the reactive GFAP-, GS-, and lectin-positive gliosis persists in the lesion site. The number of PCNA-positive cells decreases in the lesioned cortex, some of them are into blood vessels (bv), whereas they increase in the underlying ependymal zone (a). (E) At 120 days postlesion, the upregulation of GFAP-, GS-, and lectin staining persists in the lesion site whereas the PCNA staining becomes similar to controls. (F) At 240 days postlesion, all markers mentioned above return to the control situation. Asterisk in C, D, and E shows the peak-shape of the ependymal zone (a). a: ependymal zone (a); bv: blood vessel.

### *Analysis of the lesioned cerebral cortex using the Golgi–Hortega technique*

The morphological data obtained with the Golgi–Hortega method complement and support the immunohistochemical results described above. Radial glial cells were the most abundantly labeled glial type in the control and lesioned cerebral cortex, whereas typical star-shaped astrocytes were not impregnated in this study. At 1–3 days postlesion, radial glial cells were not impregnated in the lesion site whereas some neurons displayed incompletely impregnated dendrites (Fig. 5A). By contrast, at 15–30 days postlesion, radial glial cells showed hypertrophic cell bodies and processes in the lesion site (Fig. 5B). At 120–240 days postlesion, impregnated neurons and radial glial cells exhibited a morphology similar to that in controls (Fig. 5C).

### *Ultrastructural analysis of the cerebral cortex at 240 days postlesion*

Taking into account that immunohistochemical as well as Golgi–Hortega data from control (unlesioned) animals were

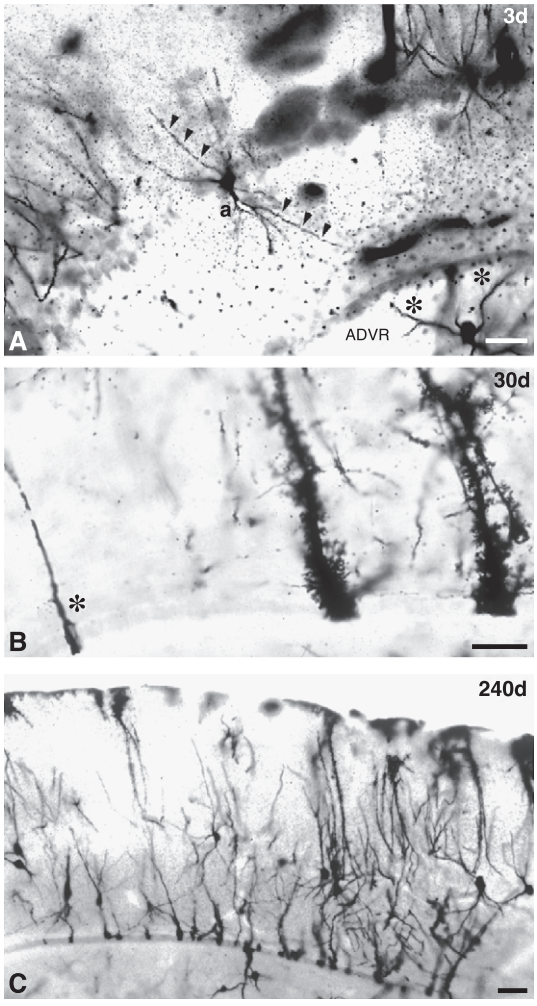


Fig. 5. Golgi–Hortega impregnation in the lesioned cerebral cortex. (A) At 3 days postlesion, a neuron displaying incomplete-impregnated dendrites (arrow-heads) is observed in the lesion site whereas a well-impregnated neuron (asterisks) is observed in the anterior dorsal ventricular ridge (ADVR). a: axon. (B) Two hypertrophic radial glial cells are impregnated on the right whereas a normal radial glia is shown on the left (asterisk), at 30 days postlesion. (C) Impregnation of neurons and radial glial cells at 240 days postlesion are similar to controls. Scale bars = 50 μm.

similar to those derived from the cortex contralateral to the lesion site, we considered the latter a valid control.

