Brain, Behavior and Evolution

Original Paper

Brain Behav Evol 2023;98:302–313
DOI: 10.1159/000535281

Received: February 25, 2022
Accepted: November 13, 2023
Published online: December 9, 2023

Entopallium Lost GFAP Immunoreactivity during Avian Evolution: Is GFAP a "Condition Sine Qua Non"?

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Keywords
Birds · Entopallium · Evolution · Interfamily differences · Lack of glial fibrillary acidic protein

Abstract
Introduction: The present study demonstrates that in the same brain area the astroglia can express GFAP (the main cytoskeletal protein of astroglia) in some species but not in the others of the same vertebrate class. It contrasts the former opinions that the distribution of GFAP found in a species is characteristic of the entire class. The present study investigated birds in different phylogenetic positions: duck (*Cairina moschata domestica*), chicken (*Gallus gallus domesticus*), and quails (*Coturnix japonica* and *Excalfactoria chinensis*) of Galloanserae; pigeon (*Columba livia domestica*) of a group of Neoaves, in comparison with representatives of other Neoaves lineages, which emerged more recently in evolution: finches (*Taeniopygia guttata and Erythrura gouldiae*), magpie (*Pica pica*), and parrots (*Melopsittacus undulatus* and *Nymphicus hollandicus*). ***Methods:*** Following a perfusion with 4% buffered paraformaldehyde, immunoperoxidase reactions were performed with two types of anti-GFAP: monoclonal and polyclonal, on floating sections. ***Results:*** The entopallium (formerly "ectostriatum," a telencephalic area in birds) was GFAP-immunopositive in pigeon and in the representatives of Galloanserae but not in songbirds and parrots, which emerged more recently in evolution. The lack of GFAP expression of a brain area, however, does not mean the lack of astroglia. Lesions induced GFAP expression in the territory of GFAP-immunonegative entopallia. It proved that the GFAP immunonegativity is not due to the lack of capability, but rather the suppression of GFAP production of the astrocytes in this territory. In the other areas investigated besides the entopallium (optic tectum and cerebellum), no considerable interspecific differences of GFAP immunopositivity were found. It proved that the immunonegativity of entopallium is due to neither the general lack of GFAP expression nor the incapability of our reagents to detect GFAP in these species. ***Conclusion:*** The data are congruent with our proposal that a lack of GFAP expression has evolved in different brain areas in vertebrate evolution, typically in lineages that emerged more recently. