Histol Histopathol (2020) 35: 1455-1471

**http://www.hh.um.es**

Histology and Histopathology
*From Cell Biology to Tissue Engineering*

# No rapid and demarcating astroglial reaction to stab wounds in Agama and Gecko lizards and the caiman *Paleosuchus* - it is confined to birds and mammals

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**Summary.** The present study proves that the rapid and demarcating astroglial reactions are confined to birds and mammals. To understand the function of post-lesion astroglial reaction, the phylogenetical aspects are also to be investigated. Considering the regenerative capabilities, reptiles represent an intermediate position between the brain regeneration-permissive fishes and amphibians and the almost non-permissive birds and mammals. Damage is followed by a rapid astroglial reaction in the mammalian and avian brain, which is held as an impediment of regeneration. In other vertebrates the reactions were usually observed following long survival periods together with signs of regeneration, therefore they can be regarded as concomitant phenomena of regeneration. The present study applies short *post-lesion* periods comparable to those seen in mammals and birds for astroglial reactions. Two species of lizards were used: gecko (leopard gecko, *Eublepharis macularius*, Blyth, 1854) and agama (bearded dragon, *Pogona vitticeps*, Ahl, 1926). The gecko brain is rich in GFAP whereas the agama brain is quite poor in this. Crocodilia, the closest extant relatives of birds were represented in this study by Cuvier's dwarf caiman (*Paleosuchus palpebrosus*, Cuvier, 1807). The *post-lesion* astroglial reactions of crocodilians have never been investigated. The injuries were stab wounds in the telencephalon. The survival periods lasted 3, 7, 10 or 14 days. Immunoperoxidase reactions were performed applying anti-GFAP, anti-vimentin and anti-nestin reagents. No rapid and demarcating astroglial reaction resembling that of mammalian or avian brains was found. Alterations of the perivascular immuno-reactivities of laminin and β-dystroglycan as indicators of glio-vascular decoupling proved that the lesions were effective on astroglia. The capability of rapid and demarcating astroglial reaction seems to be confined to mammals and birds and to appear by separate, parallel evolution in them.

**Key words:** Brain lesion, Crocodilia, GFAP, Reactive glia, Reptiles

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DOI: 10.14670/HH-18-273

## Introduction

To understand the function of post-lesion astroglial reaction, the phylogenetical aspects are also to be investigated. Considering their regenerative capabilities, reptiles have a midposition between the regeneration-permissive fishes and amphibia, and the almost regeneration-deficient mammals and birds (see e.g. Dunlop et al., 2004; Romero-Aleman et al., 2013). For a review on the regeneration in reptile (lizard) brains see Lopez-Garcia et al. (2002).

Traumatic injuries provoke an astroglial reaction in mammalian brains in a couple of days, which demarcates the tissue defect, restitutes barriers to blood and extracerebral tissues and is held as an impediment of axonal regrowth although beneficial effects have also been noted (for recent reviews, see Sofroniew, 2009; Sofroniew and Vinters, 2010). The hallmarks of this reaction are: astrocyte hypertrophy, increased production of GFAP (glial fibrillary acidic protein), and a

rearrangement of astrocyte processes toward the lesion site to form a demarcating astroglial scar. A similar reaction was also detected in birds (Canady and Rubel, 1992; Ajtai and Kálmán, 1998; Wynne et al., 2008), despite the former negative opinion of Bignami and Dahl (1976).

Whether similar reactions occur in other vertebrates the data are rather controversial. The former studies by other groups usually followed long post-lesion survival periods and it was not clear whether the glial reactions were rapid and defensive, or whether they developed later due to concomitant phenomena of regeneration. For a review see Kálmán et al. (2013). The present study allowed shorter (days 3-14) survival periods, which were proved to be enough to develop demarcating glial reactions in rat (Mathewson and Berry, 1985; Hozumi et al., 1990) and chicken (Ajtai and Kálmán, 1998).

Our former studies failed to evoke rapid glial reactions in turtle (Kálmán et al., 2013). GFAP-immunostaining did not detect true astrocytes in turtles (Onteniente et al., 1983; Kálmán et al., 1994; Lazzari and Franceschini, 2006) only thin, long ependymoglial processes. However, astrocytes seem to be essential for reactive gliosis (see e.g. Sofroniew, 2009; Sofroniew and Vinters, 2010). In lizards true astrocytes have been demonstrated (Bodega et al., 1990; Monzon-Mayor et al., 1990; Yanes et al., 1990; Lazzari and Franceschini, 2001; 2005a,b; Ahboucha et al., 2003; Lőrincz and Kálmán, 2015, 2020) as well as in caiman (Kálmán and Pritz, 2001). The post-lesion astroglial reactions of the crocodilians have never been investigated.

In our current study two species of lizards were used: gecko (leopard gecko, *Eublepharis macularius*, Blyth, 1854) and agama (bearded dragon, *Pogona vitticeps*, Ahl, 1926). The gecko brain is rich in GFAP whereas the agama's is sparse (Ahboucha et al., 2003; Lőrincz and Kálmán, 2015, 2020). The two species have relatively distant positions from each other on the phylogenetic tree (Gekkota versus Iguania subordos, Wiens et al., 2010). Crocodilians are the closest living relatives of birds, and represent the clade Archosauria whereas lizards' belong to Lepidosauria (for a recent review: Benton, 2005). As their representative, Cuvier's dwarf caiman (*Paleosuchus palpebrosus*, Cuvier 1807) was used.

The lesions were produced by stab wounds through the telencephalon. The glial reactions were investigated with different anti-GFAP, anti-vimentin and anti-nestin antibodies. Vimentin and nestin are intermediate filament proteins of immature glia which re-appear in the reactive glia in mammals (Schiffer et al., 1986; Clarke et al., 1994). Three types of positive controls were applied: a) investigation of the cerebrovascular immunoreactivity of laminin and β-dystroglycan as their alterations might be indicators of *post-lesion* glio-vascular decoupling (Krum et al., 1991; Sixt et al., 2001; Milner et al., 2008; Szabó and Kálmán, 2008); b) GFAP and vimentin immunostainings following stab wounds in rat and bird (cockatiel, *Nymphicus hollandicus*, Kerr, 1792); c) detection of vimentin immunopositivity in the agama cerebellum, to prove the reactivity our antibody with lizard vimentin.

## Materials and methods

### *Animals*

The animals were bred and obtained in pet shops; they belonged to either sex and were weighted 350 and 450 g (caiman), between 30 to 45 g (leopard gecko), or between 200 to 300 g (agama). They were supplied with food and water *ad libitum* and kept in artificial 12/12 h light-and-dark periods at 28-30°C temperature in light and 23-25°C in dark period. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Experiments were performed in accordance with the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary (22.1/3491/003/2008), the permission of Hungarian authorities (KA-1928, dated from May 31, 1916) and the European Union Directive (EU Directive 2010/63/EU).

### *Operation and perfusion*

The animals were anesthetized with hypodermic injections containing a combination of ketamine and xylazine (10 and 1 mg/kg body weight, respectively). Lesions were performed manually through the skull in lizards but through a narrow burr hole in caimans, rat and cockatiel. Brains were stabbed in the mid-section of the telencephalon and oriented according to the positions of the eyes compared to a brain in an opened skull (Fig. 1). The lesions penetrated the dorsal pallium and the dorsal ventricular ridge (DVR). The postoperative periods were 3, 7, 10 and 14 days (POD) and 2 agamas and 2 geckos were sacrified at each time point. One-one caiman was sacrified at POD 3 and 7, and one rat and one cockatiel at POD 7. Following this, all specimen were processed similarly. The animals were perfused transcardially with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Anesthesia was administered by the same method as described for the operations.

### *Immunoperoxidase reactions*

After perfusion the brains were removed carefully and immersed in the same fixative for a further 24 h. Blocks containing the stab wound were dissected out from the brains, embedded in agarose and cut into serial sections transversal to the brain axis (thickness 50 μm) using a Vibratome vibration microtome (Intracel, Shepreth Royston Herts, UK). The sections were washed in 0.1 M PBS (0.01 M phosphate buffered physiologic saline, pH 7.4, Sigma) overnight and then processed for immunohistochemical staining. The floating sections

were pre-treated at first with 3% $H_2O_2$ (for 5 min) to suppress the endogenous peroxidase activity and then with 20% normal goat serum (for 1.5 h at room temperature) to suppress the non-specific binding of antibodies. Each subsequent step was followed by a wash (30 min) in PBS between the changes of reagents.