The recovery of the lesioned cortex was indicated by the presence of ultrastructurally intact neurons and synaptic contacts in the lesioned and contralateral cortex. Neuronal somata displayed a prominent Golgi apparatus and rough endoplasmic reticulum, free ribosomes, mitochondria, and the nuclear morphology typical of transcriptionally active cells (Fig. 6A).

The scattered glial population in the cerebral cortex predominantly showed immature ultrastructural features similar to those previously described in the midbrain and subpallial nuclei of *G. galloti* during development (Monzón-Mayor et al., 1990b,c; Yanes et al., 1997). The astroglial lineage in the cerebral cortex included, apart from radial glial cells, astroblasts and immature astrocytes. Astroblasts were identified by their dilated cisternae of rough endoplasmic reticulum containing a flocculent material, glycogen granules, and numerous rosettes of free ribosomes, whereas immature astrocytes were characterized by their glycogen granules and few gliofilaments. By contrast, scanty protoplasmic astrocytes in the contralateral cortex were identified by the presence of abundant gliofilaments and glycogen granules in their cytoplasm (Fig. 6B). Oligodendrocytes with light and medium electron-dense cytoplasm were identified by the presence of typical microtubules, prominent Golgi apparatus, and the predominance of electron-dense heterochromatin in their nuclei (Fig. 6C). Cells in the ependymal zone (a) in the lesioned and contralateral cortices appeared ultrastructurally similar and resembled those described in a previous ultrastructural study on ependymal cells in the developing midbrain of *G. galloti* (Monzón-Mayor et al., 1991). Ependymal cells showed microvilli and cilia on their apical membrane, lipofuscin granules, and lipid droplets in their cytoplasm, as well as zonula adherens junctions in their lateral membranes (Fig. 6D).

