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ARTICLE

Advances of Squamata astroglia to other reptiles: numerous astrocytes and glial fibrillary acidic protein (GFAP)-free areas. A preliminary study

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ABSTRACT Squamata are diapsid reptiles. Testudines were positioned formerly to the most ancient group, Anapsida, but recently they are also classified as diapsid reptiles, although their position within this group is uncertain. The investigated species of this study involved lizards (*Timon tanginatus,* Lacertidae; *Pogona vitticeps*, Agamidae; *Eublepharis macularis*, Gekkota; *Chameleo calypratus*, Chameleonidae), snakes (*Epicrates cenchria maurus*, Boidae; *Python regius*, Pythonidae; *Pantherophis guttata* and *P. obsoletus quadrivittatus*, Colubridae), and turtles (*Testudo hermanni*, Testudinidae; *Trachemys scripta* and *Mauremys sinensis*, Emydidae; *Pelomedusa subrufa*, Pleurodira). They were overanasthetised with Nembutal and transcardially perfused with 4% buffered paraformaldehyde. Coronal sections were processed according to the immunoperoxidase protocol. Monoclonal anti-GFAP and other glial markers were used. The main astroglia were the radial ependymoglia. There were two principal advances in Squamata. First, astrocytes were frequent in several areas, although, nowhere predominated. Furthermore, considerable GFAP-poor areas were found. They were extended in *Python*, and in *Pogona* and *Chamaeleo* GFAP was almost missing throughout the brain. The Squamata share more common astroglial features with birds than the turtles, although, represents a separate branch (Lepidosauria *versus* Archosauria). In mammals and birds the GFAP-free areas are usually advanced, expanded and plastic ones. Note that Squamata display quite complex behavioural phenomena related to other reptiles. **Acta Biol Szeged 59(Suppl.3):353-360 (2015)**

KEY WORDS
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Abbreviations

A: agama; ca: anterior commissure; C: chameleon; dp: dorsal pallia; DVR/dvr: dorsal ventricular ridge; G: gecko; hy: hypothalamus; L: lacertid lizard (*Timon)*; mp: medial pallia; to: optic tract; Sb: boa; Sp: python; Sc: colubrid snake (*Pantheropis*); sp: septum; st: striatum; Tg: greek tortoise; Tc: chinese turtle.

Introduction

The present study continues to our previous studies on the astroglia of vertebrates: Chondrichthyes (Kálmán and Gould 2001; Ari and Kálmán 2008), Actinopterygii (Kálmán 1998, Kálmán and Ari 2002), birds (Kálmán et al. 1993, 1998), turtles (Kálmán et al. 1994, 1997), and caiman (Kálmán and Pritz 2001).

Former studies found that there are similar features of astroglia of mammals and birds which have the most advanced vertebrate brains. One of them the predominancy of astrocytes the other is the appereance of large brain areas free of GFAP-immunopositivity or at least very poor in it but capable of GFAP expression following lesions (Kálmán 2002). Similar areas and/or predominancy of astrocytes were not observed in either turtles or crocodilians. In them, an almost evenly dense, thin, elongated ependymoglia actually, the 'tanycytes' of Horstmann (1954) are the predominant glia as well as in most of 'anamniotes' (Kálmán 2002; Appel 2013); although, astrocytes were found in several areas of the caiman brain. Present study investigates the phenomena of astroglial evolution in lizards and snakes.

Several species of Squamata (snakes and lizards) of different taxonomic positions (Table 1) were investigated. Considering that our former studies on turtle were based on Boehringer mouse monoclonal anti-GFAP antibody which was unavailable for this new analysis, we decided to inves-

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Table 1. Species studied with their taxonomical data.

