S100 Immunoreactive Glial Cells in the Forebrain and Midbrain of the Lizard *Gallotia galloti* during Ontogeny

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ABSTRACT: **We identified S100 immunoreactive cells in the brain of the lizard *Gallotia galloti* during ontogeny using immunohistochemical techniques for light and electron microscopy. In double labeling experiments with antibodies specific for S100A1 and S100B (anti-S100) and proliferative cell nuclear antigen (anti-PCNA), myelin basic protein (anti-MBP), phosphorylated neurofilaments (SMI-31), glial fibrillary acidic protein (anti-GFAP), or glutamine synthetase (anti-GS), we detected S100-like immunoreactivity in glial cells but never in neurons. Restricted areas of the ventricular zone were stained in the hypothalamus from E32 to postnatal stages, and in the telencephalon at E35, E36, and in adults. S100 immunoreactivity was observed predominantly in scattered PCNA-negative cells that increased in number from E35 to the adult stage in the myelinated tracts of the brain and had the appearance of oligodendrocytes. Quantitative analysis revealed that all of the S100-positive glial cells were GFAP-negative, whereas most of the S100-positive glial cells were GS-positive. Ultrastructurally, most of these S100-positive/GS-positive glial cells resembled oligodendrocytes of light and medium electron density. In adult lizards, a small subpopulation of astrocyte-like cells was also stained in the pretectum. We conclude that in the lizard S100 can be considered a marker of a subpopulation of oligodendrocytes rather than of astrocytes, as is the case in mammals. The S100-positive subpopulation of oligodendrocytes in the lizard could represent cells actively involved in the process of myelination during development and in the maintenance of myelin sheaths in the adult.** J Neurobiol 57: 54–66, 2003

Keywords: **oligodendrocytes; glutamine synthetase; glial fibrillary acidic protein; proliferating cell nuclear antigen; reptiles**

INTRODUCTION

S100 proteins are abundantly expressed in the nervous system in several isoforms, the most thoroughly studied ones being S100A1 and S100B (Donato, 1999). These proteins are found in soluble (approximately 90%) and membrane-bound forms (Cocchia, 1981;

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Rickmann and Wolff, 1995). S100 family members are now considered multifunctional proteins that depend functionally on the presence of intra- and extracellular calcium. They have been implicated in the regulation of cytoskeletal dynamics and cell growth and survival (Zimmer et al., 1995), but also in the modulation of memory and learning and the pathogenesis of disorders such as Alzheimer's disease and Down's syndrome (Donato, 1999).

In mammals, S100 has been found primarily in the nucleus and cytoplasm of astrocytes, oligodendrocytes, ependymocytes, radial glia, and Schwann cells (for review see Cocchia, 1981); however, neurons were found to be S100-positive in some instances (for review see Cocchia, 1981). Although most of the studies on S100 protein in higher vertebrates have found that this protein is mainly present in mammalian astrocytes (Langley et al., 1984), a subpopulation of oligodendrocytes in rats (Rickmann and Wolff, 1995), and immature oligodendrocytes in cats (Dyck et al., 1993) and birds (Linser and Perkins, 1987) is S100-positive. Moreover, the S100 protein has also been found in ependymocytes and radial glia of mammals (Cocchia, 1981; Engel and Müller, 1989) and fish (Manso et al., 1997).

However, data on the expression of S100 in the central nervous system (CNS) of reptiles, lower amniotic vertebrates that show a remarkable capacity of CNS repair after injury (Lang et al., 1998, 2002), are lacking at present. In this respect, the fore- and midbrain of the lizard are particularly interesting: glial cells in the telencephalon appear to remain in a less differentiated stage than their counterparts in the mesencephalon (Monzón-Mayor et al., 1990a, c; Yanes et al., 1990, 1997), which corresponds with a marked capacity for regeneration in the telencephalon (López-García et al., 1992). There is, however, a scarcity of suitable glial cell markers, in particular of oligodendrocytes, required to study aspects of glial cell development and CNS repair in reptiles. Against this background, we investigated the expression of S100 in glial cells during the ontogeny of the lizard *Gallotia galloti*, and established the potential of this protein as a marker for oligodendrocytes in these animals.

MATERIALS AND METHODS

Animals, Tissue Fixation, and Paraffin Embedding

We used 30 embryos (from E32 to hatching), 10 young animals (from postnatal days 9–13), and 10 adults of *G. galloti* (Bischoff, 1982), a lizard indigenous to the island of Tenerife. The stages of embryonic development were defined according to the tables of equivalence comparing the development of *G. galloti* (Ramos-Steffens, 1980) and *Lacerta vivipara* (Dufaure and Hubert, 1961).

Fertilized eggs, postnatal, and adult animals were collected in their natural environment and maintained in compliance with local legislation. Lizards were kept in large holding tanks and fed on commercially available cat food and fruits. Animals were anesthetized with ether vapor before processing. Embryos and postnatal lizards were decapitated and their heads immersed in Bouin's fixative for 48 h. Adult animals were perfused and the dissected brains postfixed in the same fixative for 7 h. The brains were dehydrated in ethanol, embedded in paraffin, and sectioned at 10 μm in the transversal plane.

Immunohistochemistry

Detection of the S100 protein and myelin basic protein (MBP) was carried out using the avidin-biotin complex (ABC) method. Endogenous peroxidase was blocked using a solution of 10% H_2O_2 in TBS (0.05 *M* Trizma base containing 150 m*M* of NaCl, pH 7.4) for 10 min, followed by preincubation of sections in TBS containing 1% bovine serum albumin (BSA) for 3 h. Sections were then incubated in either rabbit anticow S100 antibody (Dako) diluted 1/1000 in TBS for 72 h at 4°C, or in rat anti-guinea pig MBP antibody (gift from Dr C. Linington, Münich) diluted 1/10 in TBS overnight at 4°C. Secondary antibodies were biotinylated antirabbit and antirat (Vector), both diluted 1/200 in TBS and incubated for 1 h. Finally, sections were incubated in the ABC standard kit (Vector) and the peroxidase activity was revealed with diaminobenzidine (DAB) to obtain a brown staining.

Double labeling for the S100 protein and the proliferating cell nuclear antigen (PCNA) was performed by sequential detection of the antigens. Initially, the sections were incubated in the monoclonal anti-PCNA antibody (Novocastra) diluted 1/60 in TBS for 12 h at 4°C, followed by incubation in the biotinylated antimouse antibody (Amersham) diluted 1/200 in TBS for 1 h and the avidin-peroxidase (Sigma) diluted 1/400 in TBS for 1 h. Finally, the labeling was revealed in black with DAB containing 1% ammonium nickel sulfate. After that, the sections were treated for detecting the S100 protein as described above. PCNA staining in black and S100 labeling in brown were easily distinguished under the microscope. Double labeling for the S100 protein and phosphorylated neurofilaments was performed in similar fashion but S100 reactivity was revealed first in black whereas neurofilaments were detected in brown using the monoclonal antineurofilament antibody (SMI-31; Sternberger-Meyer), diluted 1/1000 in TBS overnight at 4°C, and the same ABC method described above for the detection of the PCNA.

Double labeling for S100 protein and glutamine synthetase (GS) and for S100 protein and glial fibrillary acidic protein (GFAP) was performed by immunofluorescence

techniques. Sections were preincubated in TBS containing 1% BSA for 1 h. The polyclonal anti-S100 (Dako), monoclonal anti-GS (Chemicon), and monoclonal anti-GFAP (Sigma) antibodies were diluted 1/250, 1/500, and 1/300, respectively, in TBS and incubated for 72 h at 4°C. Secondary antibodies were antimouse-rhodamin and antirabbit-FITC, both diluted 1/100 and incubated for 2 h.

Each step in the immunohistochemical protocols described above was followed by several washes in TBS. Negative controls consisted of omitting the primary antibodies.

Electron Microscopy

For the immunogold procedure, four adult lizards were perfused in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4). The brains were dissected out and kept in the fixative for 90 min. After 45 min in the same buffer containing 50 m*M* NH_4Cl, the brains were dehydrated in methanol and embedded in Lowicryl K4M. Ultrathin sections (80 nm) were mounted on formvar-carbon coated nickel grids. Each grid was floated on a drop of 20 m*M* Tris-HCl (pH 7.4) containing 0.9% NaCl, 0.01% BSA, and 0.25% fetal calf serum (FCS) for 30 min. Then, the grids were treated for S100/GFAP and S100/GS double immunocytochemistry. Rabbit anti-S100, mouse anti-GFAP, and mouse anti-GS antibodies were diluted 1/1000, 1/300, and 1/1000, respectively, in TBS containing 0.1% BSA and 1% FCS and incubated for 24 h. Secondary antibodies were antimouse and antirabbit immunoglobulin-conjugated gold complexes (5 and 10 nm; Sigma), both diluted 1/10 in TBS containing 1% FCS and 0.05% Tween 20. Finally, the grids were counterstained in 2% uranyl acetate for 30 min. Negative controls consisted of omitting the primary antibodies.

Image Acquisition and Processing

Photomicrographs were taken using a Leitz DMRB light microscope and a Zeiss 910 electron microscope. The negatives were scanned and digitally processed in the computer using Photoshop 5.0 software.

Western Blot Analysis

Brains of *G. galloti* were homogenized in ice-cold lysis buffer (20 m*M* Tris-HCl pH 8, 137 m*M* NaCl, 10% glycerol, 1% Triton X-100, and 2 m*M* EDTA) containing proteinase inhibitors (0.15 U/mL aprotinin, 20 μM leupeptine, 1 m*M* PMSF, and 100 m*M* orthovanadate). The homogenates were centrifuged at 13,000×*g* for 15 min at 4°C. The supernatants containing the tissue extract were collected and their protein concentration measured according to Bradford (1976).

Tissue extract, positive controls consisting of bovine S100A1 and S100B proteins (Sigma), and molecular weight standards (Santa Cruz Biotechnology, Inc.) were subjected to electrophoresis on 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Separated proteins were electroblotted to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membrane was blocked for 30 min in TBS (20 m*M* Tris-HCl, 150 m*M* NaCl, pH 7) containing 3% BSA. The rabbit anticow S100 antibody (Dako) diluted 1/750 in TBS containing 3% BSA was incubated with the membrane overnight at 4°C. Binding of primary antibody and molecular weight determination were visualized using an antirabbit peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology; includes an antibody against the molecular weight standards), diluted 1/5000 in TBS containing 3% BSA, followed by enhanced chemiluminescence detection (ECL; Pierce) according to the manufacturer's instructions.

Quantitative Analysis

To quantify the numbers of S100-positive glial cells during ontogeny, we counted all labeled cells under a 40X lens in 50 transversal sections of the forebrain and midbrain, out of five lizards for each developmental stage evaluated. The mean values and standard errors were represented graphically using the PC curve fitting program Prism (Graphpad Software, Inc.).

RESULTS

The anti-S100 antiserum recognized a protein band of the same apparent molecular weight (23 KDa) as that of the S100A1 and S100B isoforms of bovine S100 in the lizard brain extracts, suggesting trans-specific immunological similarity of bovine and reptilian S100 protein (Fig. 1).

Telencephalon

Only a few cells are immunolabeled with the anti-S100 antibody in the embryonic telencephalon. At E35, small groups of S100-positive cell bodies are observed in the ventricular zone [Fig. 2(A)]. At E36, there are a few stained cell bodies in the sulcus terminalis but not in the rest of the ventricular wall [Fig. 2(B)]. In the adult telencephalon, the ependymal zones (e) (EZe) and (f) (EZf) present weakly S100-positive ependymal glial cells displaying radial processes [Fig. 2(C)], whereas the rest of the ependymal epithelium, the ependymal zone (a) (EZa) and the ependymal zone (b) (EZb), is S100-negative [Fig. 2(C)].

In adults, most of the S100-positive cells present intensely immunostained round or oval cell bodies with few processes, the latter wrapping around nerve fibers. They are located in the myelinated ventral telencephalic areas, mainly in the tractus telencephali-

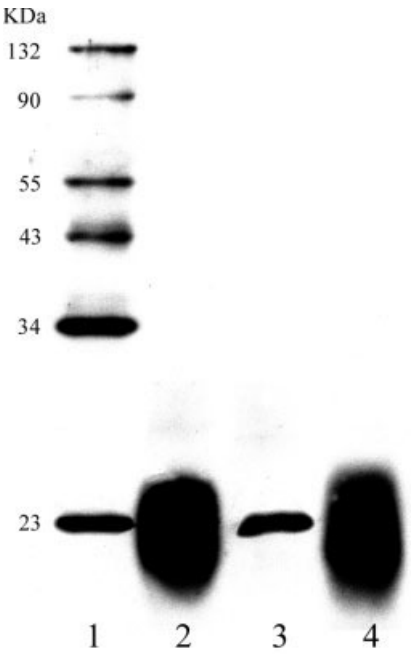

Figure 1 Western blot analysis of *Gallotia galloti* S100 protein in brain tissue extract. Lane 1 represents the molecular weight standard that reacts with the secondary antibody. The primary rabbit anticow S100 antiserum binds to a single protein band of approximately 23 KDa of molecular weight in samples of purified bovine S100A1 and S100B isoforms (lanes 2 and 4, respectively) and in the lizard brain extract (lane 3).

cus lateralis (lfb) [Fig. 2(D,E)] and tractus telencephalicus medialis (mfb) (not shown). S100-reactive cells are scarce in the telencephalic cortex and are observed mainly in the caudal telencephalic cortex in the alveus (alv) and in the medial septal nuclei (spt) (not shown). The telencephalic ventricular wall contains proliferating cells, even at the adult stage, revealed by PCNA immunostaining [Fig. 2(F)]. Double labeling for S100 and PCNA revealed that S100 immunoreactive cells were not proliferative (not shown).

Diencephalon

S100 reactive cells are present in the ventricular and intermediate zones of the hypothalamus from E32 to postnatal specimens [Fig. 3(A)]. Cells that are located between the ventricular and intermediate zones and appear to be migrating from the ventricular zone of the hypothalamus are observed at these stages [Fig. 3(A)]. Scattered S100-positive cells are found in the hypothalamus from E35 and in the optic tract (ot) and the medial forebrain bundle (mfb) from hatching to adult stages [Fig. 3(B)]. These cells are morphologically similar to those observed in the telencephalon and some cell bodies in the optic tract are located in the subpial zone [Fig. 3(C)].

S100-positive cells, similar in appearance to those described above, are scarce in the pretectum. Some stained cells with radial processes similar to those of star-shaped astrocytes [Fig. 3(D)] are observed in the stratum fibrosum et griseum superficiale (SFGS) in adult lizards.

Mesencephalon

Scattered S100-positive cells are first observed at E39 in the oculomotor nerve root (III), commissura dorsalis tegmentalis (cdt), and fasciculus longitudinalis medialis (flm). At stage E40 and around hatching, S100 immunolabeling is detected in all tegmental tracts described above, as well as in the tractus tectobulbo spinalis rectus (tbs), tractus tecto bulbaris intermedius rectus (tbi), tractus opticus lateralis (tol), tractus nuclei opticus basalis (tob), and trochlear nerve root (IV). At early postnatal and adult stages, S100-positive cells are located in the optic tectum at the stratum album centrale (SAC) and stratum opticum (SO) [Fig. 4(A,B)]. In the tegmentum, labeled cells are observed in all tracts described above [Fig. 4(A)], in the commissura colliculi (cc), lemniscus lateralis (ll), lemniscus medialis (lm), and tractus mesencephalicus trigemini (tmt) [Fig. 4(C,D)]. All scattered S100-reactive cells are nonproliferative as demonstrated in the III nerve root, where S100-positive/PCNA-negative cells are shown in a double-labeled section [Fig. 4(E)]. However, PCNA immunolabeling was present in cells of the telencephalic ventricular wall and can thus be considered a positive control [see Fig. 2(F)].

As described above, most of the S100-positive cells are seen in myelinated tegmental tracts rather than in the tectal ones. It is also noteworthy that neurons are S100-negative at all stages of development [Fig. 4(F)]. Most of the stained cells have an oligodendrocyte-like appearance in different stages of ontogeny, for example, around hatching [Fig. 5(A)], at the postnatal stage [Fig. 5(B)], and at adult stage [Fig. 5(C,D)]. These cells have the same appearance as those described in the telencephalon and diencephalon and many of the S100-positive cell processes appear to ensheath nerve fibers (Fig. 5). Double labeling reveals that most of the S100-positive cells are located in the vicinity of antineurofilament-positive nerve fibers [Fig. 5(B)]. S100 immunoreactive ependymal cell bodies are scarce and weakly stained in the mesencephalon during ontogeny (not shown).

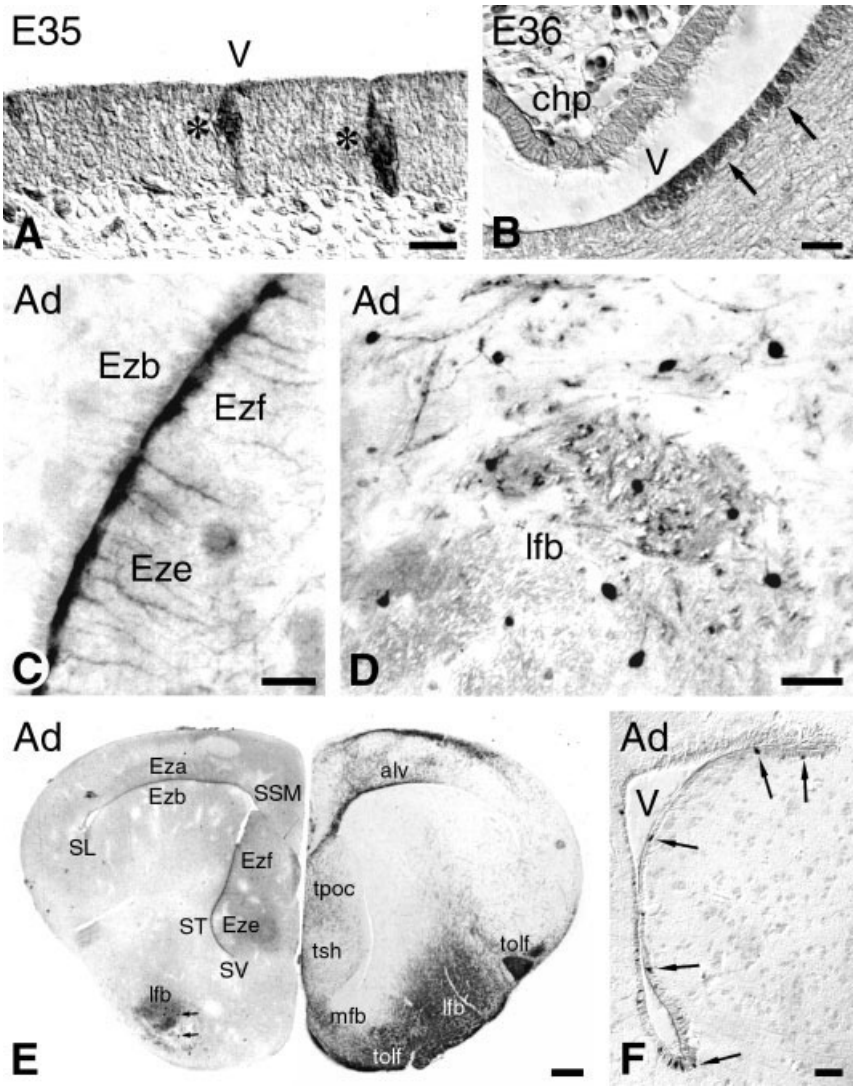

Figure 2 S100 labeling in the telencephalon during ontogeny of *Gallotia galloti*. (A,B) Nomarski pictures. Groups of stained cell bodies in the ependymal zone (a) at E35 [asterisks in (A)] and in the sulcus terminalis at E36 [arrows in (B)]. (C) Cell bodies displaying radial processes are shown in the ependymal zones (e) and (f) (EZe and EZf, respectively) in the adult lizard; however, the ependymal zone (b) (EZb) is unlabelled. (D) Scattered reactive cells in the lateral forebrain bundle (lfb) in the adult. (E) General view of a cross-section of the anterior level of the telencephalon in the adult lizard. The staining for the S100 protein and the MBP are shown in the left and right halves, respectively. The location of ependymal zones (EZa, EZb, Eze, and EZf) and sulcus lateralis (SL), septomedialis (SSM), terminalis (ST), and ventralis (SV) are indicated. Note S100-reactive cells in the lfb (arrows). (F) Nomarski picture. PCNA-positive nuclei in the lateral ventricular wall (black staining, arrows) demonstrating its proliferative capacity in the adult lizard. GFAP-positive reactivity is also shown in grey. Ad, adult; Chp, choroid plexus; V, ventricle. Bars in (A,B,C,D,F): 20 μm. Bar in (E): 250 μm.