Primary antibodies, their dilutions and final concentrations are given in Table 1. They were applied for 40 h at 4°C, thereafter the immunohistochemical reaction was further developed according to the 'ABC' peroxidase method. Both anti-GFAP and both anti-vimentin antibodies were used on both lizards and caimans. (Note: the antibodies were applied separately, not combined.) Biotinylated anti-mouse or anti-rabbit immunoglobulin (Vector), and avidin-biotinylated horseradish peroxidase (ABC) complex (Vectastain) were applied subsequently, each in a dilution of 1:100, for 1.5 h, at room temperature. The immunocomplex was visualized by diaminobenzidine (DAB) reaction: by incubation in a mixture of 0.05% 3,3'-diaminobenzidine, in 0.05 M Tris-HCl buffer (pH 7.4) and 0.01% $H_2O_2$ for 10 min, at room temperature. The sections were mounted, dried in air, covered with DePeX, and coverslipped. Control sections were incubated by substituting the primary antibody with normal serum. No structure-bound labelling was observed in these specimens.

### *Immunofluorescent reaction*

Immunofluorescent staining was only applied in the case of laminin to prevent a non-specific peroxidase reaction of endothelial cells. Following the incubation with primary anti-laminin serum anti-rabbit antibody conjugated with fluorescent Alexa 488 (Invitrogen) was applied at room temperature for 3 h. The sections were finally washed in PBS (1 h, at room temperature), mounted onto slides, coverslipped in a mixture of glycerol and bi-distilled water (1:1), and sealed with lacquer. Data on secondary antibodies of both methods are shown in Table 2.

### *Photomicrographs*

Both the bright-field and the fluorescent photomicrographs were taken by a DP50 digital camera mounted on an Olympus BX-51microscope (both from Olympus Optical Co. Ltd, Tokyo, Japan). Digital images were processed using Photoshop 9.2 software (Adobe Systems, Mountain View, CA, USA) with minimal adjustments for brightness and contrast.

## Results

The brains with lesions are shown in Fig. 1. Since gecko and agama astroglia were described in our former papers (Lőrincz and Kálmán, 2015) and others (Ahboucha et al., 2003; Lazzari and Franceschini, 2005a), the astroglial structure of intact brains is described only briefly here.

GFAP immunopositivity was found in geckos throughout the brain. The main astroglial type was radial ependymoglia (Figs. 2, 3; all the microphotographs were taken from sections incubated with Novocastra anti-GFAP.) Non-radial astroglial processes penetrated this system, and masked it almost beyond recognition, e.g. in the dorsal ventricular ridge (DVR, Fig. 2a). Astrocyte-like elements occurred only very scarcely (Fig. 2a, inset). Radial glial processes traversed the dorsal and medial pallium, but a middle zone was conspicuously

**Table 1.** Detailed data of the primary antibodies applied in the study.

| Against | Firm | Code Nr. | Dilution | Final conc. (µg/ml) | RRID |
|---|---|---|---|---|---|
| Dystroglycan† | Novocastra, Newcastle-upon-Tyne, U.K. | ncl-b-dg | 1:100 | 0.19 | AB 442043 |
| GFAP† | Novocastra, Newcastle-upon-Tyne, U.K. | ga5 | 1:100 | 100 | AB 563739 |
| GFAP†† | DAKO, Galstrup, Denmark | Z0334 | 1:200 | 56 | AB 10013382 |
| Laminin 1†† | Sigma, San Louis, MO, USA | L9393 | 1:100 | 5 | AB 477163 |
| Nestin† | Millipore, Temecula, CA, USA | MAB-353 | 1:1000 | 1 | AB 94911 |
| Vimentin clon V9† | Millipore, Billerica, MA, USA | IF01 | 1:100 | 1 | AB 2216107 |
| Vimentin clon 3B4† | Abcam, Cambridge, UK | ab28028 | 1:100 | 5 | AB 778826 |

†: mouse monoclonal, ††: rabbit polyclonal.

**Table 2.** Details of the secondary antibodies applied in the study.

| Conjugated with | Against | Host | Company | Code Nr. | Dilution | Final cc. (µg/mL) |
|---|---|---|---|---|---|---|
| Alexa Fluor 488 | Rabbit | Donkey | Invitrogen, Carlsbad, CA, USA | A31570 | 1:500 | 4 |
| Biotin | Mouse | Horse | Vector Burlingame, CA, USA | BA 2000 | 1:100 | 0.05 |
| Biotin | Rabbit | Horse | Vector Burlingame, CA, USA | BA 1000 | 1:100 | 0.05 |

light where their staining was less intense (Fig. 2a). The lesions penetrated different parts of the telencephalon, the dorsal pallium (Fig. 2a,b), the DVR, the septum (Fig. 2c,d), and the striatum (Fig. 3a-d). The lesions mutilated and disarranged the astroglial process system, but there was no other alteration. Following lesions, we did not find the characteristic marks of demarcating *post-lesion* astroglial reactions present in mammals and birds. Neither a conspicuous increase of GFAP immunoreactivity and astrocyte hypertrophy, nor a rearrangement of process system toward the tissue defect and demarcating accumulation of immunoreactive elements was observed.

In the agama telencephalon (Fig. 4a-c) GFAP immunopositive elements were found only in few areas, mainly in the medial pallium, in the septum, and in the adjacent part of the striatum. Other areas including the dorsal and lateral pallium and the DVR were almost free of GFAP. However, astrocytes were also scarcely seen (Fig. 4b). The habenula and the optic tract contained

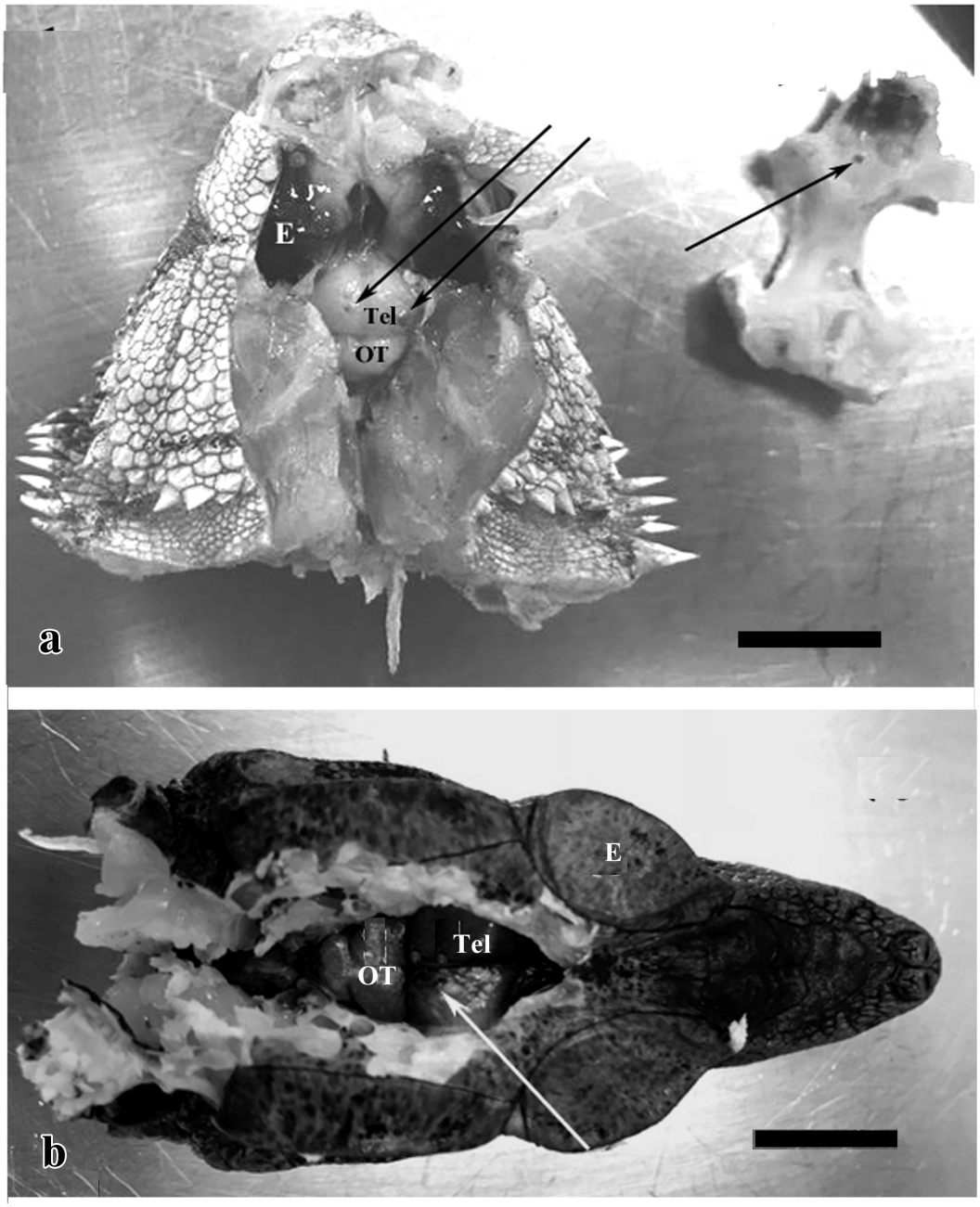


**Fig. 1.** The operation. **a.** An agama brain operated and perfused but still in situ. **b.** The roof of skull has been removed. **c.** A caiman brain operated and perfused but still in situ. Arrows point to the drill holes in the skull and the stab wounds in the brain (black arrows: agama lesions, white arrow: caiman lesion). E: eye, Tel: telencephalon, OT: optic tectum. Scale bars: 1 cm.