Classis	Ordo	Subordo	Familia	Species
Reptilia	Squamata	Scincomorpha	Lacertidae	Moroccan eyed lizard - *Timon tangitanus*
		Iguania	Agamidae	Bearded dragon - *Pogona vitticeps*
			Chamaeleonidae	Veiled chameleon - *Chamaeleo calyptratus*
		Gekkota	Eublepharidae	Leopard gecko - *Eublepharis macularius*
		Serpentes	Boidae	Columbian rainbow boa - *Epicrates cenchriamaura*
			Pythonidae	Ball python - *Python regius*
			Colubridae	Corn snake - *Pantherophis* (formerly: *Elaphe*) *guttatus*
				Yellow rat snake - *Pantherophis* (*Elaphe*) *obsoletus quadrivittatus*
	Testudines	Cryptodira	Testudinidae	Greek tortoise - *Testudo hermanni boettgeri*
			Emydidae	Yellow-bellied slider – *Trachemys* (formerly*: Pseudemys*) *scr. scripta*
				Red-eared slider - *Trachemys* (*Pseudemys*) *scripta elegans*
			Geoemydidae	Chinese stripe-necked turtle - *Mauremys* (formerly: *Ocadia*) *sinensis*
		Pleurodira	Pelomedusidae	African helmeted turtle - *Pelomedusa subrufa*

tigate more turtle species in parallel, including a representative of Pleurodira which group has not been studied earlier (Table 1).

Glial fibrillary acidic protein (GFAP), the characteristic cytoskeletal protein of astroglia (Bignami et al. 1980) of each vertebrate group has cross-reactivity to the anti-mammalian GFAP antibodies (Dahl and Bignami 1973; Dahl et al. 1985; Onteniente et al. 1983). However, according to several studies, not all astrocytes can be detected by the immunohistochemical reaction against GFAP (Connor and Berkowitz 1985; Linser 1985). Therefore, it is necessary to apply other astroglial markers, *i.e*. glutamine synthetase and S100 protein (Ludwin et al. 1976; Martinez-Hernandez et al. 1977). Our previous results (Kálmán et al. 1995) obtained on adult turtle brain suggested that vimentin-immunopositivity is also to be expected in adult reptile brains; therefore, these markers were also investigated. In this study, beside Novocastra reagent, four other anti-GFAP reagents were also tested.

Materials and Methods

Fixation and sectioning

Experimental animals (which were unable of breeding due to wounds or infertility) were obtained from breeders. They were sublethally overanasthetised with Nembutal and transcardially perfused with paraformaldehyde solution (4% in phosphate buffer-saline). Following two days postfixation, the brains were embedded into agarose and series of coronal sections (50-70 μm) were cut by Vibratome.

Immunohistochemical procedure

After a rinse in phosphate buffer the brains were embedded in agar, and then 50 μm thick serial sections were cut in the coronal plane by a Vibratome vibrating microtome. After rinsing overnight in phosphate buffer the floating sections were pre-treated with 3% H_2O_2 (for 5 min) to suppress the endogenous peroxidase activity and then incubated in 20% normal goat serum (at room temperature for 90 min) to block the non-specific antigen binding. These and all the following steps included a rinse with phosphate buffer between the change of reagents. Then primary immunoreagent (Table 2) was applied in phosphate buffer containing 0.5% Triton X-100, at 4 °C for 40 hours. As a secondary antibody, biotinylated anti-mouse or anti-rabbit immunoglobulin (Vector, Burlingam, CA, USA) was used. Then the sections were incubated with streptavidin-biotinylated horseradish peroxidase complex (Vector, Burlingam, CA, USA), diluted 1:100 in phosphate buffer and applied at room temperature for 90 min. The immunocomplex was visualized by diaminobenzidine (DAB) reaction, 0.05% 3-3'-DAB in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.01% H_2O_2 at room temperature for 5 to 10 min (until brownish color appeared). No structure-bound color product was found when the anti-GFAP antibody was omitted from the procedure. Nissl counterstaining was applied on some sections. For positive controls rat brain sections were applied.

Results

General observations

This study does not demonstrate complete mappings on the different species studied. The common and the different features of the GFAP-immunopositive astroglial architecture are emphasized in parallel in the species, therefore, the results are arranged according to the brain areas, not the species.

The immunostaining revealed that the main astroglial type is the radial ependymoglia. Non-radial long fibers penetrated

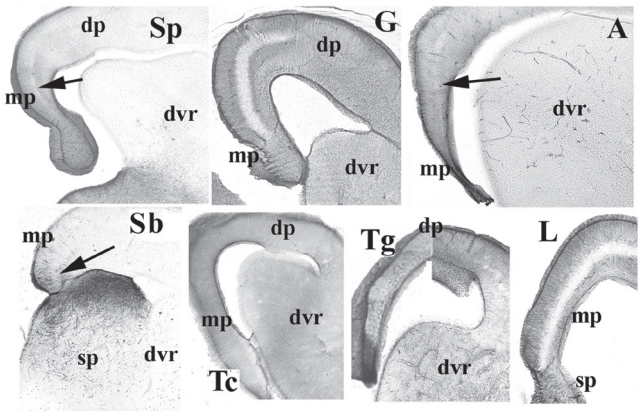

Figure 1. Cross-sections at the interventricular foramen. Three different distribution of GFAP are observed in the medial and dorsal pallium. In gecko and lacertid lizard these areas are full of GFAP-immunopositive radial, trans-pallial glial processes even a middle zone is conspicuously light. In snakes and agama this pattern is confined to the end of the medial pallium, the other areas are free of GFAP. In turtles these areas are evenly densely rich in GFAP.

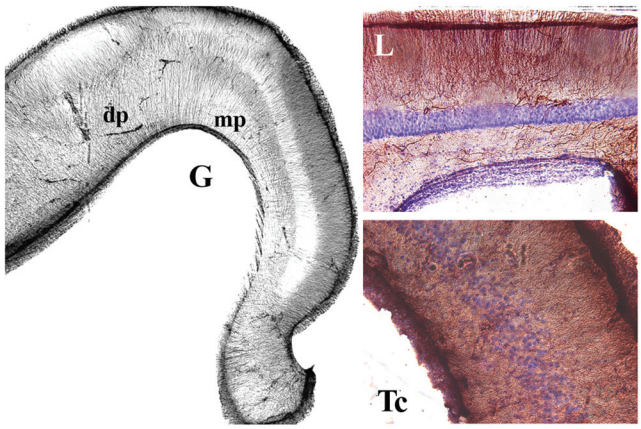

Figure 2. Enlarged parts of medial pallium. In gecko the radial arrangement is well visible. The glial processes traverse the GFAP-poor light zone but here the density of both their branching and staining is weaker. Counterstaining according to Nissl demonstrates (in lacertid lizard) that the 'light' area is occupied by neurons. Similar neuronal layer is found in turtles but does not alter the distribution of GFAP. In gecko there is another GFAP-free ellipsoid area in the dorsal pallium. Their septum is also poor in GFAP.

this system around the large vessels. In snakes and lizards astrocyte-like elements occurred in several areas, *e.g.*, in the pallium and the striatum, but nowhere predominated. GFAP-free areas were also found. Nothing similar was found in turtles. The Squamata may form three groups. In gecko and *Timon* GFAP immunopositivity was found throughout the brain, with some exceptions (see later). As an intermediate group GFAP-free areas were extended in snakes, in the dorsal pallium, in the septum, in the dorsal ventricular ridge and in the hypothalamus. The opposite end was represented by the chameleon and agama in which the GFAP was almost absent, only in a few areas GFAP immunopositive elements were found.

Telencephalon

Three different patterns of GFAP immunopositivity were observed in the medial and dorsal pallium: 1) In gecko and lacertid lizard (*Timon*) these areas were full of GFAP-immunopositive radial, trans-pallial glial processes but a middle zone was conspicuously light. 2) In snakes and agama this pattern was confined to the end of the medial pallium, the other areas were free of GFAP. 3) In turtles the pallium was evenly densely rich in GFAP. In gecko there was another GFAP-free ellipsoid area in the dorsal pallium (Fig. 1).

In higher magnification the radial arrangement of glial processes was well visible (Fig. 2). These processes traversed the GFAP-poor light zone but here the density of both their branching and staining was weaker. Counterstaining according to Nissl demonstrated that the 'light' area is occupied by neurons. Similar neuronal layer is found in turtles but does not alter the distribution of GFAP.

