Glial fibrillary acidic protein and vimentin immunoreactivity of astroglial cells in the central nervous system of adult *Podarcis sicula* (Squamata, Lacertidae)

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(*Accepted 11 July 2000*)

ABSTRACT

The present immunoperoxidase cytochemical study describes the distribution of glial intermediate filament molecular markers, glial fibrillary acidic protein (GFAP) and vimentin, in the brain and spinal cord of the adult lizard, *Podarcis sicula*. GFAP immunoreactivity is abundant and the positive structures are mainly represented by fibres of different lengths which are arranged in a rather regular radial pattern throughout the CNS. They emerge from generally immunopositive radial ependymoglia and are directed from the ventricular wall towards the meningeal surface. The glial fibres give origin to endfeet which are apposed to the blood vessel walls and subpial surface where they form the continous perivascular and subpial glia envelopes, respectively. In the optic tectum and spinal cord, star-shaped astrocytes coexist with radial glia. In the spinal cord, cell bodies of immunopositive radial glia are displaced from the ependyma. While vimentin immunoreactive elements are almost completely absent in the brain except for a few diencephalic radial fibres, the spinal cord ependyma exhibits a clearly vimentin positivity and no GFAP staining. In the *Podarcis* CNS the immunocytochemical response of the astroglial intermediate filaments appears typical of mature astroglia cell lineage since it fundamentally expresses GFAP immunoreactivity. Moreover, this immunocytochemical study shows that the *Podarcis* fibre pattern with predominant radial glial cells is morphologically more immature than in avians and mammalians, a condition suggesting that reptiles represent a fundamental step in the phylogenetic evolution of vertebrate astroglial cells.

Key words: Reptiles; intermediate filaments; radial glia.

INTRODUCTION

Radial glia consists of pear or spindle-shaped cells whose bodies are located in the ependymal or periependymal layer. Their long radial cytoplasmic processes spread over the central nervous system (CNS), terminating on the vascular and pial surfaces with endfeet which constitute respectively the perivascular glial layer and the membrana gliae limitans externa (Monzon-Mayor et al. 1990; Yanes et al. 1990; Elmquist et al. 1994; Lazzari et al. 1997). Radial glia is considered not only the most primitive form of glia in phylogenesis (Onteniente et al. 1983; Miller & Liuzzi, 1986) but also an ontogenetically immature type of glia since it is the first to appear during ontogeny (Levitt & Rakic, 1980; Monzon-Mayor et al. 1990). While in developing mammals it progressively reduces itself and therefore is virtually absent in the adult (Pixley & De Vellis, 1984; Elmquist et al. 1994), in lower vertebrates it persists throughout the entire life cycle (Ebner & Colonnier, 1975; Lazzari et al. 1997).

Glial fibrillary acidic protein (GFAP) is a reliable specific molecular marker of mature cells of the astroglial lineage (Dahl & Bignami, 1985). It is expressed in gliofibrils of vertebrate astrocytes of all types (typical star-shaped fibrous or protoplasmic astrocytes, Bergmann glia, periependymal radial glia and tanycytes) (see Wasowicz et al. 1994; Wicht et al. 1994; Naujoks-Manteuffel & Meyer, 1996, for reviews). In each vertebrate group GFAP shows cross reactivity to antimammalian GFAP antibodies

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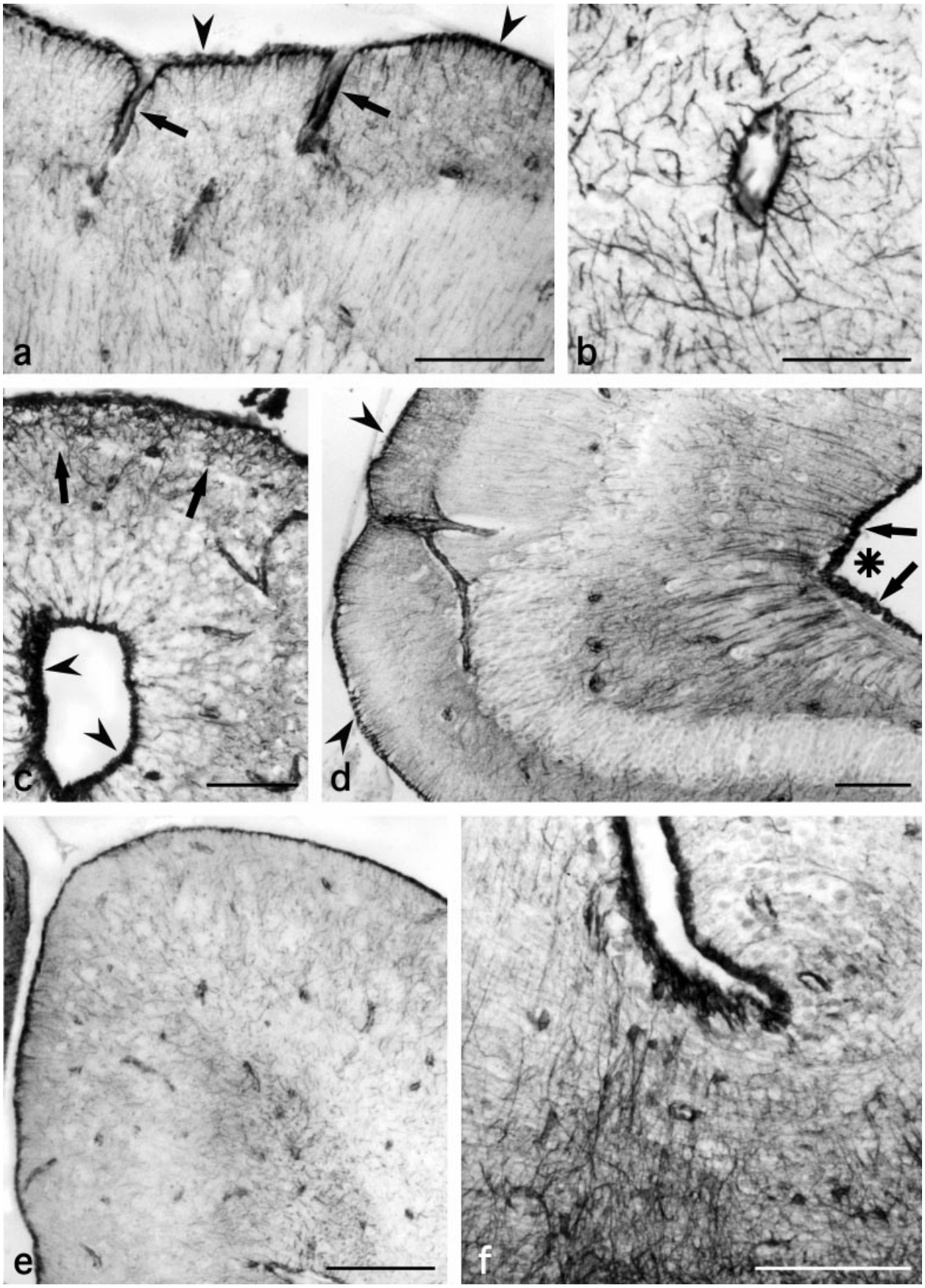

Fig. 1. Immunocytochemical detection of GFAP in *Podarcis sicula* telencephalon and diencephalon. (*a*) Radial fibres give origin to glial endfeet (arrowheads) located on the submeningeal surface of the telencephalon. The endfeet layer generated by other radial fibres on the surface of longitudinally sectioned vessels (arrows) penetrating into the nervous tissue is connected to the submeningeal glial endfeet stratum.

(Onteniente et al. 1983; Dahl et al. 1985; Bodega et al. 1994) demonstrating that GFAP is of considerable stability in its molecular and antigenic characteristics throughout vertebrate phylogenesis. Nevertheless, ontogenetic and phylogenetic implications result not only from the presence of GFAP in the different astroglial cell types, but also from the relative proportion of these astrocytic subtypes and their regional distribution in the CNS of different vertebrates (Elmquist et al. 1994; Monzon-Mayor et al. 1998).

Even though vimentin has been found in immature cells of the astroglial lineage in mammals (Elmquist et al. 1994; Pulido-Caballero et al. 1994) and reptiles (Monzon-Mayor et al. 1990; Yanes et al. 1990), it is still present in adult glial cells in teleosts and amphibians (Zamora & Mutin, 1988; Cardone & Roots, 1990; Rubio et al. 1992; Lazzari et al. 1997). Furthermore, vimentin is highly conserved in phylogenesis as suggested by the cross reaction of antibodies produced against mammalian vimentin with the corresponding protein in birds and amphibians (Bennett et al. 1978; Szaro & Gainer, 1988; Zamora & Mutin, 1988; Bodega et al. 1994). Thus the knowledge of phylogenetic and ontogenetic relations between the different cell types belonging to the glial cell lineage might be facilitated by comparative studies on glial cells in the CNS of species which are members of different vertebrate classes.

Despite the considerable progress in glial cell research in mammals in recent decades and the fact that reptiles are phylogenetically the earlier amniotes, studies on reptilian glial cells are still relatively scarce (Dahl & Bignami, 1973; Onteniente et al. 1983; Kalman et al. 1994). In particular, with regard to the saurian CNS, immunocytochemical studies on the cytoskeletal components of glial cells have only been performed in *Gallotia galloti* (Monzon-Mayor et al. 1990, 1998; Yanes et al. 1990), *Anolis carolinensis* (Dahl et al. 1985), *Lacerta sicula* (Lauro et al. 1991) and *Lacerta lepida* (Bodega et al. 1990, 1994).

The aim of the present work is to study the presence and distribution of the intermediate filament specific molecular markers, GFAP and vimentin, in the CNS of the lacertid *Podarcis sicula* using light immunoperoxidase cytochemistry.

MATERIALS AND METHODS

Adult lizards, *Podarcis sicula*, (15–20 cm total length) of both sexes were kept in terraria at room temperature in natural light-dark cycle and fed ad libitum. The animals were deeply anaesthetised with ethyl ether and perfused transcardially with 20 ml of physiological saline (0.9% NaCl) containing heparin (3 IU/ml) at 4 °C and subsequently with 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C. Perfusion solutions were delivered by a Minipulse 3 peristaltic pump (Gilson) operating at a constant flow of 5 ml/min. The brain and spinal cord were then removed and postfixed by immersion in the same fixative for an additional 1 h at 4 °C. After washing overnight in 0.1 M phosphate buffer, pH 7.4, at 4 °C, specimens were dehydrated with ethanol and embedded in Paraplast plus (Sherwood Medical; melting point 55–57 °C). Coronal sections (10 μm thick) were mounted on poly-L-lysine (Sigma) coated slides and dried. In the subsequent processing all incubation and washing solutions were used at room temperature. The sections were deparaffinised with xylene, hydrated, pretreated for 20 min with 1% H_20_2 in 0.05 M phosphate buffer with 0.15 M NaCl (PBS), pH 7.4, to remove endogenous peroxidase activity, and finally preincubated in PBS containing 10% normal goat serum (NGS; Vector), 1% bovine serum albumine (BSA; Sigma) and 0.1% Tween 20 (Merck) for 30 min to reduce nonspecific background staining. The sections were incubated in a moist chamber on a floating plate for 3 h in either a polyclonal rabbit anticow GFAP antiserum (1:500; Dakopatts) or a monoclonal mouse antibovine vimentin antibody (1:3; Boehringer). Antibodies were diluted in PBS containing 3% NGS, 1% BSA and 0.1% Tween 20. After rinsing in PBS with 0.1% Tween 20, the sections were incubated in the secondary antibody for 1 h: HRP-conjugated goat antirabbit IgG (1:200; Vector) for GFAP and biotinylated goat antimouse IgG (1:250; Vector) for vimentin. After rinsing in 0.1% Tween 20 in PBS, for vimentin immunostaining the sections were incubated in the avidin-biotin-HRP complex (ABC kit, Vector) for 1 h. For both immunodetections the sections were then rinsed for 10 min in 0.1 M phosphate buffer, pH 7.4, and treated with the

(*b*) GFAP-immunopositive glial fibres terminate with endfeet on the surface of a cross sectioned vessel in the telencephalon. (*c*) In the olfactory bulb a strong immunoreaction is present in the ependyma (arrowheads). Radial fibres (arrows) are evident in the superficial zone. (*d*) Telencephalic wall at the level of the sulcus septomedialis (asterisk): the ependyma (arrows), the glia limitans externa (arrowheads) and the vascular wall are intensely stained. Radial glial fibres are clearly seen especially in the periependymal and submeningeal regions. (*e*) In the anterior dorsal ventricular ridge thin fibres are evenly arranged and they form a clear perivascular glial coating. (*f*) In the ventral diencephalon the ependymal tanycytes show a strong immunoreaction. A glial fibre texture is located ventrally to the third ventricle. Bars, 100 μm (*a*, *c*, *d*, *f*); 50 μm (*b*); 200 μm (*e*).

diaminobenzidine method modified by Adams (1981). The sections were then dehydrated in ethanol, cleared in xylene and coverslipped with Permount (Fisher Scientific). Negative controls were obtained by omission of the primary antibodies, replaced by 3% NGS.

RESULTS

GFAP-like immunoreactivity

In *Podarcis* brain, the general pattern of GFAP-immunopositivity was fundamentaly represented by long and thick fibres which ran from cell bodies located at the ventricular surface to the meningeal layer, thus indicating their tanycytic character. These radial glia fibres gave origin to the external glial limiting membrane (Fig. 1*a*) and the perivascular glial coating (Fig. 1*a*, *b*).