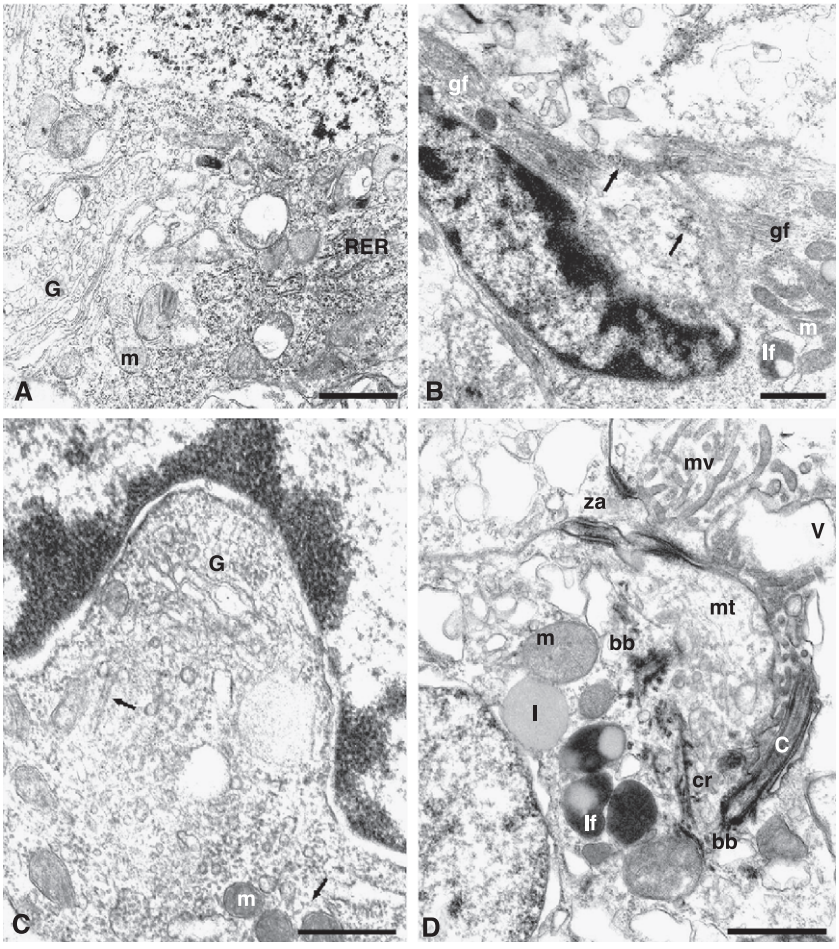


Fig. 6. Ultramicrographs in the lesioned and contralateral cerebral cortex at 240 days postlesion. (A) A neuronal cell body from the lesion site shows features of cells specialized in synthesis and secretion of proteins: abundant euchromatin, developed rough endoplasmic reticulum (RER), and Golgi complex (G). (B) In the contralateral cerebral cortex, a protoplasmic astrocyte shows gliofilaments (gf), glycogen (arrows), lipofuscin granules (lf), and mitochondria (m). (C) Oligodendrocyte from the lesion site showing perinuclear heterochromatin, typical microtubules (arrows), Golgi complex (G), and mitochondrias (m). (D) Cells from the underlying ependymal zone (a) to lesion site show cilias (c), basal bodies (bb), ciliar roots (cr), microvilli (mv), zonula adherens (za), lipid droplets (l), and lipofuscin granules (lf). m: mitochondria; mt: microtubules. Scale bars = 1 μm.

*Quantification of PCNA-positive cells in the lesioned telencephalon*

In the lesioned cortex, the increase in numbers of PCNA-positive cells was most significant at 15 days postlesion (Fig. 7A), as opposed to 30 days postlesion in the underlying ependymal zone (a) (Fig. 7B). This ependymal zone contained significantly more proliferating cells than its contralateral counterpart in controls, 15, 30, 120 days postlesion (Fig. 7B, asterisks). Among these four groups, the increase in numbers of PCNA-positive cells at 15 and 30 days postlesion was the most significant.

In the ependymal zones (b), (e), and (f) ipsilateral to the lesion, the variation in the mean number of PCNA-positive cells among the different groups and between both hemispheres was not statistically significant (not shown).

In the sulcus lateralis (SL) ipsilateral to the lesion, the number of PCNA-positive cells was significantly higher at 30 days postlesion than at 1 and 15 days postlesion (Fig. 7C). Moreover, it contained significantly higher numbers of

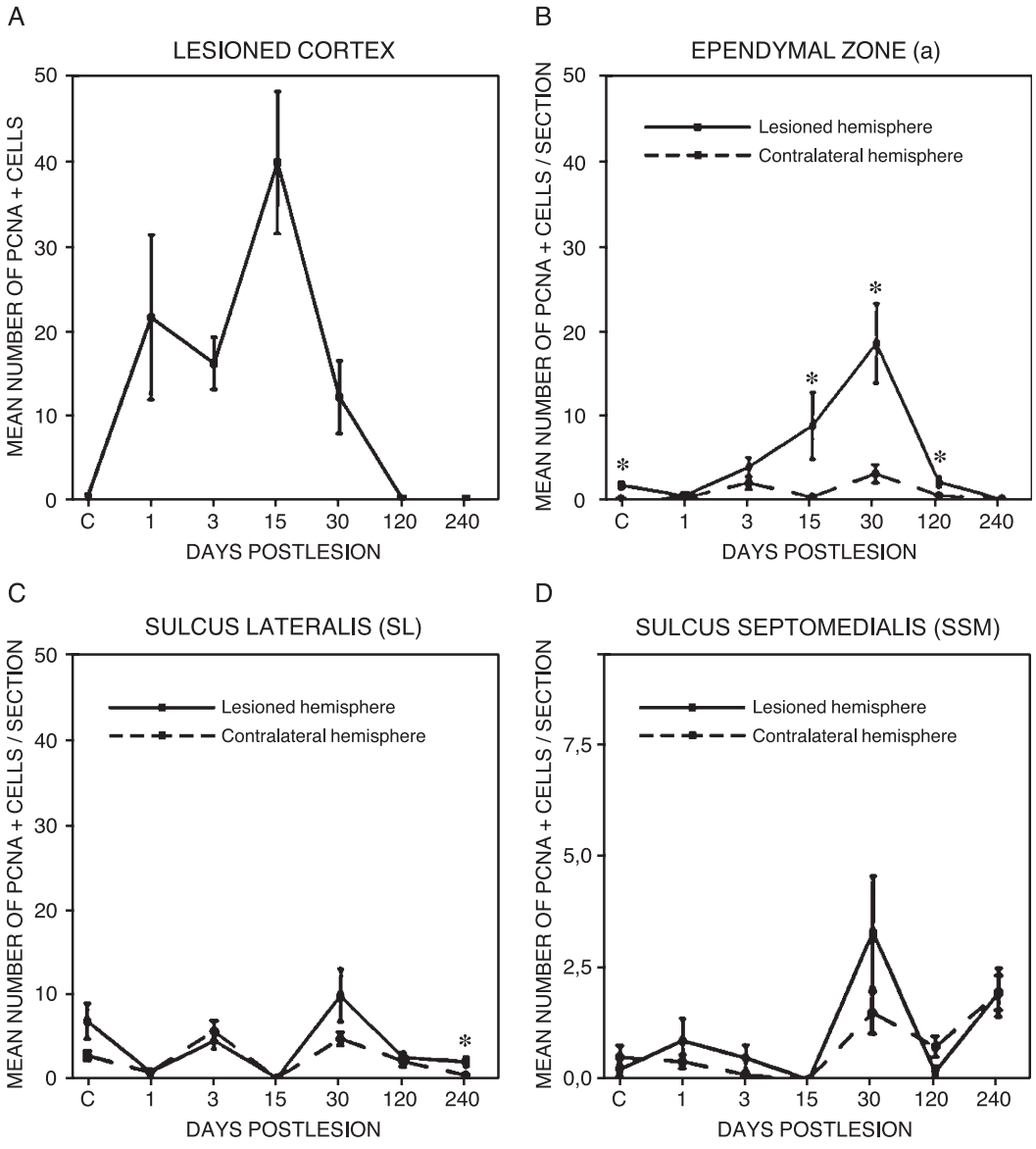


Fig. 7. Quantification of PCNA-positive cells in several telencephalic areas of control (c) and lesioned lizards. (A) In the lesion site, PCNA-positive cells are significantly increased from 1 to 30 days postlesion peaking at 15 days postlesion. (B) Comparison between the ependymal zone (a) of both hemispheres. The ependymal zone (a) ipsilateral to the lesion is significantly more proliferating than the contralateral in controls, 15, 30, and 120 days postlesion (asterisks). Among these four groups, the proliferative responses at 15 and 30 days postlesion are the most significant. (C) Comparison between the sulcus lateralis (SL) of both hemispheres. The SL ipsilateral to the lesion is significantly more proliferating than the contralateral at 240 days postlesion (asterisk). The SL ipsilateral to the lesion at 30 days postlesion is the most proliferating. (D) Comparison between the sulcus septomedialis (SSM) of both hemispheres showing no significant differences at any time postlesion. The SSM ipsilateral to the lesion at 30 and 240 days postlesion is the most proliferating. Standard errors are shown by the vertical lines. The significance level is $P \leq 0.05$; $n = 3$ animals/group.

PCNA-positive cells than the contralateral SL at 240 days postlesion (asterisk in Fig. 7C).

The sulcus septomedialis (SSM) ipsilateral to the lesion showed more proliferating cells at 30 and 240 days postlesion than at the other stages (Fig. 7D); however, the Wilcoxon test indicated no significant differences between the SSM of both hemispheres in any group.

In the sulcus terminalis (ST) and ventralis (SV) ipsilateral to the lesion, the number of proliferating cells was significantly lower at 15 days postlesion than in the other groups, but no significant differences were observed between both hemispheres in any group (not shown).

According to the above data, the most significant increases in the number of PCNA-positive cells in the lesioned telencephalon occurred at 15 and 30 days postlesion: in the lesion site at 15 days postlesion, in the underlying ependymal zone (a) at 15 and 30 days postlesion, and in the two closest sulci (SL and SSM) at 30 days postlesion. It is noteworthy that the number of proliferating cells in the SL and SSM was still significantly increased at 240 days postlesion.

## Discussion

### *Glial response after injury in the cerebral cortex of the lizard*

After a physical lesion in the central nervous system (CNS) of adult vertebrates, a reactive gliosis involving reactive astrocytes, microglia, macrophages, and meningeal cells is observed (Hirsch and Bähr, 2001). As it was expected, the first reactive cells in this study were PCNA+/lectin+ and PCNA−/lectin+ microglia/macrophages. Previous studies on lectin staining in the lizard (Nácher et al., 1999a; Plaza-Pérez, 2002) and our data on GFAP and GS staining support our view that these lectin-positive cells are not astrocytes, unlike those lectin-positive cells that were observed during postnatal development of the mouse cortex (Cooper and Steindler, 1986a,b). In addition, some periventricular cells were lectin-positive in intact lizards (Nácher et al., 1999a; Plaza-Pérez, 2002), as well as at all control and postlesion stages analyzed in this study (e.g., see Fig. 3G). Interestingly, another microglial marker, nucleoside diphosphatase (NDP-ase), also stains a population of periventricular cells in the intact cortex of the lizard *P. hispanica* (López-García et al., 1994). Although the functional aspect of these findings remains unknown, they could be linked to the role of radial glial cells in phagocytosis of cellular debris described in the lesioned cortex of *P. hispanica* (Nácher et al., 1999b) and to the role of periventricular cells in cellular recognition in the intact lizard.

Apart from the role of microglia/macrophages in the immune response, it has been postulated that these cells contribute to a "cytokine network" that influences the composition of the glial scar and thus regulates the regenerative success after CNS injury (Giulian et al., 1994). The morphological changes of reactive lectin-positive microglia/macrophages from the predominantly ameboid appearance at 1–3 days postlesion (Figs. 3E, F) to the predominance of the most mature forms (e.g., primitive ramified and ramified) at 240 days postlesion were also described during the ontogeny of the cerebral cortex of *G. galloti* (Plaza-Pérez, 2002), suggesting that the microglial response to the lesion recapitulates a similar pattern to that observed during development. At 1–3 days postlesion, the decrease in GS and GFAP immunoreactivity along the incision line and in the underlying ependymal zone (a) (Fig. 2D, trapezoid), as well as the absence of Golgi-impregnated radial glial cells in the lesion site, might be consequences of the damage and loss of the local radial glial cells. Along similar lines, the Golgi method showed changes in neurons in the lesion site, such as the incomplete impregnation of dendrites (Fig. 5A), that might indicate a process of cell degeneration.

It is generally accepted that reactive astrocytes are the main contributors to gliosis. Reactive astrocytes are characterized by hypertrophic morphology, proliferation, migration, and upregulation of certain proteins such as GFAP, S100 (Hirsch and Bähr, 2001, review), and GS (Benton et al., 2000). These characteristics, except upregulation of S100 (see below), were observed from 15 to 120 days postlesion in radial glial cells instead of astrocytes, since radial glial cells are the most common glial type in the cerebral cortex of the lizard. Despite the absence of GFAP-positive astrocytes in the intact cerebral cortex (Yanes et al., 1990), scarce PCNA+/GFAP+ reactive astrocytes were observed in the lesion site indicating that they developed in response to the lesion, as has been described because of neurotoxin-induced damage to the cerebral cortex of the lizard *P. hispanica* (Font et al., 1995). By contrast, scattered glial cells expressing S100 or GS labeling in the intact cerebral cortex persisted and did not show any changes in numbers or immunolabeling properties in response to the lesion. We have previously identified most of these S100-positive cells in the brain of *G. galloti* as a subpopulation of oligodendrocytes in myelinated tracts (Romero-Alemán et al., 2003), which also were GS-positive. Accordingly, the S100 staining in the intact cerebral cortex was scarce and almost restricted to the alveus. In the present context, a reactive and proliferative response of the S100-positive oligodendrocytes and GS-positive oligodendrocytes/astrocytes to the cortical lesion might not be of a scale large enough to be detected by our methods.

Our results are indicative of an important role of radial glial cells in the reactive gliosis in the lizard cerebral cortex. Hypertrophic morphology of radial glia was observed using the Golgi–Hortega method (Fig. 5B). Double immunolabeling for PCNA/GS and PCNA/GFAP in the lesion site at 15–30 days postlesion (Figs. 2E, F) revealed the proliferation and upregulation of GFAP and GS in reactive radial glial cells. The pseudostratified appearance of the underlying ependymal zone (a) and its peak-shape (Figs. 2E, F and

3A, C) might be consequences of these marked changes of the radial glial cells. These data strongly suggest that the reactive radial glial cells in the telencephalic cortex are involved in the repair of the damaged blood–brain barrier, as reactive astrocytes are in mammals (Lindsay, 1986). Upregulation of GS in radial glial cells of *G. galloti* could be induced by neurotoxic effects of glutamate, an excitatory neurotransmitter present in the telencephalic cortex of lizards (Martínez-Guijarro et al., 1991), in an attempt to protect the nervous tissue, as Gorovits et al. (1997) suggested for the rat retina.

Reactive gliosis was a unilateral and transitory event in the lesioned cerebral cortex of the lizard, since the labeling of GFAP, GS, PCNA, and lectin was reduced to control levels by 240 days postlesion. Furthermore, the ultrastructural data of the lesion site at 240 days postlesion not only corroborated the return of the glial structure to normal conditions according to previous ultrastructural studies in *G. galloti* (Monzón-Mayor et al., 1990b,c; 1991, Yanes et al., 1997), but also showed intact neuronal somata (Fig. 6A) and synaptic connections that strongly suggest the functional recovery of the tissue.

*Proliferative response in the lesioned cortex and in the lateral ventricular wall*

The increase in the numbers of mitotic cells in the lesioned cortex was one of the first responses to the lesion and was observed between 1 and 30 days postlesion. Many PCNA-positive cells were lectin-positive microglia/macrophages but other populations were GFAP-, GS-, S100-, and lectin-negative. These proliferating cells might be mesodermal cells, based on the following: Firstly, the repair of the blood–brain barrier is likely to be associated with proliferation of meningeal and endothelial cells. Secondly, the presence of PCNA-positive cells in blood vessels at the lesion site (Fig. 2F) might indicate the hematogenous origin of many of these proliferating cells, which would be in line with the monocytic origin of microglia (Kaur et al., 2001). Finally, PCNA-positive cells in the lesioned cortex of *G. galloti* possibly do not belong to the neuronal lineage since the proliferating stem cells that continuously cause new neurons in the cerebral cortex of adult lizards are in the ventricular wall (Font et al., 2001, review). In addition, the identification of the majority of PCNA-positive cells in the regenerating cortical parenchyma of *P. hispanica* as lectin-positive microglial cells (Nácher et al., 1999a) agrees with our results and supports this interpretation.

The presence of dividing cells in the lesioned cortex was transitory since they were not detectable beyond 120 days postlesion (Figs. 3A, B). By contrast, 260 days after the ablation of the dorsal telencephalic cortex of the lizard *Lacerta viridis*, increased cell division rates are still observed in the lesion site (Minelli et al., 1978). The greater severity of the lesion applied in *L. viridis*, and possibly species-specific differences, are likely reasons why the regenerative process in *L. viridis* is more prolonged than in *G. galloti*.