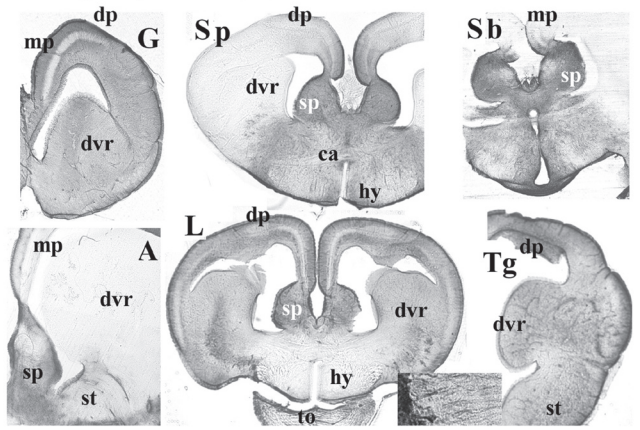

Figure 3. Cross-sections at the anterior commissure. Within a brain the adjacent areas may have strikingly different GFAP-staining in snakes and lizards. In agama, boa and python the DVR has hardly any GFAP immunopositivity, whereas the striatum is intensely stained (inset shows the radial glial pattern). Identical areas have different GFAP immunopositivity in different species: the staining patterns of DVR, striatum, and hypothalamus are just opposite in python and lacertid lizard. Similar differences can you see in the case of the septum (see also Fig. 4). The GFAP-immunopositivity of a part of brain may alter throughout the series of sections (see, *e.g.*, the agama septum, here and in Fig. 4).

The septum had an intense staining in most species (Fig. 3 and 4) but not in the agama and chameleon. In geckos the anterior septal nuclei formed a characteristic thickening. In it, mainly rostrally, the GFAP-immunopositivity was rather weak. The dorsal ventricular ridges (DVRs) also displayed

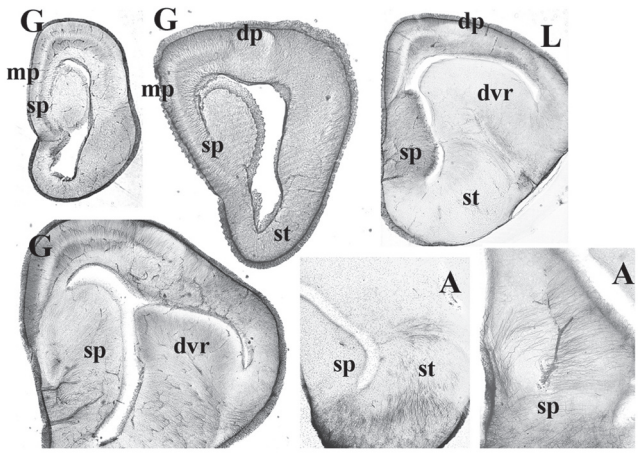

Figure 4. In front of the anterior commissure. The septum had an intense staining in most species (see also Fig. 3), but not in the agama. In geckos the anterior septal nuclei formed a characteristic thickening, in which, mainly rostrally, the GFAP immunopositivity was rather weak.

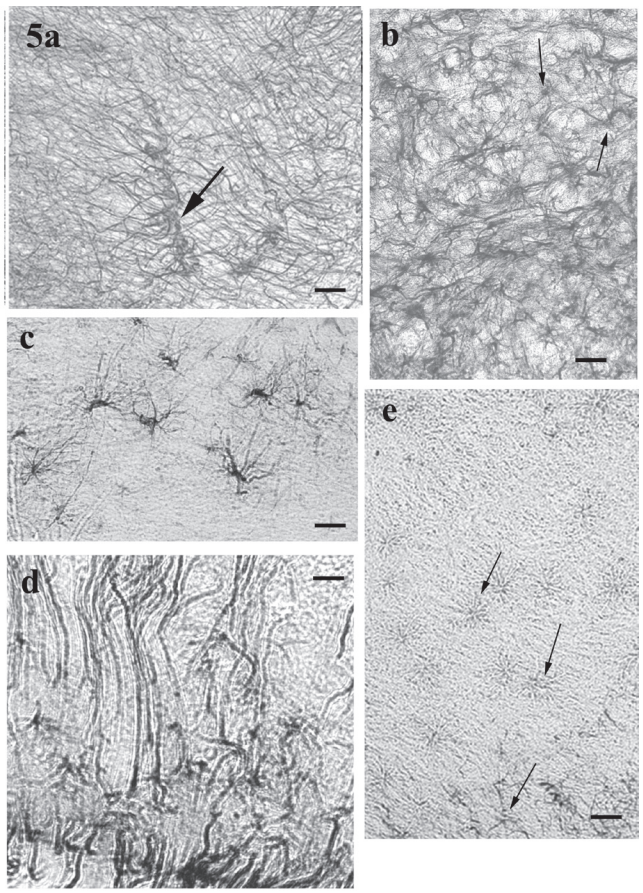

Figure 5. The fine pattern. a) The radial pattern was sometimes masked beyond recognition by other processes, mainly around the vessels (arrow) (colubrid snake, *Pantherophis*, DVR). Scale bar: 20 μm; b) Sometimes it was almost insolvable (arrows) whether astrocytes or superpositions of processes are in view (*Timon*). Scale bar: 10 μm; c) In some areas the presence of astrocytes is obvious (mesencephalon, chameleon). Scale bar: 10 μm; d) Long radial processes may also emerge from astrocytes, not only ependyma ('radial astrocytes', chameleon, mesencephalon). Scale bar: 10 μm; e) Faint contours (arrows) of unstained astrocytes (colubrid snake, *Pantherophis,* medulla)? Scale bar: 10 μm.

different density of GFAP-immunopositivity. Whereas lacertid lizard (*Timon*), gecko and turtles had densely stained DVR, in python, boa, agama and chameleon DVR was almost free of GFAP-immunopositivity (Fig. 3 and 4). We observed three main tendencies: 1) Within a brain the adjacent areas had strikingly different GFAP-staining in snakes and lizards. In agama, boa and python the DVR had hardly any GFAP immunopositivity, whereas the striatum was intensely stained. In turtles, however, both area had intense staining; 2) Identical areas had different GFAP immunopositivity in different species: the staining patterns of DVR, striatum, and hypothalamus are just opposite in python and lacertid lizard. Similar differences can you see in the case of the septum (Fig. 3 and 4); 3) The GFAP-immunopositivity altered throughout the series of sections, see *e.g.*, the agama septum (Fig. 3 and 4).

Sub-telencephalic brain parts

In lizards and snakes in the thalamus GFAP was rather confined to the borders of the nuclei, and the hypothalamus was rather negative whereas turtles had a rather even distribution except fot the prosencephalic fascicles.

In most of the species studied, the tectum was GFAP-immunopositive except for the Chamaeleon. The layered structure and, mainly medially, a radial glial pattern was recognizable, mainly in the gecko. Tegmentum was rather immunonegative in all the species examined.

In the medulla, the dorsoventral nuclei have usually an intense staining as vell as the median glial septum. The radial pattern is usually recognizable (agama, python and boa). The reticular formation has a reticular glial pattern rather extended in turtles and gecko but confined in the lacertid lizard *Timon,* agama, and snakes; except for colubrid ones (*Pantherophis*), where the pattern was not delineated by the immunostaining at all. The medial longitudinal fasciculus is recognizable, the white matter tracts are usually lighter than their environment. In the cerebellum Bergmann-fibers proved to be GFAP-immunopositive.

The fine pattern

In every species local modifications usually masked the basic system, the radial ependymoglia. This basic pattern, however, was sometimes masked beyond recognition by processes,

Table 2. The primary antibodies used in this study.