Distribution and Quantification of S100-Positive Cells

As described above, most of the S100-positive cells observed in the brain during ontogeny of *G. galloti* are located in the myelinated tracts. The quantitative analysis reveals a biphasic increment in the number of these S100-positive glial cells during brain development in the diencephalon and mesencephalon (Fig. 6). In the diencephalon, stained glial cells are observed from E35 to the postnatal stage and then rapidly increase in number until reaching the adult stage. In the mesencephalon, labeled cells are first observed at

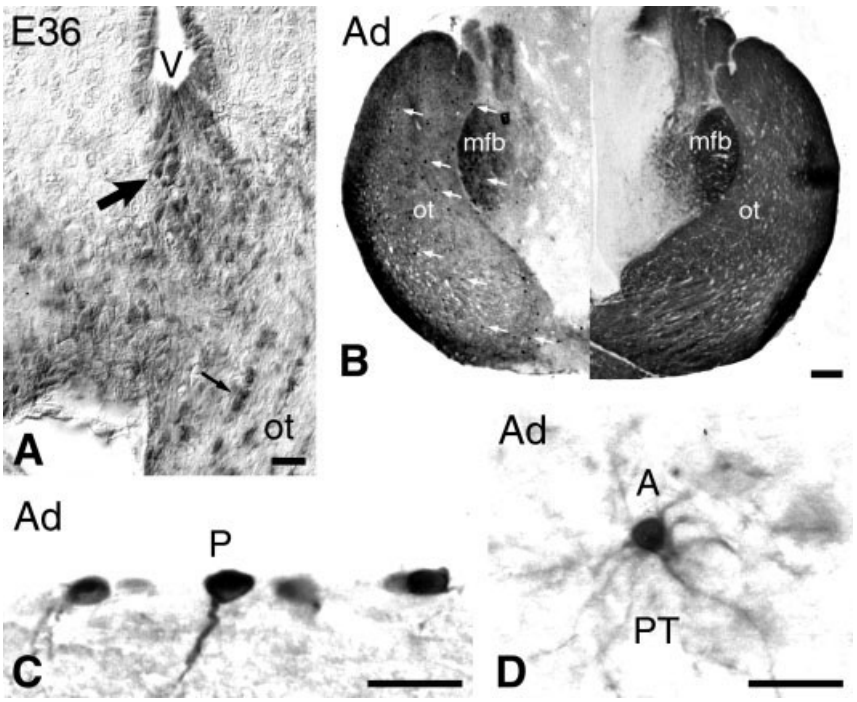

Figure 3 S100 staining in the diencephalon during ontogeny of *Gallotia galloti*. (A) Nomarski picture. Hypothalamus at E36. Some labeled cells seem to separate from the ventricular zone (thick arrow), others are shown in the optic tract (ot) (thin arrow). (B) General view of a cross-section of the diencephalon in the adult lizard (Ad). Labeling for the S100 protein and the MBP are shown in the left and right halves, respectively. Note S100-positive cell bodies in the optic tract (ot) and medial forebrain bundle (mfb) (arrows). (C) Reactive cell bodies attached to the pia matter (P) in the optic tract. (D) Stained cell with a typical astrocyte-like appearance (A) in the pretectum (PT). Ad, adult; V, ventricle. Bars in (A,C,D): 20 μm. Bar in (B): 150 μm.

E39 in the mesencephalic tegmentum, increasing slightly in number from this period to the hatching stage, but a striking increment in the number of labeled cells occurs from hatching to adult stages. In contrast, few scattered S100-positive cells are observed in the telencephalon, and only in adult lizards. These results indicate that S100-positive glial cells arise late in embryonic development and are present mainly in adult lizards.

Double Labeling for S100/GS and S100/GFAP in Adult Lizards

A quantitative analysis of double immunofluorescence labeling for S100/GS reveals that 70% of S100-positive glial cells located in myelinated tracts are also GS-positive in the adult brain [Figs. 7, 8(A,B,C,D)]. There are numerous S100-negative/GS-positive glial cells [Fig. 8(A,B,C,D)] scattered throughout fiber tracts, while others are localized in the ventricular wall, displaying radial processes (not shown). It is noteworthy that S100-positive cells are GFAP-negative [Fig. 8(E,F)].

Immunogold Double Labeling for S100/GFAP and for S100/GS in the Adult Mesencephalon

Using the immunogold technique with gold particles of different sizes (5 and 10 nm), electron microscopy was carried out to corroborate the absence of S100 protein in GFAP-positive cells, to identify the S100-reactive cells by their ultrastructural features, and to determine their stage of differentiation.

S100-negative/GFAP-positive labeling was observed in the glial filaments of astrocytes [Fig. 9(A)] and in radial glial processes [Fig. 9(B)]. Rarely, ependymal cells showed gold particles of both sizes in the cytoplasm, indicating the presence of both S100 and GFAP proteins (not shown).

We also identified a subpopulation of oligodendrocytes showing light or medium cytoplasmic density, containing microtubules as well as gold particles corresponding to anti-S100 and anti-GS labeling in the cytoplasm and nucleus [Fig. 9(C)]. These cells were closely associated with S100-negative myelin sheaths [Fig. 9(C,D)].