In the olfactory bulb and peduncle the ependymal layer was uniformly GFAP-immunopositive and gave origin to wavy glial processes organised into a dense superficial texture (Fig. 1*c*). In the telencephalic cortex, where the neural wall thickness did not increase, 3 zones could be distinguished schematically (Fig. 1*d*): a rather regularly radial-organised deep (periependymal) zone; a superficial (submeningeal) zone; and an irregular middle zone with a fine texture. In the thickened parts of the telencephalon (septum, ventral striatum, anterior dorsal ventricular ridge) this scheme became less evident (Fig. 1*e*). The different regions of the telencephalic wall showed immunostaining of different intensity. In particular, the ependymal cell bodies and corresponding radial processes showed intense staining in the sulci of the lateral ventricles (Fig. 1*d*), whereas in the zones between sulci, the radial ependymoglia showed smaller cell bodies with thin and processes that were not easily distinguishable.

In the diencephalon the narrow dorsoventrally lengthened ventricle was clearly outlined by an intensely GFAP-immunostained ependymal layer giving origin to radial processes directed to the outer surface (Fig. 1*f*).

In the mesencephalon the ependyma showed a more intense GFAP-immunopositivity in its ventral part, especially at the sulcus limitans (Fig. 2*a*). The superficial part of the optic tectum was characterised by radial processes and star-shaped astrocytes which appeared in approximately 2 layers (Fig. 2*b*). While some fine GFAP-positive radial fibres were observed in the middle region of the optic tectum (Fig. 2*b*), they were more evident in the tegmentum, particularly in the most ventral part.

In the medulla oblongata the raphe was intensely immunostained, the medial longitudinal fascicle was well circumscribed and the marked ependymal cells, particularly those lateral to the colliculum, appeared as tanycytes with thin processes (Fig. 2*c*).

The cross-sectioned spinal cord showed clear GFAP-immunopositivity with thin radial oriented immunopositive processes evident throughout the section (Fig. 3*a*). Radial astrocytes proper were detected only in the spinal cord. They were characterised by cell bodies displaced away from the ependymal layer into a periependymal position, preferentially aggregated in the dorsal and lateral part of the grey matter (Fig. 3*b*). They gave origin to the radial processes directed towards the spinal cord surface. Star-shaped astrocytes were evident in the ventral horns at the boundary between white and grey matter (Fig. 3*c*). Other astrocytes appeared in the middle of the lateral column (Fig. 3*d*) with numerous processes directed to the grey matter or the meningeal surface. Ependymal cells showed no GFAP-immunopositivity.

No specific reaction was seen in the control sections.

Vimentin-like immunoreactivity

In *Podarcis* brain, a clear antivimentin immunoreactivity was detectable only in the marginal zone of the ventral part of the diencephalon as thin fibres oriented radially towards the meningeal surface (Fig. 4*a*). Vimentin-positive cell bodies were not found in the adult *Podarcis* brain.

In the spinal cord, the ependymal cells showed clear vimentin immunoreactivity and the most dorsal ones sent long processes to the pial surface along the glial medial septum (Fig. 4*b*, *c*). The ependymocytes located in the ventral region gave origin to thin processes which lined the dorsolateral and medial parts of the medial longitudinal fasciculus (Fig. 4*b*).

Control experiments showed no staining.

DISCUSSION

In the *Podarcis* CNS immunoperoxidase cytochemistry has demonstrated not only the presence of different astroglial lineage cell types (ependymal radial glia or tanycytes throughout the brain, radial glia proper or radial astrocytes in the spinal cord with their cell bodies displaced from the ependymal layer, and star-shaped astrocytes in the optic tectum and spinal cord), but also that the staining intensity is not identical for the same cell type. Therefore, as has been reported by Monzon-Mayor et al. (1990, 1998) in