dense systems of GFAP-immunopositive glial processes (Fig. 4d,e). Following lesions, hallmarks of demarcating astroglial reactions were not seen in agamas either. The GFAP-free areas remained devoid of GFAP-immunoreactive elements, which did not appear around the tissue defect. The lesions mutilated the process

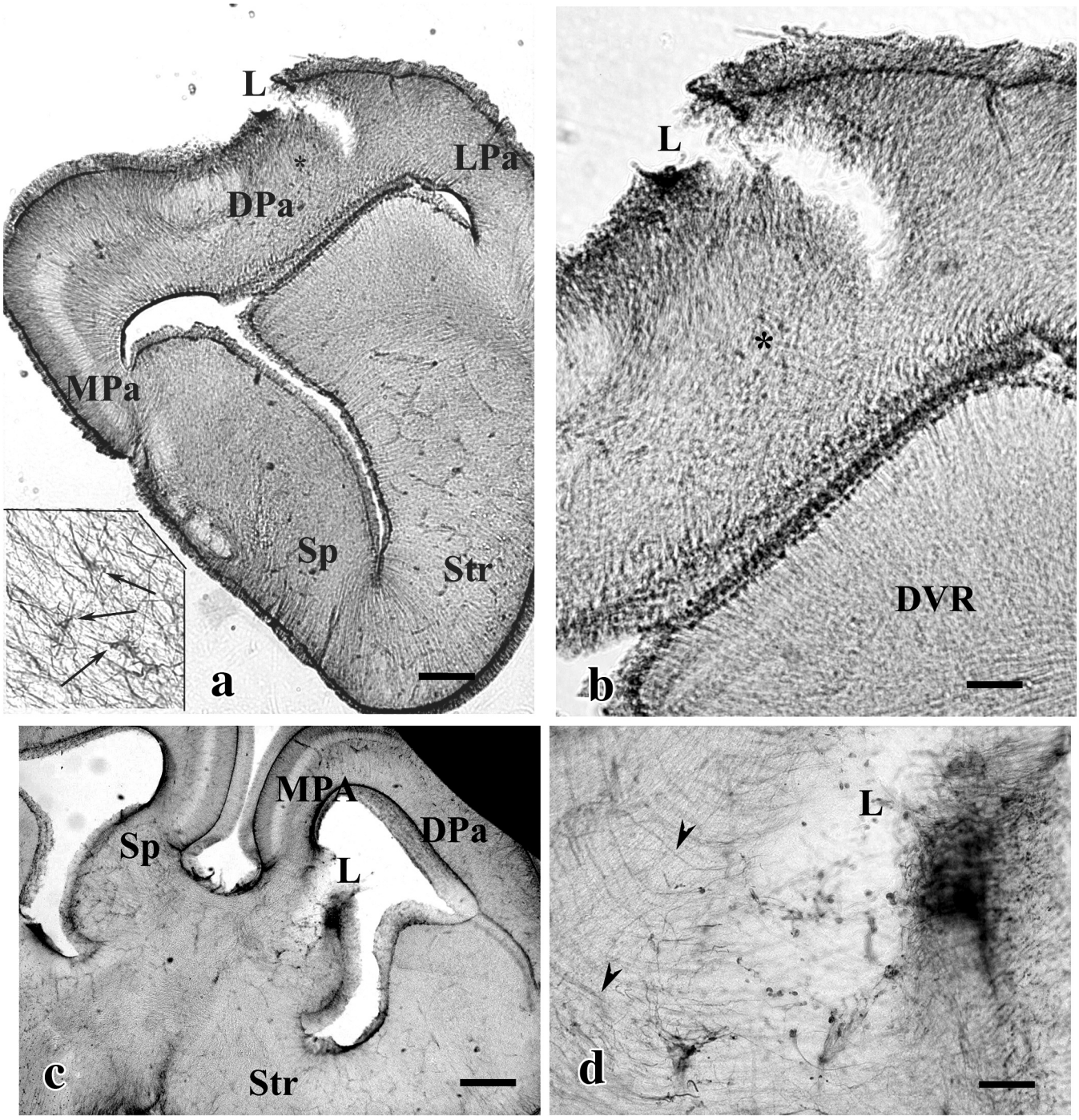


**Fig. 2.** Distributions of GFAP-immunopositive elements following lesions in gecko brains, POD3 and POD7. There is no severe astroglial accumulation, hypertrophy or demarcation around the lesions (L). DPa: dorsal pallium, DVR: dorsal ventricular ridge, LPa: lateral pallium, Sp: septum, Str: striatum. Note the GFAP-poor middle layer in the medial pallium (MPa). **a, c.** General views on sections with lesion at POD3 and POD7. Inset in panel **a**, scare astrocyte-like structures (arrows) at asterisks in the pallium. **b, d.** Enlarged details adjacent to the tissue defect. Arrowheads in panel d): glial processes. Scale bars: a, 150 µm; b, d, 80 µm.; c, 200 µm; inset, 40 µm.

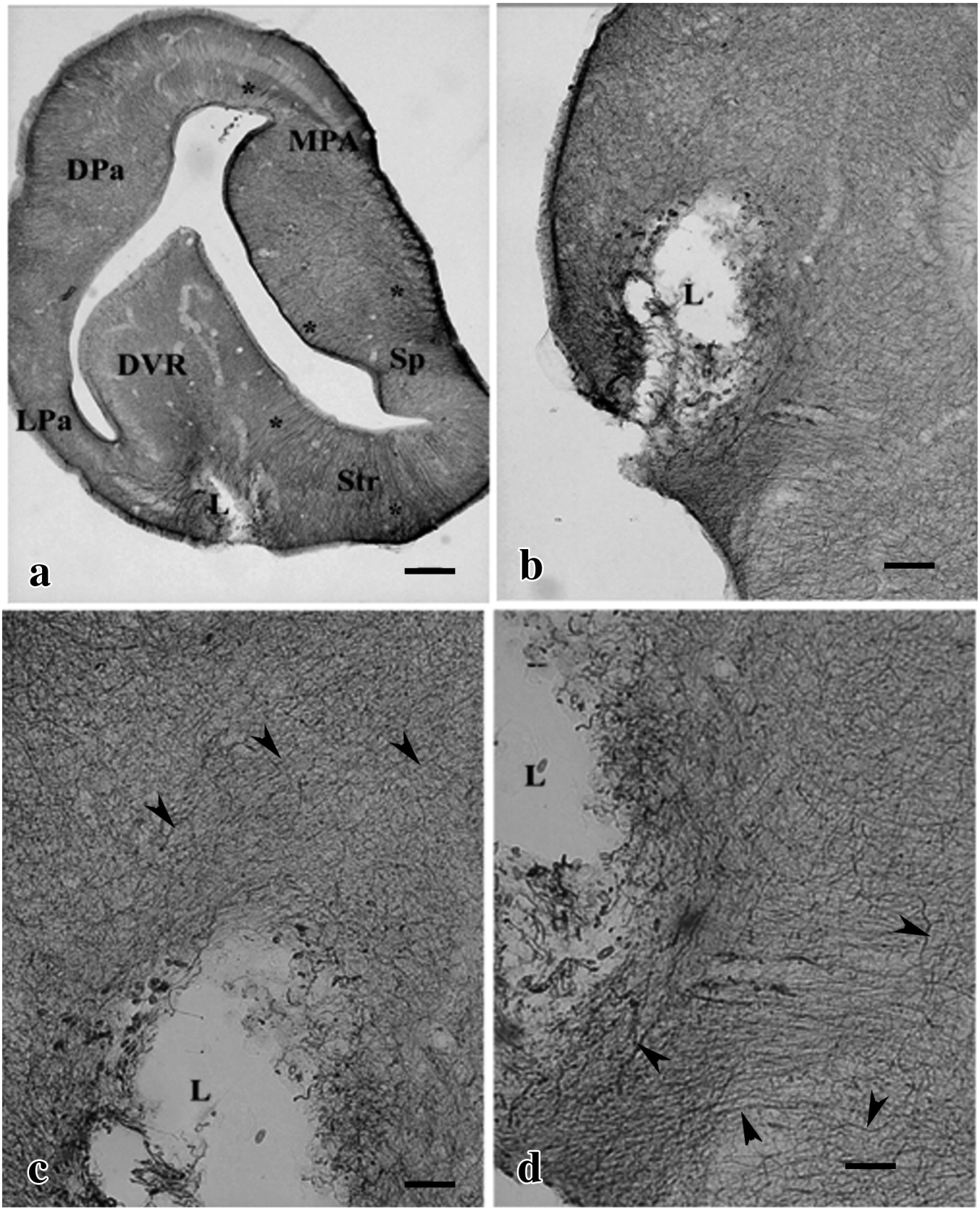


**Fig. 3.** Distributions of GFAP-immunopositive elements following lesions in gecko brains, POD14. There is no severe astroglial accumulation, hypertrophy or demarcation around the lesions (L). DVR: dorsal ventricular ridge, DPa: dorsal pallium, LPa: lateral pallium, MPa: medial pallium, Sp: septum, Str: striatum. **a.** General view on the section with lesion. Note the staining of resting astroglia (asterisks). **b.** The area of lesion enlarged. **c, d.** Details of panel **b**, enlarged. Arrowheads point to glial processes. Scale bars: a, 200 µm; b, 80 µm; c, d, 40 µm.