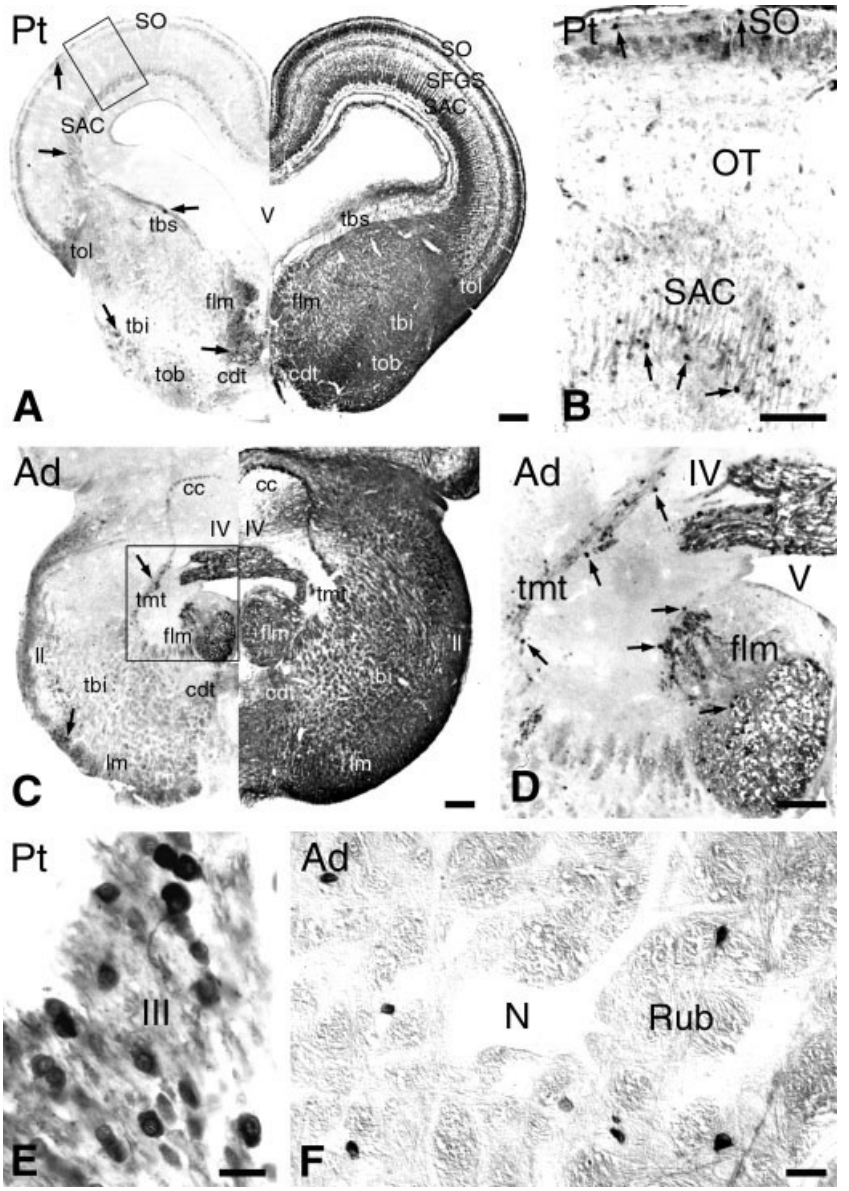

Figure 4 S100 labeling in the mesencephalon at postnatal and adult stages. (A–D) General view of a cross-section of the medial and caudal mesencephalon [(A) and (C), respectively]. The left half reveals the S100 reaction and the right half shows the MBP staining. Note scattered S100-positive cell bodies (arrows) in the SAC, SO (A,B), tbs, tol, flm, tbi, tob, cdt (A), as well as in tmt, cc, ll, lm, and IV (C,D). The insets in (A) and (C) are shown in (B) and (D), respectively. (E) S100-positive/PCNA-negative cells in the III nerve root. (F) Nomarski picture. Scattered S100-positive cell bodies and S100-negative neuron (N) in the ruber nucleus (Rub). Ad, adult; Pt, postnatal; V, ventricle. Bars in (A,C): 200 μm. Bars in (B,D): 100 μm. Bars in (E,F): 20 μm.

DISCUSSION

S100-Positive Oligodendrocytes and Myelination

In this study, we analyzed the distribution of S100-positive glial cells during development of the lizard, *G. galloti*. This is of interest because there are at present few comparative data available on the development of glial cells in these lower amniotes, and because lizards, like anamniotic vertebrates (fish and amphibians) have a remarkable capacity for CNS repair and axon regeneration after injury (López-García et al., 1992; Lang et al., 1998, 2002), and it is widely assumed that the properties of glial cells, in particular oligodendrocytes, play a crucial role in this regeneration process.

Oligodendrocytes were also of interest in this con-

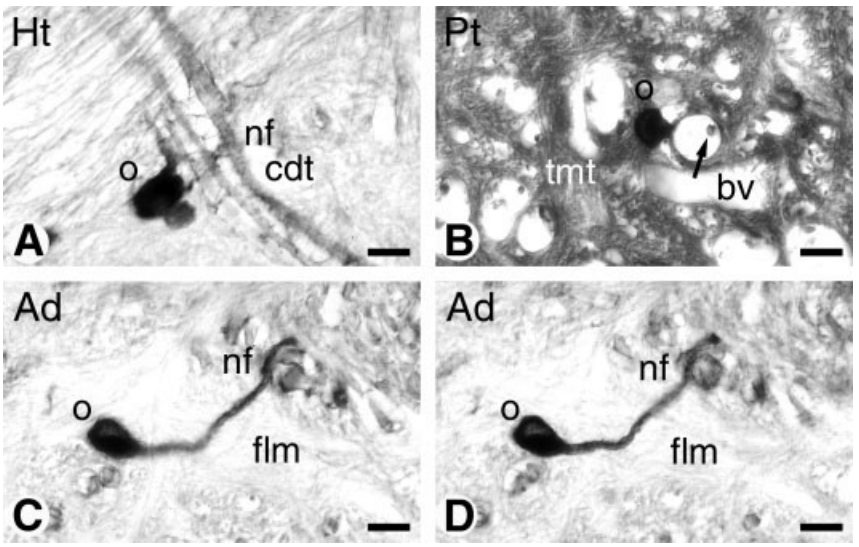

Figure 5 S100-positive oligodendrocyte-like cells in the mesencephalon during ontogeny. (A) Cell body and processes surrounding longitudinally cut nerve fibers (nf) of different thickness in the cdt, at hatching. (B) S100-positive/SMI-positive double labeling in the tmt at postnatal stage. A S100-positive cell body is shown in black whereas SMI-positive nerve fibers are in grey. Note a transversally cut nerve fiber (arrow) and a blood vessel (bv) next to the stained cell body. (C,D) Stained cell in the flm at adult stage. Note details of its process surrounding a transversally cut nerve fiber (nf). Ad, adult; Ht, hatching; o, oligodendrocyte; Pt, postnatal. Bars: 10 μm.

text because several oligodendrocyte markers established in mammals, such as MBP, proteolipid protein (PLP), the O4 antigen, myelin-associated glycoprotein (MAG), and carbonic anhydrase (CAII), proved unsuitable for staining oligodendrocyte cell bodies in tissue sections in the lizard using a variety of tissue processing and staining protocols, and therefore, specific markers for oligodendrocyte cell bodies in reptiles are lacking. It is noteworthy that immunolabeling for MBP, PLP, and O4 has been successful in cultured

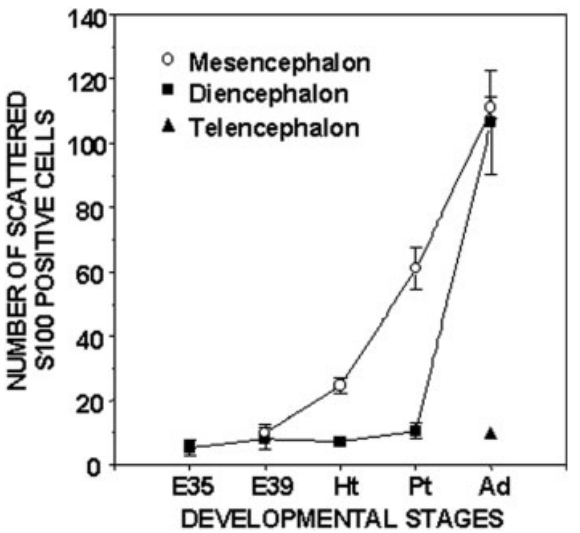

Figure 6 Mean number of scattered S100-positive cells in the telencephalon, diencephalon, and mesencephalon during ontogeny of *Gallotia galloti*. Labeled cells predominate late in development and mainly in adult specimens. Data are expressed as means ± SE, $n = 5$. Average SE (standard error) is 16% of the corresponding mean. Ad, adult; Ht, hatching; Pt, postnatal.

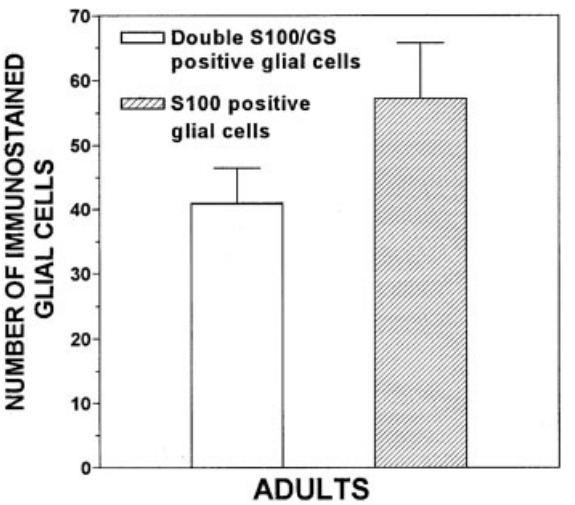

Figure 7 Quantification of the cells exhibiting both S100/GS immunoreactivity expressed as a percentage of the total number of S100-positive cells in the adult lizard. Data are means ± SE, $n = 5$. Average SE (standard error) is 14% of the corresponding mean. Seventy percent of the S100-positive cells are also GS-positive.

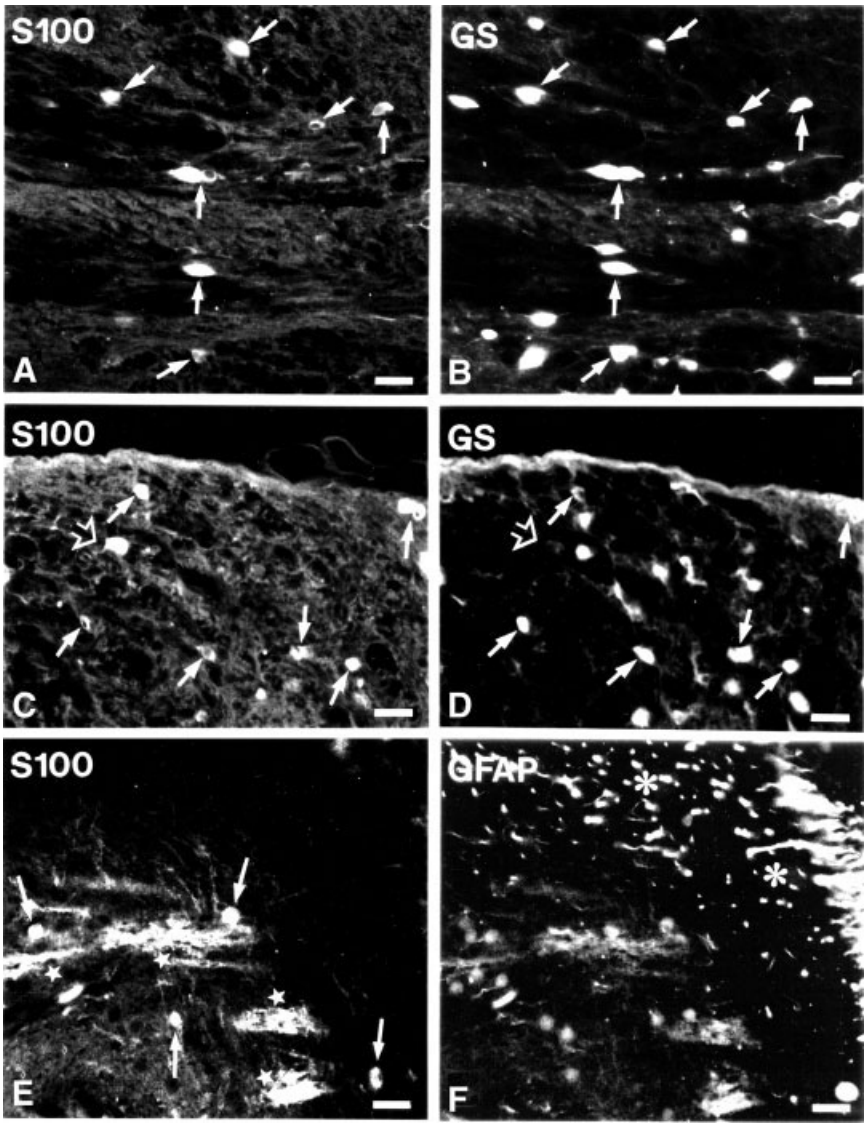

Figure 8 Double immunofluorescence labeling in the diencephalon and mesencephalon of adult *G. galloti* with antibodies that recognize the S100 protein (A,C,E), the glutamine synthetase (B,D), and the glial fibrillary acidic protein (F). (A,B) S100-positive/GS-positive cell bodies (arrows) in the optic tract. Many more S100-negative/GS-positive cell bodies are also present (B). (C,D) The S100-positive/GS-negative cell bodies (open arrows) are more unusual than S100-positive/GS-positive cell bodies (arrows) in the flm of the mesencephalon. (E,F) S100-positive cell bodies (arrows) and processes (stars) in the SAC (E) are GFAP-negative (F). Note S100-negative/GFAP-positive transversally cut radial glia processes [asterisks in (F)]. Bars: 20 μm.

oligodendrocytes of *G. galloti* (Lang et al., 1998), indicating that the antibodies in question recognize their respective antigens in the lizard. Several reasons could account for the failure to detect these antigens in oligodendrocyte cell bodies in tissue sections, such as insufficient tissue penetration of the antibodies used. However, it appears most likely that myelin proteins such as MBP and PLP are not present in oligodendrocyte cell bodies in detectable quantities and are either transported rapidly from the cell body into the cellular processes (Hartman et al., 1979) or are translated from their mRNAs outside the cell body in the myelin-forming processes, as has been suggested by Zeller et al. (1985).

In the absence of a specific marker for oligodendrocytes to counterstain S100-positive cell bodies, we have verified S100 expression in these cells ultrastructurally. Our previous studies on oligodendrocytes were essentially ultrastructural work (Monzón-Mayor et al., 1990b; Yanes et al., 1997), and that information