Against	Type	Supplier	Code	Dilution	Final concentration (µg/ml)
GFAP	Mouse*	Novocastra, Newcastle, UK	ga5	1:100	100
	Rabbit**	DAKO, Galstrup, Denmark	Z0334	1:100-500	6-28
	Mouse*	Biosciences, San Diego, CA, USA	GA5	1:100	5
	Rabbit**	Sigma, Saint Louis, MO, USA	G9269	1:100-500	***
	Rabbit**	Santa Cruz, San Diego, CA, USA	Sc-32956	1:100	2
Glutamine synthetase	Mouse*	Transduction Labs., Erembodegem, Belgium	610518	1:100	2.5
	Rabbit**	Novus Biologicals, Littleton, CO, USA	NB-110-41404	1:1000	***
S100	Rabbit**	Sigma, San Louis, MO, USA	s-2644	1:100	81
	Mouse*	Abcam, Cambridge, UK	B-32.1	1:100	***
Vimentin	Mouse*	Abcam, Cambridge, UK	Vim3B4	1:100	***
	Mouse*	Calbiochem, Darmstadt, Germany	IF01-100UG	1:1000	0.5
	Chicken**	Novus Biologicals, Littleton, CO, USA	NB300-223	1:1000	20

* monoclonal, ** polyclonal, *** original concentration is not given by the supplier

mainly around the vessels (Fig. 5a). In some areas it was almost insolvable whether astrocytes or superpositions of processes are in view (Fig. 5b). In some areas the presence of astrocytes was obvious (Fig. 5c). Long radial processes also emerged from astrocytes, not only ependyma ('radial astrocytes', Fig. 5d). In some GFAP-free areas faint contours suggested the presence of unstained astrocytes (Fig. 5e).

Effect of other markers

When in some areas GFAP immunopositivity was not detected with the Novocastra monoclonal mouse anti-GFAP antibody, other markers were also applied (Table 2). Though four other anti-GFAP reagents tested, only the other mouse-derived (Biosciences) reagent resulted in similar staining as the Novocastra product. Anti-glutamine synthetase and anti-S100 antibodies visualized only a small fragment of the glial processes, mainly radial ependymoglia in the tectum and around the dorsal and ventral sulci beside the DVR. These antibodies worked well in the controll sections cut from rat brains.

Antigenes did not reveal new localisations of GFAP; the immunopositivity was more conspicuous due to a weaker background staining. Those anti-vimentin antibodies which were raised against mammalian vimentin were ineffective. With anti-vimentin antibody raised against bird vimentin the preliminary results were encouraging.

Discussion

Comparison of present and former results

The main Squamata astroglial type is radial ependymoglia, with several local modifications. However, there are two principal differences from turtles. In different Squamata species considerable GFAP-poor or -free areas were found. Furthermore, true astrocytes were found in several areas, although, nowhere predominated (true astrocytes are stellate-shaped cells with equally-sized processes, independent from the ependyma).

True astrocytes have been demonstrated at least in some areas in lizards, Iguanidae (*Anolis carolinensis*, Dahl et al. 1985; *Anolis sagrei*, Lazzari and Franceschini 2005a; Lacertidae, *Gallotia galloti*, Monzon-Mayor et al. 1990a; Yanes et al. 1990; *Lacerta lepida*, recently *Timon lepidus*, Bodega et al. 1990; *Podarcis sicula*, Lazzari and Franceschini 2001), geckos (*Eublepharus macularius*, Lazzari and Franceschini 2005b; *Tarentola mauretanica*, Ahboucha et al. 2003). Only one data referred to snake (*Elaphe quadrivirgata*, recently *Pantherophis quadrivirgata*, Onteniente et al. 1983). No former study emphasized the occurrence of large GFAP-poor or GFAP-free areas.

In the turtle brains (including that of the Pleurodira *Pelomedusa*) the findings corresponded to that found formerly in *Trachemys* (formerly: *Pseudemys*) *scripta elegans* (Kálmán et al. 1994) and *Mauremys leprosa* (Kálmán et al. 1997): no GFAP-free areas and no astrocytes were found. Independent studies with GFAP-immunostaining have also not detected astrocytes (*Clemmys japonica*, Onteniente et al. 1983; *T. scripta elegans*, Dahl et al. 1985, Kriegstein et al. 1986; *Trionyx sinensis*: Lazzari and Franceschini 2006).