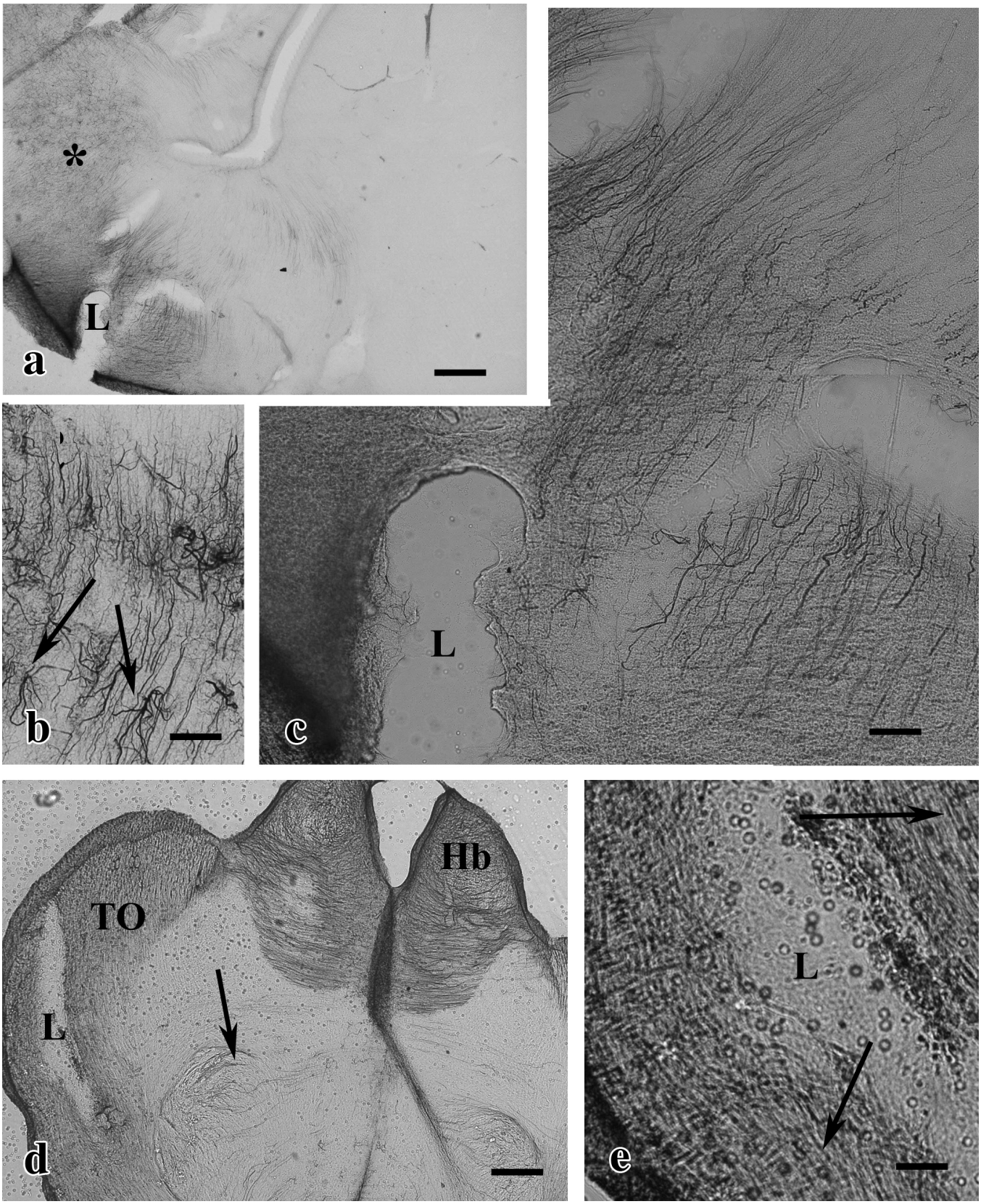


**Fig. 4.** Distributions of GFAP-immunopositive elements following lesions in agama brains. There is no severe glial accumulation, hypertrophy or demarcation around the lesions (L). **a.** Ventral part of telencephalon, POD 10; asterisk labels area with GFAP-immunopositive processes and astrocytes see enlarged in panel **c**. **b.** Enlarged part of panel **a**, at the asterisk; arrows point to astrocytes. **c.** Enlarged part of panel a) at the lesion. **d.** Thalamus, POD7, note the GFAP-rich habenula (Hb). Arrow points to glial process in a GFAP-poor area. The lesion is in the optic tract (TO). **e.** Enlarged part of panel **d**; arrows point to GFAP-immunopositive processes following the course of optic axons but not oriented to the lesion. Scale bars: a, d, 200 µm; b, 20 µm; c, 60 µm; e, 80 µm.

systems, but did not evoke their re-arrangement toward the tissue defect, an example of which can be seen in the optic tract (Fig. 4e). In the dorsal pallium of agama (Fig. 5a) GFAP immunopositive, irregular and densely packed processes (Fig. 5b) were found already at POD3 and persisted even in POD14 (Fig. 5c).

The caiman astroglial architecture has been described in spectacled caiman (*Caiman crocodilus*,