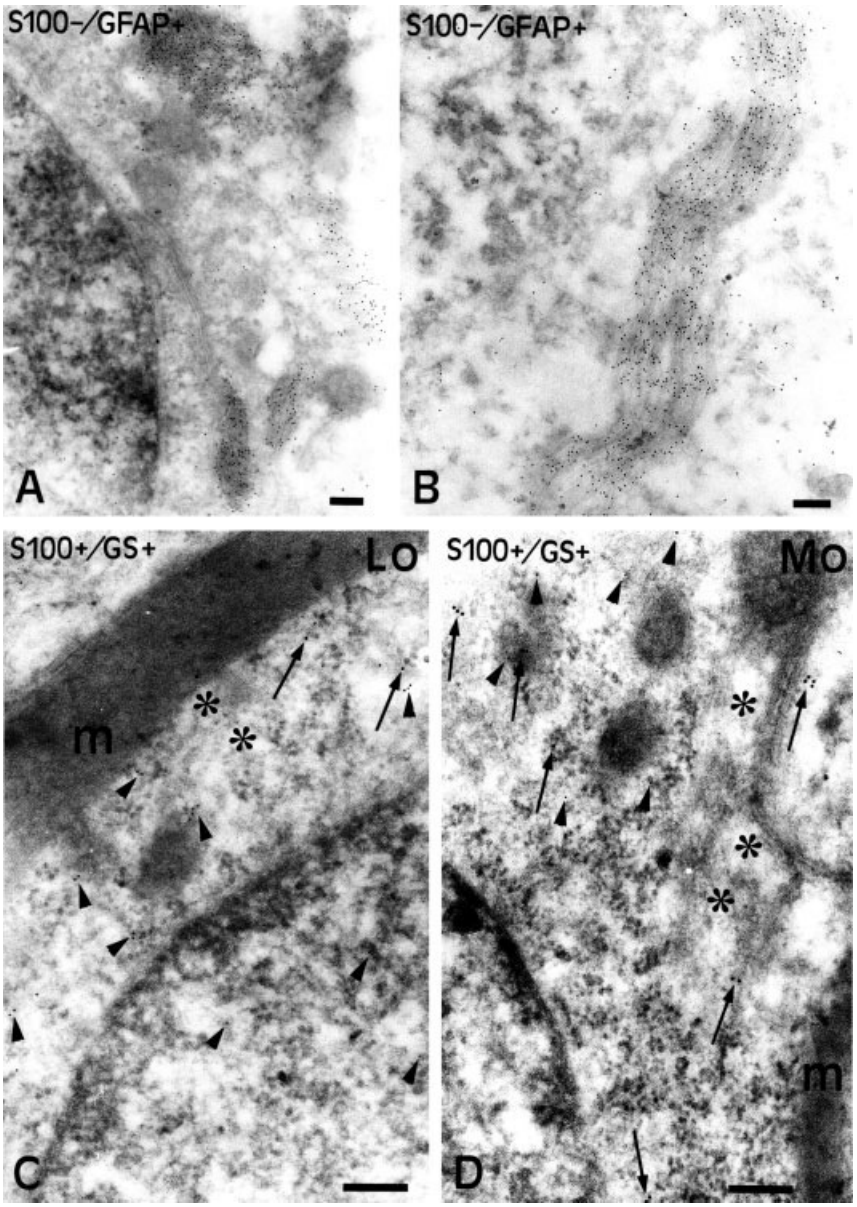

Figure 9 Double immunogold labeling for S100/GFAP and S100/GS in the mesencephalon of adult *Gallotia galloti*. (A,B) S100-negative (10 nm) and GFAP-positive (5 nm) astrocyte (A) and radial glia process (B). (C,D) S100-positive (5 nm, arrowheads) and GS-positive (10 nm, arrows) oligodendrocytes of light [Lo, in (C)] and medium [Mo, in (D)] electron-density. Note microtubules (asterisks) in their cytoplasm and unlabelled myelin sheaths (m). Bars: 0.2 μm.

supported the identification of S100-positive cells, in this study, as light and medium oligodendrocytes.

It is generally accepted that oligodendrocytes are responsible for myelination in the CNS and it is therefore not surprising that the rapid increase in the number of glial cells that precedes the onset of myelination and continues during the phase of rapid myelination is mainly due to proliferation and differentiation of oligodendrocytes (Vaughn, 1969; Sturrock, 1982a, b; Skoff et al., 1976a, b). However, the S100-positive oligodendrocytes were PCNA-negative during the ontogeny of the forebrain and midbrain of *G. galloti*, indicating that these cells are postmitotic. S100B is synthesized during the G1 phase and accumulates in the G0 phase in glioma cells (Donato, 1999), and this may be the case in our material, too. S100-positive cells are observed in myelinated tracts after the onset of myelination in the mesencephalon and telencephalon of the lizard, similar to findings in the optic tectum of the chick (Linser and Perkins, 1987). The myelin sheaths were S100-negative because S100 is mainly a cytosolic protein. Ultrastruc-

tural studies have suggested that active oligodendrocytes as well as those oligodendrocytes with a light to medium appearance of cytoplasm, but not dark oligodendrocytes, are involved in the myelination process during development (Parnavelas et al., 1983; Monzón-Mayor et al., 1990b, 1998). Because they are identified in myelinated tracts as light and medium oligodendrocytes, the observed S100-positive cells are likely to be differentiating oligodendrocytes that are involved in the myelination process during development. Active oligodendrocytes were observed from E37 to hatching in the mesencephalon of the lizard (Monzón-Mayor et al., 1990b), and this coincides with the presence of S100-positive cells during these stages of development, but the identification of S100-positive cells as active oligodendrocytes needs to be verified ultrastructurally.

The predominance of light to medium S100-positive oligodendrocytes late in development and mainly in the adult lizard, as well as their location in myelinated tracts, suggests that they could have a function in the maintenance of the myelin sheaths in the adult lizard. The occurrence of S100-positive oligodendrocytes in the SAC and SO but not in other myelinated tracts of the optic tectum, as well as the absence of these cells in the developing telencephalon, suggests that these S100-positive cells represent a subpopulation of the oligodendrocytes in the lizard.

The predominance of S100-positive oligodendrocytes in caudal zones (mesencephalon) in contrast to the rostral zones (diencephalon and telencephalon, see Fig. 6) in the brain of *G. galloti* is also evident when compared with the expression patterns of other glial markers such as MBP, GFAP, glutamine synthetase, and vimentin (Monzón-Mayor et al., 1990a, c; Yanes et al., 1990, 1992).These data might reflect the more immature properties of glial cells in the rostral parts of the lizard brain.

With regard to the GS immunostaining in this study, S100-positive oligodendrocytes are a heterogeneous subpopulation because most of them are also GS-positive (see Fig. 7). Therefore, S100 seems to be a good marker of those GS-positive oligodendrocytes described previously in the adult *G. galloti* (Monzón-Mayor et al., 1998). The presence in our study of S100 in the nucleus could be related to its involvement in stimulation of RNA polymerase as suggested by Miani et al. (1973) in rabbits and chickens.

S100-Positive Ependymal Glial Cells, Radial Glia, and Astrocytes

The transient S100 staining in restricted areas of the ventricular zone in the diencephalon, from E32 to postnatal specimens, and in the telencephalon at stages E35 and E36, may be related to the role of the S100B protein in cell differentiation (Zimmer et al., 1995), because these zones give rise to new cells in *G. galloti* (Yanes et al., 1988). Along this line, oligodendrocyte precursors originating from restricted ventricular zones in the brain have been described in chicks and mice (Ono et al., 1998; Spassky et al., 2000). The identification of stained cells in the ventricular zone as precursors of S100-positive oligodendrocytes and the elucidation of the origin of S100-positive scattered cells require further studies that exceed the scope of this article. In adult specimens of *G. galloti,* we also observed S100 immunostaining in restricted areas of the ventricular wall in the telencephalon and mesencephalon. These S100-positive cells, which correspond to ependymocytes and radial glia (Yanes et al., 1988; Monzón-Mayor et al., 1990a), might serve different functions from those unlabelled cells in the same ventricular wall.

S100 protein is considered mainly an astrocyte marker in mammals (Langley et al., 1984), but in the lizard, only a very small subpopulation of S100-positive glial cells in the pretectum is similar to star-shaped astrocytes. Moreover, we observed S100-positive/GFAP-negative and S100-negative/GFAP-positive glial cells during the development of the mesencephalon that could correspond to oligodendrocytes and astrocytes, respectively. S100-positive/GFAP-positive astrocytes are absent in *G. galloti*, except in the optic nerve and chiasm (Arbelo et al., unpublished results), and after optic nerve transection, the S100 protein and GFAP are both up-regulated in the reactive astrocytes (Arbelo et al., unpublished results). The up-regulation of S100 after optic nerve injury in the lizard clearly warrants a more detailed study of the role of this protein in CNS repair and axon regeneration.

CONCLUSIONS

In this study, the S100 protein was detected in glial cells of the lizard *G. galloti*. Our findings indicate that, unlike the situation in the mammalian CNS, most of these S100-positive glial cells represent a subpopulation of postmitotic oligodendrocytes of light and medium cytoplasmic density, located in myelinated tracts during later developmental stages and in the adult lizard. The presence of the S100 protein in a small subpopulation of GFAP-negative astrocytes and in some ependymal and radial glial cells reflects the heterogenous expression and/or function of S100 proteins in the CNS.