Mitogenic factors produced by the reactive glia in the lesioned lizard cortex might increase the rate of cell division in the lesion site, as has been suggested for the mammalian cortex (Giulian et al., 1994), as well as in the adjacent ependymal zones, as suggested O'Hara and Chernoff (1994) in the spinal cord of amphibians. In this study, the marked increase in dividing cells in the underlying ependymal zone (a) and the two sulci nearest to the lesion site, SSM and SL, might indicate that these zones cause new neurons and glial cells involved in the repair of the lesion site and adjacent areas. Many of these proliferating cells showed GFAP- and/or GS-positive processes indicating that they were radial glial cells. At 240 days postlesion, the proliferative activity in the SSM and SL was the only feature observed in our study that did not return to the normal conditions. Probably it represents a residual response to the cortical lesion that suggests that the complete recovery of this system is very slow, as has also been documented in the regenerating optic nerve this species (Lang et al., 1998, 2002) and other reptiles (Beazley et al., 1997; Rio et al., 1989).

The lifelong persistence of dividing cells in the lateral ventricular wall of the intact brain of *G. galloti* (Yanes-Méndez et al., 1988a,b) was confirmed in this study by PCNA labeling and is in line with the findings of previous studies in other lizard species (Font et al., 2001, review). The continuous neurogenesis observed in the cerebral cortex of adult lizards (López-García et al., 1992) are very likely important factors contributing to the regenerative success in the cerebral cortex of these animals, as suggested by Holder and Clarke (1988). However, regrowth of transected axons through the optic nerve of *G. galloti* was observed in the absence of neurogenesis in the corresponding retina (Lang et al., 2002). These data suggest that while cell division and neurogenesis might be a prerequisite for tissue repair in the cerebral cortex of the lizard, this is not the case as far as regrowth of severed retinal ganglion cells axons is concerned.

The possibility that radial glial cells generate new neurons in the cerebral cortex of this lizard species should be taken into account, since it has been recently documented in the telencephalon of other reptiles (Font et al., 2001, review; Weissman et al., 2003, review). Basically, these studies showed BrdU+/GFAP+ periventricular cells that transformed into BrdU-positive cells that had ultrastructural features of neurons. Some authors consider radial glial cells to be radial neuronal cells (Morest and Silver, 2003). Currently, this possibility cannot be confirmed in our material due to the lack of neurogenic stem cell marker antibodies that cross-react with their respective antigens in reptiles. However, this study shows radial glial cells expressing not only GFAP but also glutamine synthetase (GS). The glutamate recycling as part of the glutamate/glutamine cycle is one of the most firmly established functions for mature astrocytes (Brunet et al., 2004). More-

over, we have demonstrated in previous studies that during the ontogeny of the lizard brain, GS is expressed in radial glial cells, astrocytes, and oligodendrocytes but never in neurons (Monzón-Mayor et al., 1990a, 1998). In adult mammals, radial glial cells in the dentate gyrus and astrocytes in the subventricular zone of the forebrain have shown proliferative and neurogenic capacities as well as expression of glial markers (e.g., GFAP, vimentin) (Weissman et al., 2003, review) as also have shown radial glial cells in the telencephalon of adult reptiles (Font et al., 2001, review; Weissman et al., 2003 review). During early stages of *G. galloti* development, radial glial cells change from expressing vimentin to GFAP-containing intermediate filaments, as do astrocytes (Monzón-Mayor et al., 1990d; Yanes et al., 1990). Only residual vimentin-positive subpial endfeet persist in the adult lizard brain (Monzón-Mayor et al., 1990d; Yanes et al., 1990). Taken into account all data mentioned above, we conclude that radial glial cells in the adult lizard are able to play roles of mature astrocytes and maintain some of their original properties during early development (e.g., guidance of migrating neurons) (García-Verdugo et al., 1986). We hypothesize that the multiple properties of radial glial cells could be closely related to the plasticity and regenerating abilities of the adult lizard CNS.

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