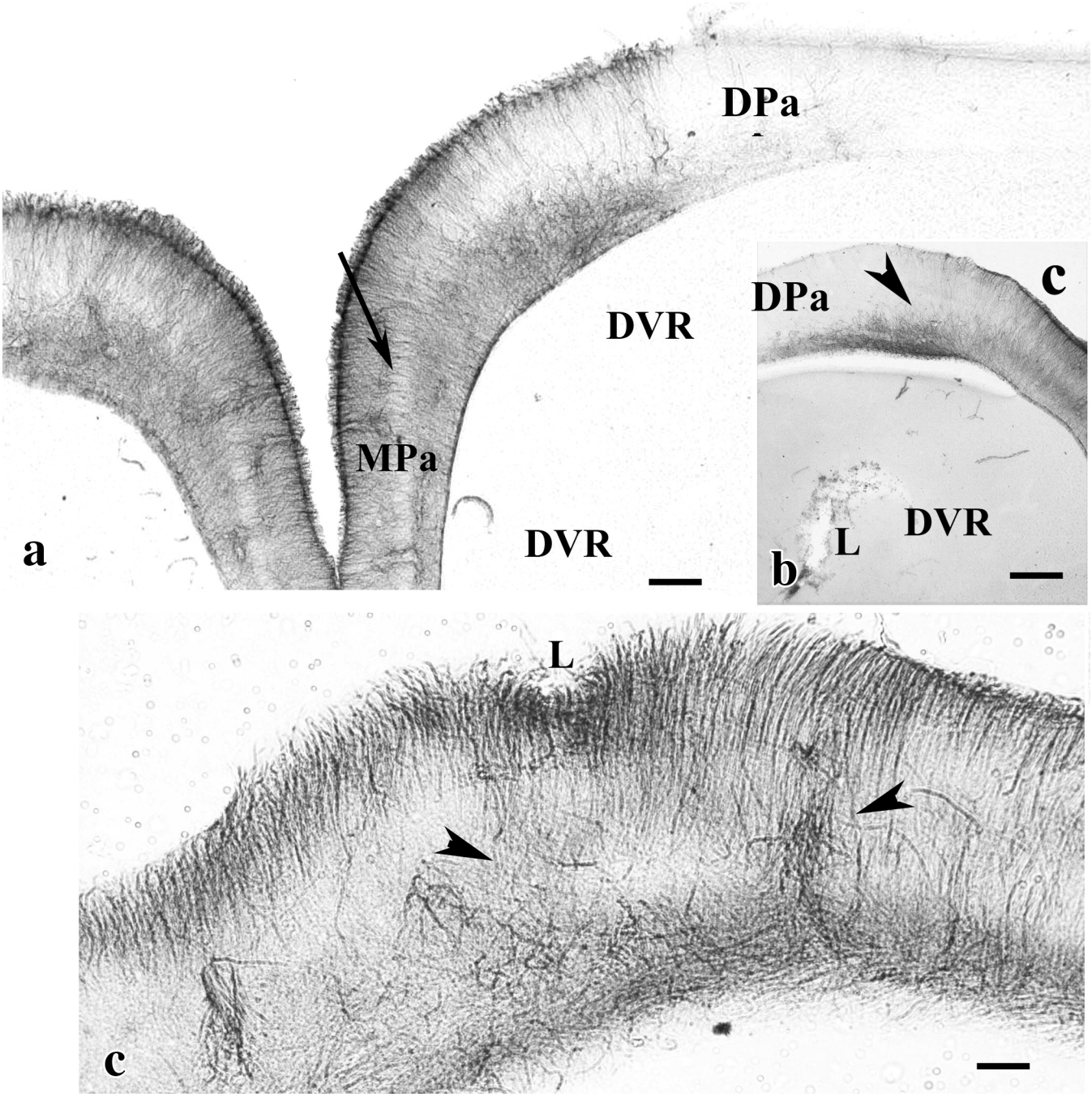


**Fig. 5.** Distributions of GFAP-immunopositive elements following lesions in agama pallia. DPa, MPa - dorsal and medial pallia, DVR -dorsal ventricular ridge, L -lesion. **a.** In the intact agama pallium the GFAP-immunopositive process pattern is regular and confined to the medial and dorsomedial pallium and leaves a middle zone free (arrow). **b.** A section adjacent the lesion site. A GFAP-immunopositive process population appears already at POD3 (arrowheads). These processes do not respect the middle zone and they are not parallel to each other although their courses are roughly perpendicular to the pial surface. **c.** The disarranged processes were still visible at POD14 (arrowhead). Note that there is no accumulation of GFAP-immunopositive elements around the tissue defect (L) in the DVR. Scale bars: a, 100 µm; b, 200 µm; c, 60 µm.

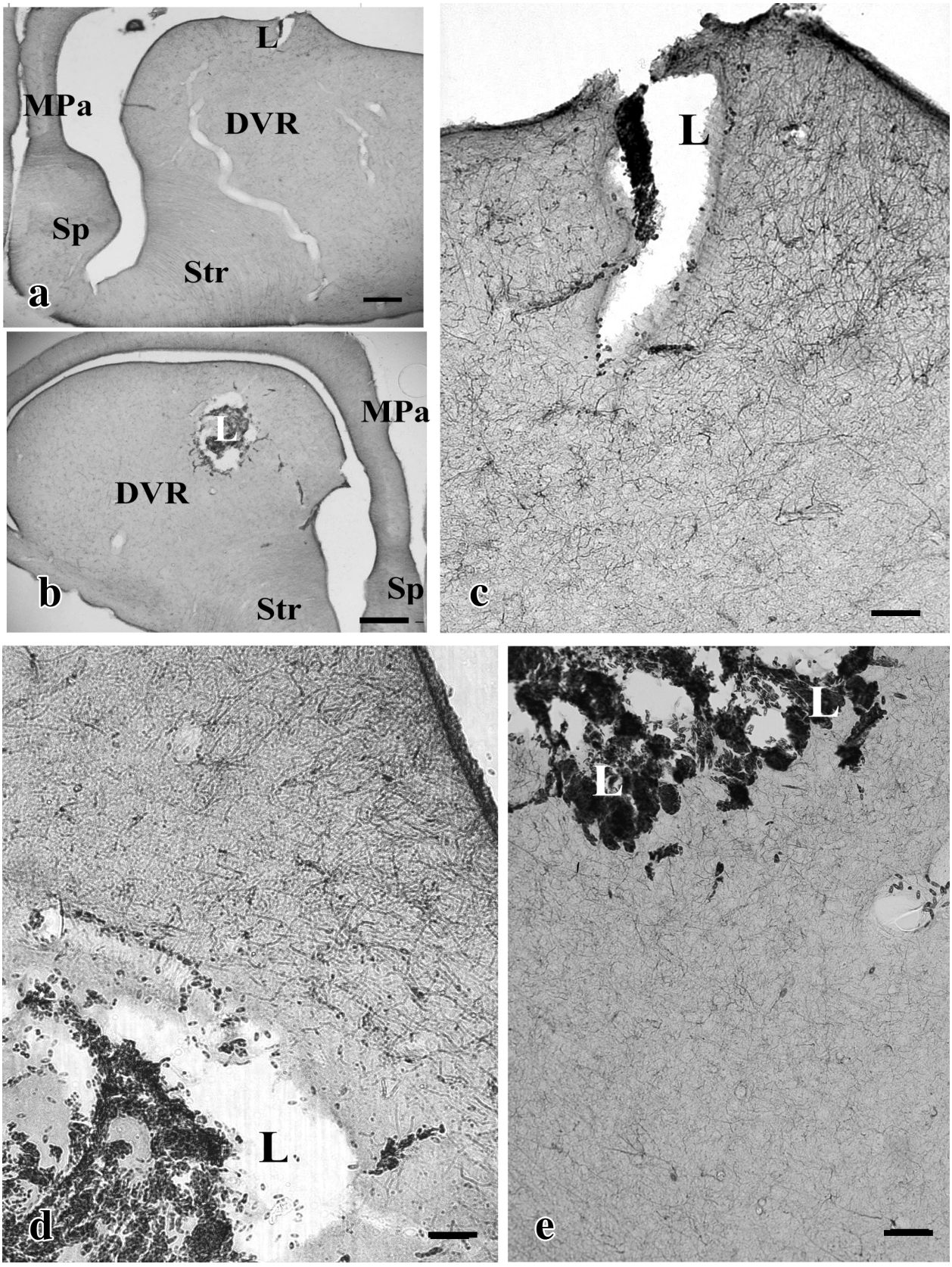


**Fig. 6.** Distributions of GFAP-immunopositive elements following lesions in caiman brains. There is no severe glial accumulation, hypertrophy or demarcation around the lesions (L). DVR: dorsal ventricular ridge; MPa: pallium; Sp: septum; Str: striatum. **a.** Telencephalon, POD3. **b.** Telencephalon, POD7. **c.** Enlarged detail of panel **a**. **d.** Enlarged detail of panel **c**. **e.** Enlarged detail of another lesion. Scale bars: a, b, 700 µm; c, d, e, 80 µm.