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REFERENCES

Bischoff W. 1982. Die innerartliche Gliederung von Gallotia galloti (Dumeril D. Bibron 1839) (Reptilia, Sauria: Lacertidae) auf Tenneriffa, Kanarische Inseln. Bonn Zool Beitr 33:361–381.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Cocchia D. 1981. Immunocytochemical localization of S-100 protein in the brain of adult rat. An ultrastructural study. Cell Tissue Res 214:529–540.

Donato R. 1999. Functional roles of S100 proteins, calcium binding proteins of the EF-hand type. Biochim Biophis Acta 1450:191–231.

Dufaure JP, Hubert J. 1961. Table de développment du lézard vivipare (Lacerta vivipara jacquin). Arch Anat Microsc Morphol Exp 50:309–327.

Dyck RH, Van Eldik LJ, Cynader MS. 1993. Immunohistochemical localization of the S-100b protein in postnatal cat visual cortex: spatial and temporal patterns of expression in cortical and subcortical glia. Dev Brain Res 72: 181–192.

Engel AK, Müller CM. 1989. Postnatal development and vimentin-immunoreactive radial glia cells in the primary visual cortex of the cat. J Neurocytol 18:437–450.

Hartman BK, Agrawal HC, Kalmbach S, Shearer WT. 1979. A comparative study of the immunohistochemical localization of basic protein to myelin and oligodendrocytes in rat and chicken brain. J Comp Neurol 188:273–290

Lang DM, Monzón-Mayor M, Bandtlow CE, Stuermer CAO. 1998. Retinal axons regeneration in the lizard Gallotia galloti in the presence of CNS myelin and oligodendrocytes. Glia 23:61–74.

Lang DM, Romero-Alemán M, Arbelo-Galván JF, Stuermer CAO, Monzón-Mayor M. 2002. Regeneration of retinal ganglion cell axons in the lizard Gallotia galloti is not linked to neurogenesis. J Neurobiol 52:322–335.

Langley OK, Ghandour MS, Gombos G. 1984. Immunohistochemistry of cell markers in the central nervous system. In: Lajtha A, editor. Handbook of Neurochemistry. New York: Plenum Publishing Corporation, p 545–611.

Linser PJ, Perkins M. 1987. Gliogenesis in the embryonic avian optic tectum: neuronal-glial interactions influence astroglial phenotype maturation. Dev Brain Res 32:227–290.

López-García C, Molowny A, Martínez-Guijarro FJ, Blasco-Ibañez JM, Luis de la Iglesia JA, Bernabeu A, García-Verdugo JM. 1992. Lesion and regeneration in the medial cerebral cortex of lizards. Histol Histopath 7:725–746.

Manso MJ, Becerra M, Becerra M, Anadon R. 1997. Expression of a low-molecular-weight (10 KDa) calcium binding protein in glial cells of the brain of the trout. Anat Embryol 196:403–416.

Miani N, Michetti F, De Renzis G, Caniglia A. 1973. Effect of a brain-specific protein (S100 protein) on the nucleolar RNA polymerase activity in isolated brain nuclei. Experientia 29:1499–1501.

Monzón-Mayor M, Yanes C, Ghandour MS, de Barry J, Gombos G. 1990a. Glial fibrillary acidic protein and vimentin immunohistochemistry in the developing and adult midbrain of the lizard Gallotia galloti. J Comp Neurol 295:569–579.

Monzón-Mayor M, Yanes C, James JL, Sturrock RR. 1990b. An ultrastructural study of the development of oligodendrocytes in the midbrain of the lizard. J Anat 170:43–49.

Monzón-Mayor M, Yanes C, Tholey G, de Barry J, Gombos G. 1990c. Immunohistochemical localization of glutamine synthetase in mesencephalon and telencephalon of the lizard Gallotia galloti during ontogeny. Glia 3:81–97.

Monzón-Mayor M, Yanes-Méndez C, De Barry J, Capdevilla-Carbonell C, Renau-Piqueras J, Tholey G, Gombos G. 1998. Heterogeneous immunoreactivity of glial cells in the mesencephalon of a lizard: A double labeling immunohistochemical study. J Morphol 235:109–119.

Ono K, Tsumori T, Kishi T, Yokota S, Yasui Y. 1998. Developmental appearance of oligodendrocytes in the embryonic chick retina. J Comp Neurol 398:309–322.

Parnavelas JG, Luder R, Pollard SG, Sullivan K, Lieberman AR. 1983. A qualitative and quantitative ultrastructural study of glial cells in the developing visual cortex of the rat. Philos Trans R Soc Lond B Biol Sci 301:55–84.

Ramos-Steffens A. 1980. Tabla del desarrollo embrionario de Lacerta gallotia galloti (periodo de organogénesis), y aspectos de su reproducción. Minor thesis, Facultad de Biología, Universidad de la Laguna, Spain.

Rickmann M, Wolff JR. 1995. S100 immunoreactivity in a subpopulation of oligodendrocytes and Ranvier's nodes of adult rat brain. Neurosci Lett 186:13–16.

Skoff RP, Price DL, Stocks A. 1976a. Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. I. Cell proliferation. J Comp Neurol 169:291–312.

Skoff RP, Price DL, Stocks A. 1976b. Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. II. Time of origin. J Comp Neurol 169:313–334.

Spassky N, Olivier C, Pérez-Villegas E, Goujet-Zalc C, Martínez S, Thomas JL, Zalc B. 2000. Single or multiple oligodendroglial lineages: A controversy. Glia 29:143–148.

Sturrock RR. 1982a. Gliogenesis in the prenatal rabbit spinal cord. J Anat 134:771–793.

Sturrock RR. 1982b. Cell division in the normal central nervous system. Adv Cel Neurobiol 3:3–33.

Vaughn JE. 1969. An electron microscopic analysis of gliogenesis in rat optic nerve. Z Zellforsch 94:293–324.

Yanes C, Monzón-Mayor M, de Barry J, Gombos G. 1992. Myelin and myelinization in the telencephalon and mesencephalon of the lizard Gallotia galloti as revealed by the immunohistochemical localization of myelin basic protein. Anat Embryol 185:475–487.

Yanes C, Monzón-Mayor M, Ghandour MS, de Barry J,

Gombos G. 1990. Radial glia and astrocytes in developing and adult telencephalon of the lizard Gallotia galloti as revealed by immunohistochemistry with Anti-GFAP and Anti-Vimentin antibodies. J Comp Neurol 295:559–568.

Yanes C, Monzón-Mayor M, Gombos G, Renau-Piqueras J, Sturrock RR. 1997. Glial cells in the lizard Gallotia galloti subpallial nuclei during ontogeny: An ultrastructural study. J Morphol 233:1–13.

Yanes-Méndez C, Martín-Trujillo JM, Pérez-Batista MA, Monzón-Mayor M, Marrero A. 1988. Ependymogenesis of the lizard basal areas. I. Ependymal zones. Z mikrosk-anat Forsch Leipzig 102:555–572.

Zeller NK, Behar TN, Dubois-Dalcq ME, Lazzarini RA. 1985. The timely expression of myelin basic protein gene in cultured rat brain oligodendrocytes is independent of continuous neuronal influences. J Neurosci 5:2955–2962.

Zimmer DB, Cornwall EH, Landar A, Song W. 1995. The S100 protein family: History, function, and expression. Brain Res Bull 37:417–429.