Comparison of Squamata to birds and mammals

Lizards and snakes (Squamata) share more common astroglial features with birds than the turtles and crocodilians (Kálmán and Pritz 2001). Some representatives (*e.g.*, agama, snakes) have so extended GFAP-free areas which match the avian ones. However, there are two meaningful differences in the homologuous areas: the molecular layer of cerebellum and the upper layers of tectum contain GFAP in lizards but not

in birds (Linser 1985; Roeling and Feirabend 1988; Kálmán et al. 1993). Those areas which are GFAP-free in mammals and birds are desribed usually as the advanced, expanded and plastic ones (Butler and Hodos 2005). Note that Squamata display quite complex behavioural phenomena related to other reptiles (Zug et al. 2001). Mammals and birds belong to different amniote clads: Synapsida and Diapsida, respectively. Their similar glial features most likely evolved independently, during parallel, separate evolutions (Kálmán 2002) because they have not found in turtle or caiman so far examined (Kálmán et al. 1994; Kálmán and Pritz 2001). On the other hand, Squamata represents a separate diapsid clad divergent from that the crocodiles and birds (Lepidosauria *versus* Archosauria). Whereas lizards of different taxonomic positions had rather different distribution of GFAP and glial architecture (Ahboucha et al. 2003), but no considerable differences were found in turtles, including the Pleurodira *Pelomedusa subrufa*.

Other glial markers

Vimentin immunopositivity was repeatedly documented in the brain of different lizards: Iguanidae (*Anolis sagrei*, Lazzari and Franceschini 2005a), Lacertidae (*Gallotia galloti*, Monzon-Mayor et al. 1990a; Yanes et al. 1990; *Podarcis sicula*, Lazzari and Franceschini 2001), and gecko (*Eublepharus macularius*, Lazzari and Franceschini 2005b). Our preliminary findings obtained on adult turtle brain (*T. scripta elegans*) by an antibody presented by P. Viklicky (Lukas et al. 1989) is supported by the observations of Lazzari and Franceschini (2006, *Trionyx sinensis*). The present results with anti-vimentin antibody raised against bird vimentin are promising. It is immportant to emphasize that usually different monoclonal anti-vimentin antibodies are applied in mammalian and bird brain (Bohn et al. 1992; Gereben et al. 1995). Further experimental evidence is required in this field.

In their experiments, Monzon-Mayor et al. (1990b, 1998) used anti-chicken glutamine syntethase to detect glial elements in *Gallotia galloti* (this was not available for our study). Scarce data were found with S100 (Romero-Aleman et al. 2003).

Importance of turtle phylogeny

Formerly turtles were classified as anapsids, therefore, the closest living relatives of the extinct stem-amniotes (Carroll 1988; Reiner 1990). This opinion has recently been challenged by several studies based on classic fossil data and DNA analyses which point to that turtles are diapsids, like the other extant reptiles and the birds, although their position within this group is uncertain (Zardoya and Meyer 1998; Shen et al. 2011; Carroll 2013). Critical analyzis of these contradictory opinions is beyond the scope of this study (for a detailed review, see Kálmán et al. 2013). In either case the turtle glial structure seems to be the simplest one among reptiles since no astrocytes intermingle with the ependymoglia, which have a rather even density. The turtles are the sole extant Amniota group without true astrocytes (*Sphenodon*, which is nearest to the stem of Lepidosauria, has not been investigated).

Surveying the divergent opinions there are two possibilities:

- The turtles emerged earlier than lepidosaurs and archosaurs separated from each other. In this case they may have preserved a glial system ancestral to that of the other extant reptiles.

- The turtles emerged following that lepidosaurs and archosaurs separated from each other. In this case absence of astrocytes in them may be a secondary phenomenon, not an ancestral characterisctics. We have to mention that according to some opinions the occurrance and frequency of astrocyte depends rather the brain structure than the phylogenetical position (Wicht et al. 1994).

Conclusion

The glial structure of Squamata seems to be most advanced among reptiles. So conspicuous differences within a taxonomic ordo have not found yet in other vertebrates. Further studies are requested, how the differencies of astroglia have formed in the different Squamata, and what is their functional (behavioural) importance.

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