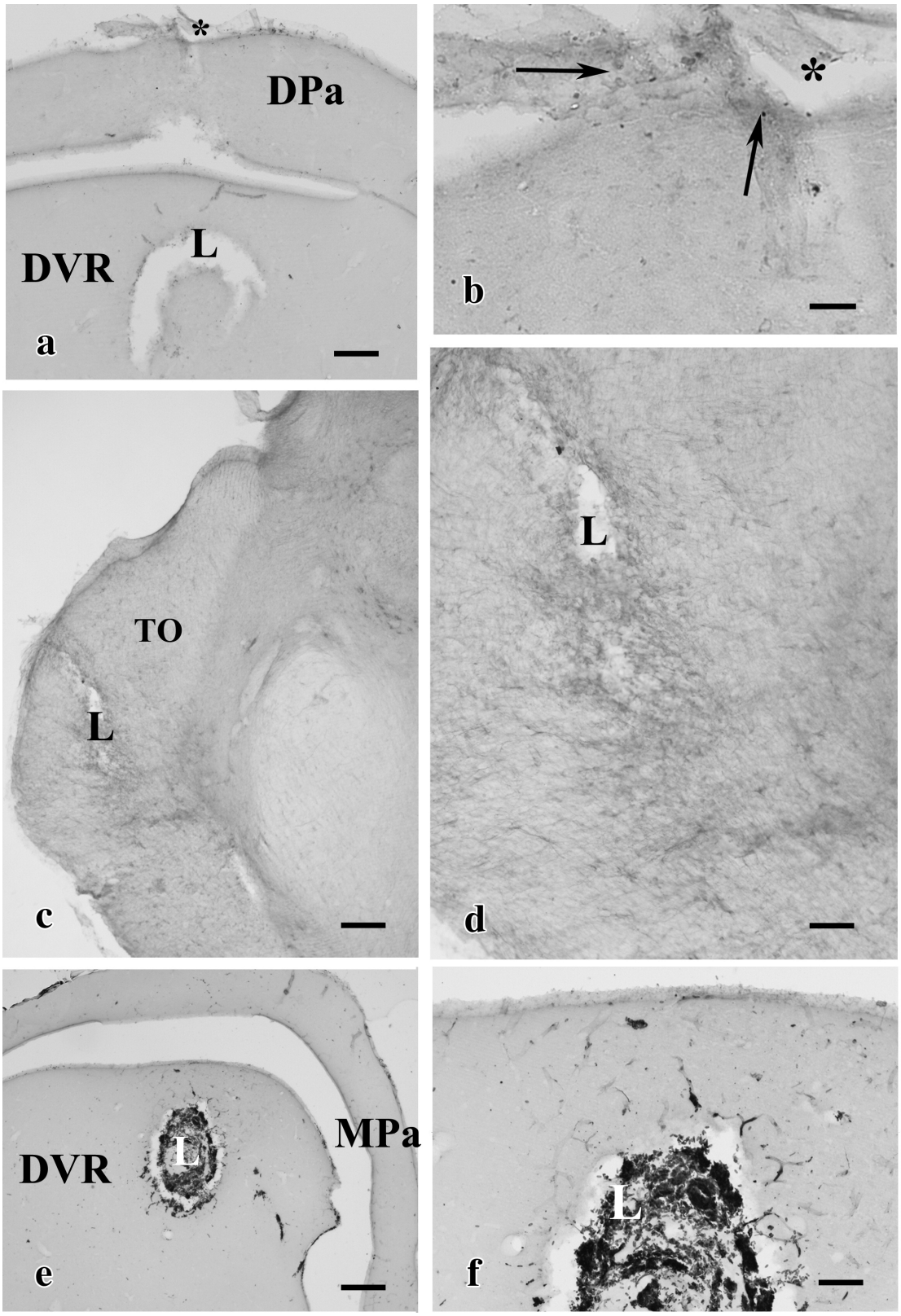


**Fig. 7.** Reactive gliosis was not revealed with vimentin immunohistochemistry either. These photomicrographs were taken following reactions with antibody of clone 3B4 but reactions with antibody of V9 also resulted in negative. **a.** Detail of agama telencephalon, similar to that seen in Fig 5b. DPa: dorsal pallium; DVR: dorsal ventricular ridge; L: the site of lesion; asterisk: the pallial part of lesion, see enlarged in panel b. **b.** Detail around asterisk in panel a) enlarged. Note the red blood cell remained after lesion (arrows). **c.** Detail of agama thalamus, similar to that seen in Fig. 4d. L: the site of the lesion, TO: optic tract. **d.** The tissue defect enlarged from panel c. **e.** Detail of caiman telencephalon, similar to that seen in Fig. 6b. DVR: dorsal ventricular ridge; MPa: medial pallium; L: the site of the lesion. **f.** The tissue defect enlarged from panel e. Scale bars: a, 200 µm; b, 50 µm; c, 100 µm; d, 40 µm; e, 500 µm; f, 140 µm.

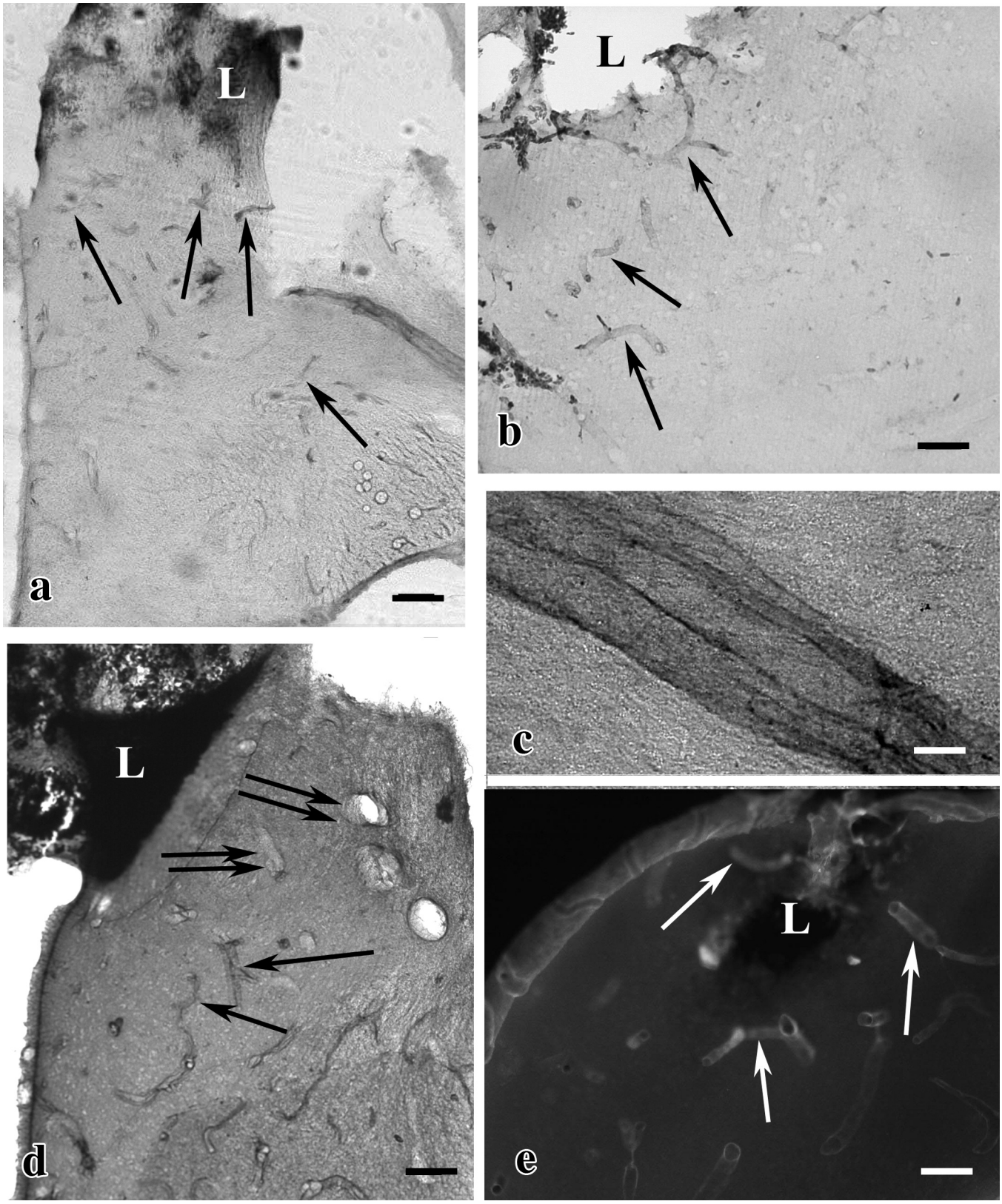


**Fig. 8.** Positive control I. Immunohistochemical detection of cerebrovascular laminin and β-dystroglycan following lesions. **a.** Immunoperoxidase reaction for laminin in gecko, POD3, DVR. Note that vessels are labelled (arrows) only around the lesion (L). **b.** Immunoperoxidase reaction for laminin in caiman, POD3, DVR. Note that vessels are labelled (arrows) only around the lesion (L). **c.** Enlarged vessel immunopositive to laminin, gecko, DVR. **d.** Immunoperoxidase reaction for β-dystroglycan, POD3, gecko, dorsal pallium. Vessels are labeled (arrows) only at a distance, but not (double arrows) adjacent to the damaged tissue (L - lesion). **e.** Immunofluorescent reaction for laminin, POD3, gecko, dorsal pallium. Vessels are labelled (arrows) only around the lesion (L). Scale bars: a, b, 300 µm; c, 20 µm; d, e, 200 µm.

Linneaus, 1758) (Kálmán and Pritz, 2001), therefore, the few differences that we found in *Paleosuchus* are only mentioned here. The density of radial processes was similar in the striatum and in the DVR. The radial glia was densely interwoven with fine non-radial processes (Fig. 6), giving a general impression indicative of a three-dimensional network rather than a uni-directional radial system. The particularly thick radial processes