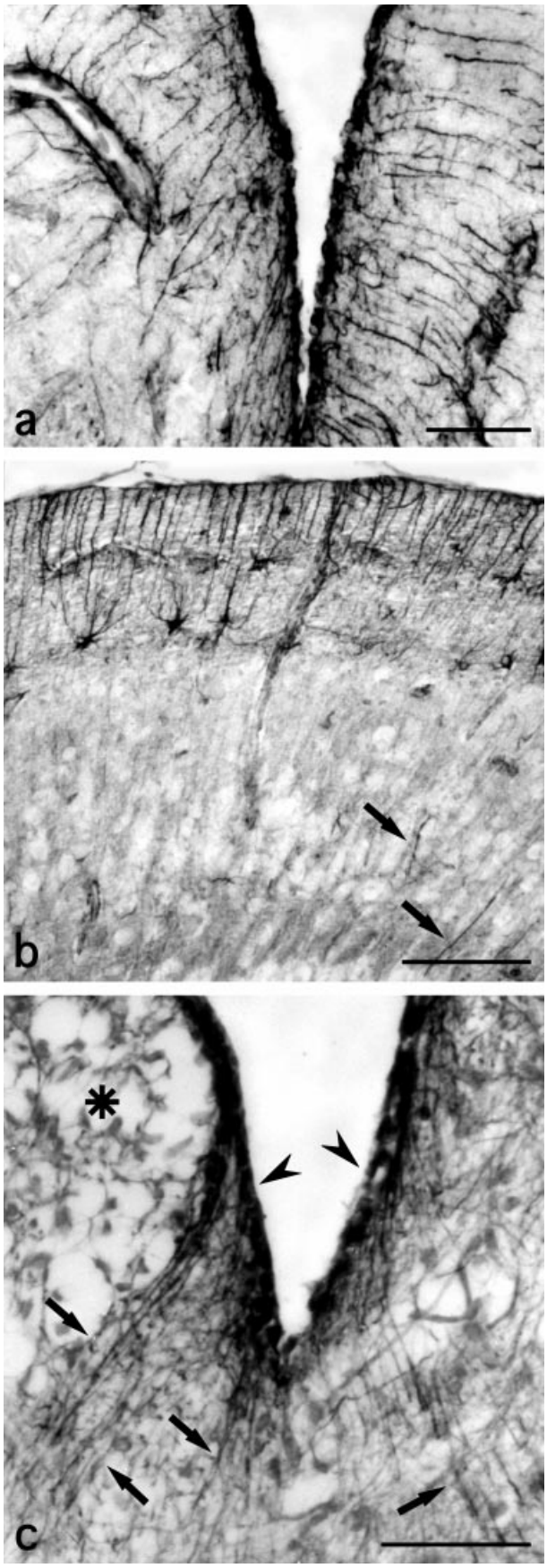

Fig. 2. GFAP-immunoreaction in *Podarcis sicula* mesencephalon and medulla oblongata. (*a*) At the sulcus limitans level tanycytes show strong staining in both their ependymal cell bodies and radial processes. (*b*) In the superficial region of the optic tectum radial glial processes reach the submeningeal surface; some star-shaped astrocytes are clearly evident. A few radial fibres are observed in deeper layers of the optic tectum (arrows). (*c*) Immunostained ependymal tanycytes (arrowheads) in the medulla oblongata, laterally to the colliculum (asterisk) with radially oriented processes (arrows). Bars, 50 μm (*a*, *c*); 100 μm (*b*).

Gallotia galloti, our results indicate a certain degree of heterogeneity both in the immunological and morphological characteristics of glial cells of *Podarcis sicula*.

Previous studies in mammals have shown that, while the radial glia fibre system is present during development, it disappears postnatally (Voight, 1989; Elmquist et al. 1994). Moreover in mammals, even though vimentin-immunopositive glial structures can be detected from the first embryonic stage, this antigen is progressively replaced by GFAP during maturation (Pixley & De Vellis, 1984; Elmquist et al. 1994). Conversely, in *Podarcis* as in *Gallotia*, radial glial elements are still present in adults (Monzon-Mayor et al. 1990; Yanes et al. 1990), even if during lacertian development the molecular composition of the intermediate filaments in these cells changes. In fact in the embryonic *Gallotia galloti*, vimentin appears as the main component of the intermediate filaments in radial glial structures (Monzon-Mayor et al. 1990; Yanes et al. 1990). However, in *Podarcis* as in *Gallotia*, vimentin-immunoreactive glial structures have almost completely disappeared in the adult except for a few fibres and endfeet in the tegmentum of *Gallotia* (Monzon-Mayer et al. 1990) and in the ventral diencephalon and spinal cord ependyma of *Podarcis*.

The present immunocytochemical study reveals that the GFAP-immunoreactivity of *Podarcis* CNS is intense. This is in accordance with studies performed on other reptiles (Dahl & Bignami, 1973; Onteniente et al. 1983; Dahl et al. 1985; Monzon-Mayor et al. 1990; Yanes et al. 1990; Kalman et al. 1994).