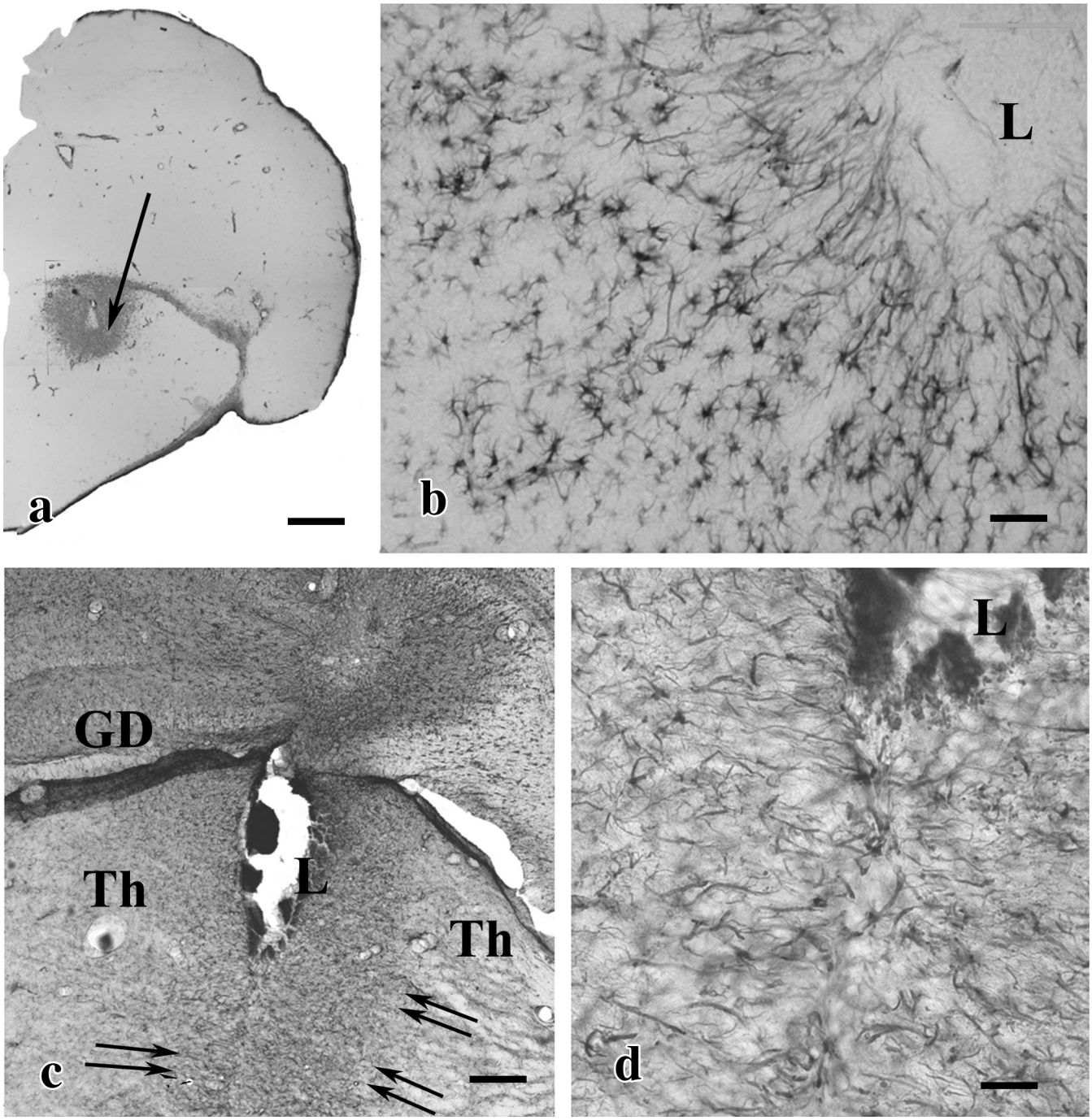


**Fig. 9.** Positive control II. GFAP-immunopositive astroglial reactions around stab wounds in rat and bird (cockatiel). **a.** Astroglial reaction in cockatiel telencephalon around a stab wound (arrow). Immunohistochemical reaction against GFAP. **b.** Enlarged part of panel **a**. L: the site of the lesion. **c.** Astroglial reaction in rat brain. Immunohistochemical reaction against GFAP. GD: gyrus dentatus; L: lesion, Th: thalamus, double arrows: the territory of the glial reaction. **d.** Enlarged part of astroglial reaction in rat brain, L: lesion. Scale bars: a, 1 mm; b, d, 50 µm; c, 250 µm.

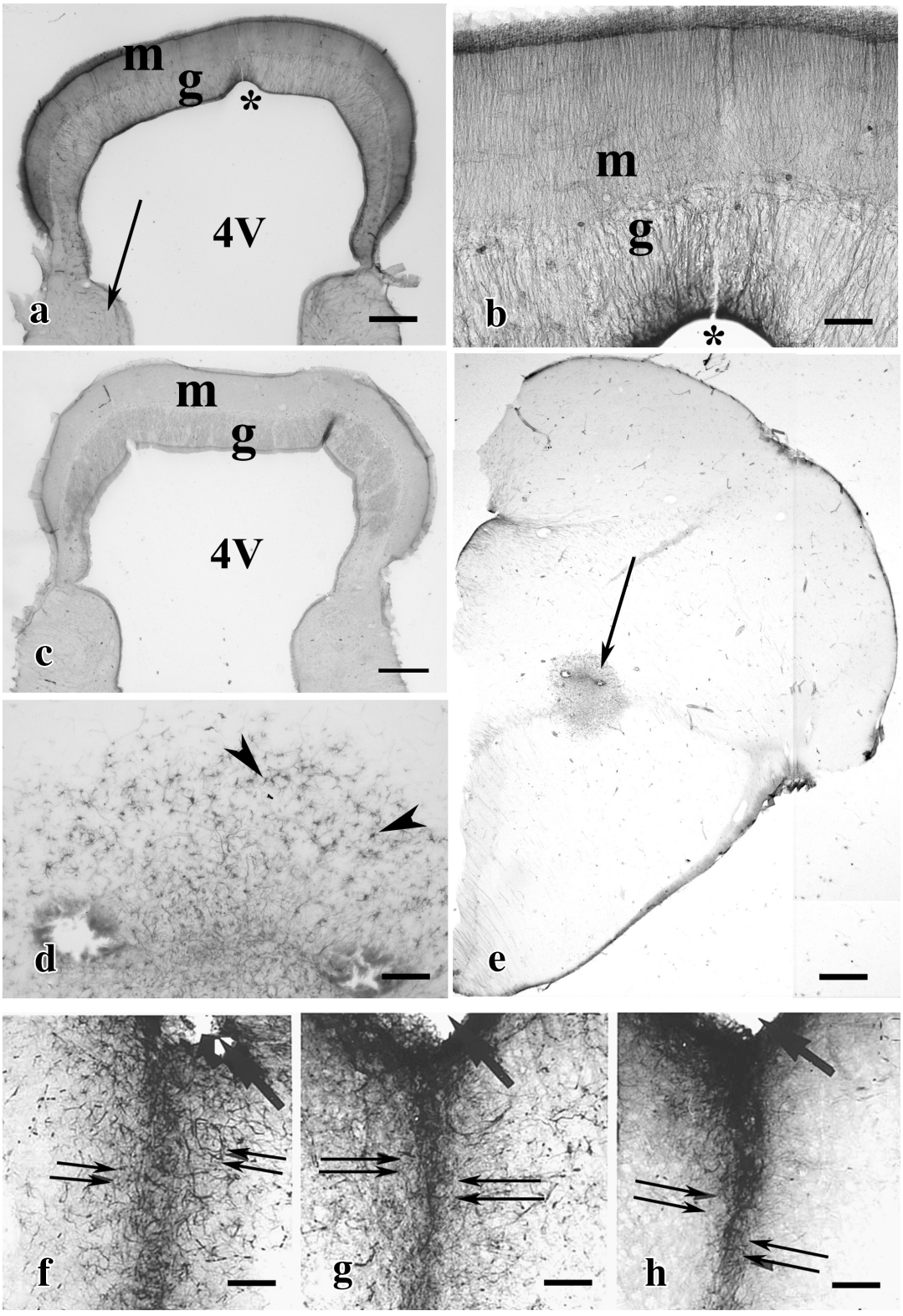


**Fig. 10.** Positive control III: the effectivity of the vimentin staining. **a.** Vimentin immunopositivity in agama cerebellum applying antibody of 3B4 clone. The glial systems are different in the molecular and granular layers (g, m). The joining part of the brain stem (cerebellar peduncle, arrow) contains no immunopositive glia. Asterisk marks the detail enlarged in panel b. 4V: 4th ventricle. **b.** Detail of panel a) (see asterisk) enlarged. Note the fine, dense system of Bergmann glia in the molecular layer (m) in contrast to the less densely packed but coarser processes of the granular layer (g). Marks like in panel a. **c.** In contrast to the anti-vimentin of 3B4 clone, the anti-vimentin of V9 clone was unable to detect vimentin immunopositivity in the agama cerebellum. **d.** The anti-vimentin antibody of 3B4 clone detected reactive glia in cockatiel. Note the astrocytes (arrowheads). The anti-vimentin of V9 clone was unable to detect vimentin immunopositivity in the cockatiel cerebellum either (not shown). **e.** The position of the lesion seen in panel d. The lesion (arrow) is identical with that seen in Fig. 9a. **f.** Astroglial reaction in rat brain. Immunohistochemical reaction against GFAP. Arrow: the lesion, double arrows: the astroglial reaction along the lesion track. **g.** Astroglial reaction in rat brain. Immunohistochemical reaction against vimentin with antibody V9. Marks as above. **h.** Astroglial reaction in rat brain. Immunohistochemical reaction against nestin. Marks as above. Scale bars: a, c, 600 µm; b, 120 µm; d, 80 µm; e, 640 µm; f-h, 160 µm.

described in *C. crocodilus* were not found in *Paleosuchus*. The lesions were found in the DVR (Fig. 6a,b), leading to the mutilation and disarrangement of the astroglial process system (Fig. 6c-e), but the characteristic phenomena of reactive astrogliosis seen in mammals and birds (see later) were not found.

When anti-vimentin (Fig. 7) or anti-nestin antibodies were applied, no labeling was found around the tissue defect or in the intact parts of telencephalon.

Some characteristic *post-lesion* phenomena known in mammals, however, were also found in the lizard and caiman brains and served as positive control. Around the site of lesion the vessels were delineated by immunopositivity to laminin, whereas in the distant brain areas they remained immunonegative (Fig. 8a-c). In contrast, the β-dystroglycan immunopositivity, which delineated the vessels in the intact brain areas, disappeared from the peri-lesional vessels (Fig. 8d). The immunoperoxidase results were supported by immunofluorescent detection of laminin (Fig. 8e). The lesions caused GFAP-immunopositive astroglial reactions in rat and cockatiel (Fig. 9).

The effectivity of anti-vimentin of clone 3B4 on reptiles was proved by the immunopositivity of the cerebellum (Fig. 10a,b). The molecular layer contained a dense system of fine processes like Bergmann glia in contrast to the less dense system of coarse processes in the granular layer. Other brain parts contained no or very scarce vimentin-immunopositive processes. Anti-vimentin antibody of clone V9 was not effective (Fig. 10c) even in the cerebellum. The effectivity of anti-vimentin and anti-nestin antibodies on reactive glia was also controlled: in cockatiel brain the anti-vimentin antibody of 3B4 clone was capable to detect reactive glia (Fig. 10d,e), whereas in rat the anti-vimentin clone V9 and the anti-nestin antibodies detected it (Fig. 10f-h).

## Discussion

### *Rapid versus late astroglial reactions have different functions*

Our former (goldfish: Kálmán and Ajtai 2000; turtle, shark and rays: Kálmán et al., 2013) and present results indicate that rapid and demarcating post-lesion astroglial reaction seen in mammals and birds does not occur in other vertebrates. ('Rapid' means a couple of days in this case, see Mathewson and Berry, 1985; Hozumi et al., 1990).

The astroglial reactions that have been described in teleost fish species (Bignami et al., 1974; Anderson et al., 1984; Wolburg and Kastner, 1984; Nona and Stafford, 1995) and amphibians (Stensaas and Feringa, 1977; Reier, 1979; Scott and Foote, 1981) were usually accompanied with axonal regeneration. The survival periods were rather long (weeks-months), therefore it is almost impossible to decide whether the astroglial 'reactions' were rapid, defensive and demarcating and/or rather concomitant phenomena of regeneration attempts (Bignami et al., 1974; Anderson et al., 1984; Nona and Stafford, 1995).

In Squamata brains Rio et al. (1989), Lang et al. (2002) and Romero-Aleman et al. (2004, 2010, 2013) found astroglial phenomena following 2-week or longer postoperative periods and attributed them to regeneration. Gu et al. (2015) demonstrated the differences between the *in vitro* reactions of mammalian and lizard astroglia and found the latter similar to the immature mammalian astroglia rather than the mature one.

### *The length of survival period*

The time periods used in the present experiment were long enough for the complete formation of reactive gliosis in case of mammals (Mathewson and Berry, 1985; Hozumi et al., 1990). The question arises, whether the astroglial reaction requires a longer *post-lesion* period in poikilotherm animals than in homoiotherms. In several studies accumulation of macrophages, proliferation of various cells, and closures of tissue defects were found within a few post-lesion days in poikilotherm animals (Endo et al., 2007: Hui et al., 2010; Baumgart et al., 2012; especially in lizards: Romero-Aleman et al., 2004). These observations proved that a long postoperative period is not a *sine qua non* for *post-lesion* cellular reactions in poikilotherm animals either. Therefore, it may be surmised that the absence of GFAP-immunoreactive astroglial reaction is not to be attributed to the brevity of the survival period designed. An astroglial reaction could not even fulfill its barrier task if its formation takes a long period.

### *Results with anti-vimentin and anti-nestin antibodies*

The lack of post-lesion glial reaction was also supported by the fact that it was also not detected with anti-vimentin antibodies. Vimentin immunopositive astroglia almost disappear from the lizard brains by adulthood (Monzon-Mayor et al., 1990; Yanes et al, 1990; Lazzari and Franceschini, 2001, 2005a,b, and present study). However, in our experiments the staining of cerebellar glia proved the capability of 3B4 clone anti-vimentin to detect lizard vimentin. Here it delineated a glial system similar to that found in turtle (Kálmán et al., 1994), caiman (Kálmán and Pritz, 2001), and different lizards (Lőrincz and Kálmán, 2015, 2020). Note that 3B4 anti-vimentin antibody has been recommended for use in bird brain whereas V9 antibody in mammalian brain (Bohn et al., 1992; Gereben et al., 1995). The effectivity of anti-nestin antibody was proved in lesioned rat brain. According to Cole and Lee (1997) nestin does not occur in birds. It may explain its ineffectivity in bird and also in reptile brains.

### *Presence of astrocytes is necessary but not sufficient*

In radial glia a single nucleus controls the full length of the cell, however, the astrocytes form a more versatile

network, which can adapt better to local demands (Connor and Berkowitz, 1985; Mugnaini, 1986). This advantage could be due to the rapid and demarcating astroglial reactions seen in mammals and birds. Astrocytes can be present but not predominant in lizard brains. According to some opinions (Sofroniew, 2009; Sofroniew and Vinters, 2010), even the mammalian brain glial reaction and scar formation only develops when new astrocytes recruited by the lesion pervade the territorial system of resident astrocytes. On the other hand, the persistence of radial glia in the mature brain of fishes, amphibia and reptiles may promote regeneration (see e.g. Singer et al., 1979).

### *Cerebralization, blood-brain barrier and astroglial reactivity*

The lack of rapid and demarcating astrogliosis in reptiles supports that it evolved independently in mammals and birds, which correlates with their very complex homeothermic brains. The progressing cerebralization raised a demand of progressing demarcation of the central nervous system, which provides "stabilization of the intracerebral ionic homeostasis to eliminate its alterations influencing adversely the electrophysiologic processes" (Abbott, 2005). On the contrary, increasing effectiveness of the immune system also requested a progressive isolation of the central nervous system to reduce the risk of autoimmune attacks. As a consequence, whereas in fishes, amphibians and reptiles, blood-borne macrophages eliminate tissue debris (Dowding and Scholes, 1993; Abbott, 1995; Sofroniew, 2009), in birds and mammals the removal of tissue debris activates astrocytes, and results in gliosis (Lawson and Perry, 1995; Merrill and Benveniste, 1996).

### *The results with laminin and β-dystroglycan prove the effect of lesion*

The alterations were similar to previous findings in mammals (Szabó and Kálmán, 2004, 2008; Kálmán et al., 2011). To explain these phenomena, we agree with the opinions of Krum et al. (1991) and Milner et al. (2008). The astroglial and vascular basal laminae fuse and, therefore, 'cover' the laminin epitopes (Krum et al., 1991). A *post-lesion* separation of the glial and vascular basal laminae (Krum et al., 1991; Sixt et al., 2001) allows the laminin epitopes to be accessible to immunoreagents. For a more detailed discussion see Szabó and Kálmán (2004).

The peroxidase produced in vessels may be attributed to the endogenous peroxidase activity of endothelium. However, vessels were also visualized by immunofluorescent reactions in our experiments.

The immunoreactivity of β-dystroglycan delineates the vessels of intact brain (Uchino et al., 1995; Zaccaria et al., 2001), but it is not present in the damaged brain tissue (Milner et al., 2008; Liu et al., 2015). The β-dystroglycan unit is localized in the perivascular astroglial end-feet and has an important role in the glio-vascular coupling (Correale and Villa, 2009; Wolburg et al., 2009). The *post-lesion* disappearance of the β-dystroglycan immunoreactivity is attributed to the cleavage of β-dystroglycan by matrix metalloproteinases (Milner et al., 2008). Therefore, the alterations of cerebrovascular β-dystroglycan and laminin immunoreactivities may indicate alterations in glio-vascular coupling. It proves that despite the lack of reactive gliosis the lesion had effects on the astroglia.

### *Conclusions*

No demarcating astroglial reaction occurred in lizards and caiman, although glio-vascular alterations proved the effect of brain injuries.

These and our former results prove that the rapid and demarcating post-lesion astroglial reaction is confined to mammals and birds. This phenomenon probably appeared by parallel evolution in these two groups.

*Acknowledgements.* Laboratory assistance of A. Oz and S. Deak is highly appreciated.

*Ethics.* All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Experiments were performed in accordance with the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary (22.1/3491/003/2008), the permission of Hungarian authorities (KA-1928, dated from May 31, 1916) and the European Union Directive (EU Directive 2010/63/EU).

*Conflict of Interest.* The authors declare that there is no potential conflict of interest with respect to the research, authorship, and/or finances.

*Funding.* The work was supported by the scientific budget of the Semmelweis University (Excellence Program for Higher Education of Hungary, FIKP-2018).

*Author Contributions.* All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by David Lorincz and Mihaly Kalman. The first draft of the manuscript was written by Mihaly Kalman and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Accepted October 27, 2020.