The absence of GFAP-positive star-shaped astrocytes in *Podarcis* telencephalon is in accordance with observations on *Gallotia* (Yanes et al. 1990) where only GFAP-positive ependymal cell bodies and their radial fibres are observed in the telencephalon. The same GFAP-immunolocalisation is found in the turtle, the CNS of which lacks typical astrocytes and possesses only a radial glial pattern (Kalman et al. 1994). Conversely, GFAP-positive star-shaped astrocytes are observed in the snake hippocampus (Onteniente et al. 1983).

Nevertheless, GFAP-positive typical star-shaped astrocytes are found together with radial glial elements in the mesencephalon of both *Gallotia* (Monzon-

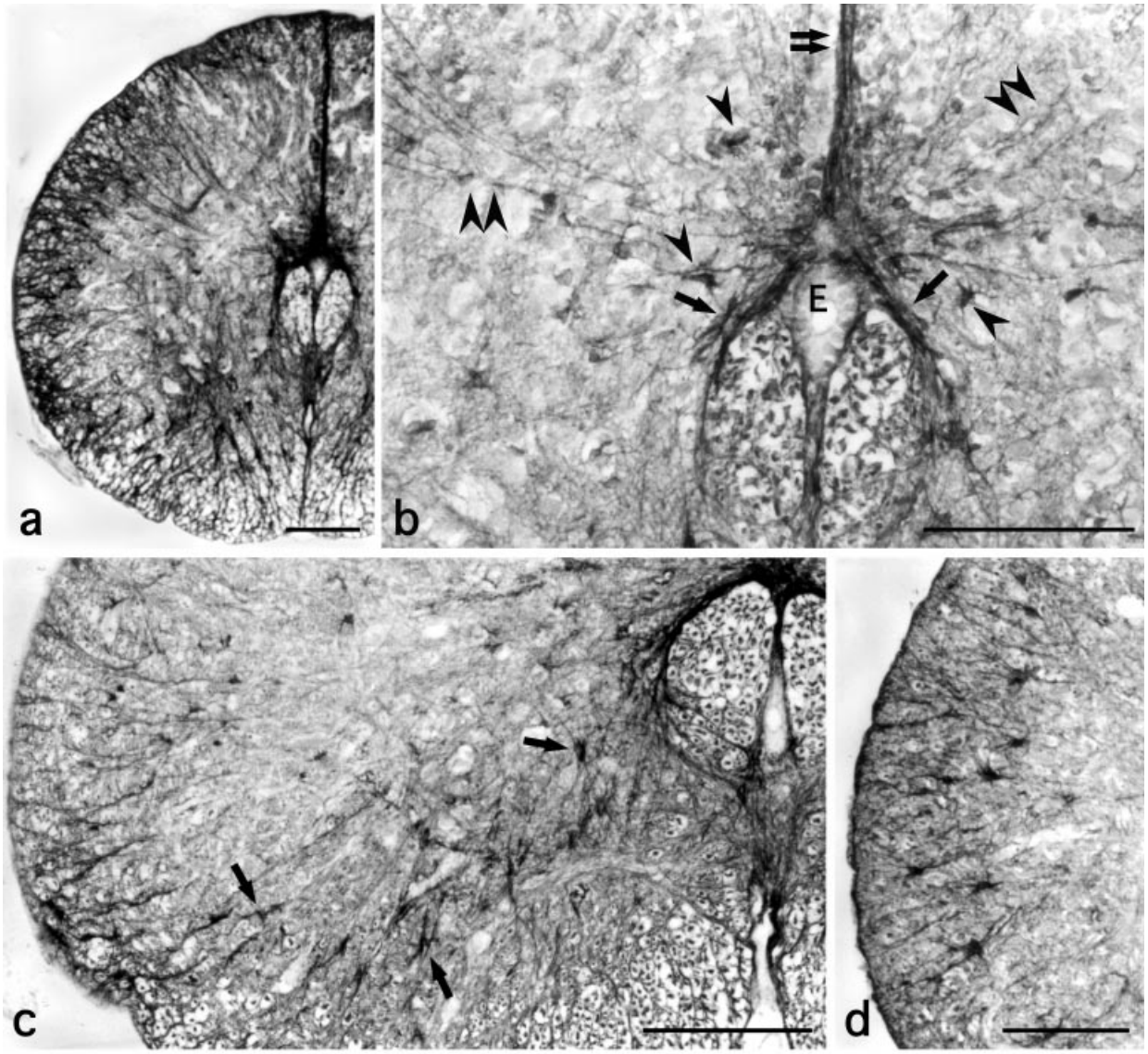

Fig. 3. GFAP expression in the spinal cord of *Podarcis sicula*. (*a*) Low magnification of spinal cord showing general distribution of GFAP immunoreaction in the grey and white matter. (*b*) At higher magnification the ependyma (E) is immunonegative and aggregates of immunopositive radial glial cells are located dorsally to it extending their processes to the glial medial septum (double arrows), medial longitudinal fasciculus (arrows) and laterally (double arrowheads) to the meningeal surface. Some radial glial cells are displaced laterally (arrowheads). (*c*) Star-shaped astrocytes (arrows) in the lower part of the spinal cord ventral horn. (*d*) Star-shaped astrocytes in the lateral column of the spinal cord. Bars, 100 μm (*a*, *b*, *d*); 200 μm (*c*).

Mayor et al. 1990, 1998) and *Podarcis* (present study). This condition should be interpreted by assuming that in Lacertidae the telencephalon is more primitive than the mesencephalon, which in reptiles attains the highest phylogenetic development. In the *Podarcis* optic tectum, the radially oriented fibres are particularly evident in the superficial layers and it is interesting to find the typical star-shaped astrocytes in these same layers. At present, some relationship between the 2 structures cannot be excluded. Moreover, the intensity of the GFAP-immunoreactivity does not appear uniform throughout the ependymal layer of *Podarcis*. In particular, it is intense at the sulcal level and appears faint in the optic tectum and moderate in the tegmentum. Where the ependymal cell bodies show more immunoreactivity, their radial fibres are well marked.

With regard to the spinal cord in mammals, while the ependyma is mainly GFAP-immunonegative (Bodega et al. 1994; Elmquist et al. 1994), the ependymal vimentin expression shows a species-related pattern because it is positive in the opossum (Elmquist et al. 1994) and rat (Bodega et al. 1994) and negative in sheep (Bodega et al. 1994).

Considering intermediate filament associated proteins in the lacertid spinal cord, Lauro et al. (1991)

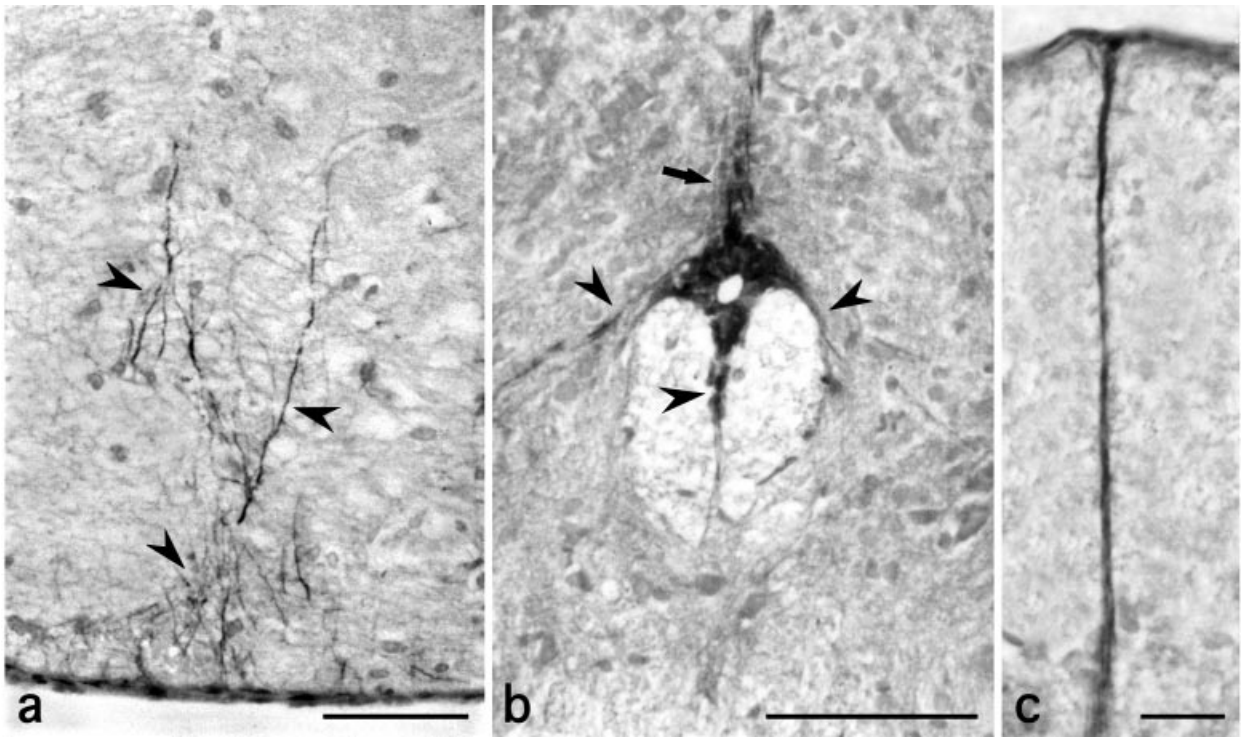

Fig. 4. Immunohistochemical detection of vimentin in the CNS of *Podarcis sicula*. (*a*) Some immunopositive radial fibres (arrowheads) in the superficial zone of the hypothalamus. (*b*) Clear immunoreaction in the ependymal cells of the spinal cord. Some fibres (arrowheads) outline the dorsolateral and medial surface of the medial longitudinal fasciculus; others (arrow) are dorsally directed to the glial medial septum. (*c*) The superficial part of the glial medial septum is clearly immunostained. Bars, 50 μm (*a*); 100 μm (*b*); 20 μm (*c*).

found that in *Lacerta sicula* ependymal cells only rarely expressed a vimentin-positive cytoskeleton and mostly showed intense GFAP-immunopositivity. Our results in *Podarcis* spinal cord are not in accordance with these findings since we found an intense ependymal vimentin-positivity and a substantial GFAP-immunonegativity. Our results also differ from *Lacerta lepida* (Bodega et al. 1994) which did not show any GFAP- and vimentin-immunoreactivity at the ependymal level of the spinal cord. Nevertheless, in accordance with Bodega et al. (1994) we must emphasise the importance of methodology as it is possible that some of the differing results reported in literature could be ascribed not only to species dependent patterns but also to methodological differences.

In the mammalian spinal cord the fibrous glia of the white matter appears intensely GFAP-immunopositive, while it is less reactive in the grey matter (Dahl & Bignami, 1985). This tendency is already noticeable in *Podarcis* spinal cord as most star-shaped astrocytes, with well developed GFAP-positive processes, are clearly located in the white matter or the adjacent grey matter.

The ependymal cells of adult higher vertebrates mostly show absence of GFAP-immunoreaction (Bodega et al. 1994), whereas transient GFAP expression has been demonstrated during development (Roessman et al. 1980). But the scarce GFAP-immunopositive ependymal cells that have been reported in adult higher vertebrates provides evidence of tanycytic features (Levitt & Rakic, 1980; Chouaf et al. 1989; Bodega et al. 1990). According to Bodega et al. (1994) we can assume that GFAP expression in ependymal cells shows an inverse relation with phylogenesis, i.e. lower vertebrates express more GFAP ependymal immunoreactivity than higher ones. Moreover, although in lower vertebrates GFAP can be expressed by both ependymal cell types (ependymocytes and tanycytes), in higher vertebrates it can be observed only in tanycytes which progressively decrease in more highly evolved groups. Furthermore, the occurrence of star-shaped astrocytes would appear to be closely connected to the decrease in GFAP ependymal expression in phylogenesis. In fact, reptiles are the first vertebrates showing astrocytes proper, and in their spinal cord, where many astrocytes are present, the ependyma is substantially GFAP-immunonegative whereas in the optic tectum, which contains various star-shaped astrocytes, the ependymal layer shows a weaker GFAP immunoreaction.

During CNS development the vimentin-GFAP shift has been reported in reptiles (Monzon-Mayor et al. 1990; Yanes et al. 1990), birds (Tapscott et al. 1981;

Kalman et al. 1998) and mammals (Oudega & Marani, 1991; Elmquist et al. 1994). Nevertheless vimentin is still expressed in the ependymal cells of some regions of adult CNS in mammals (Chouaf et al. 1989; Oudega & Marani, 1991; Yamada et al. 1992), birds (Alvarez-Buylla et al. 1987; Kalman et al. 1998), reptiles (Lauro et al. 1991; present study), amphibians (Zamora & Mutin, 1988; Lauro et al. 1991; Lazzari et al. 1997) and fish (Cardone & Roots, 1990; Rubio et al. 1992; Bodega et al. 1993).

If vimentin expression is related to a mesenchymal character of cells, in accordance with the conclusions of Lauro et al. (1991) the presence of a mesenchymal molecular marker in cells of neuroectodermal origin would be indicative of a degree of histogenetic indetermination.

Even though GFAP expression in vertebrates seems to follow a phylogenetic pattern, vimentin expression appears to have a species-dependent distribution. In any case the relationship occurring between vimentin and GFAP in the CNS during evolution and ontogeny is not yet clear and therefore further studies are needed about the characteristics of glial lineage cells.

ACKNOWLEDGEMENTS

This study was supported by grants from the Ministero della Ricerca Scientifica e Tecnologica.

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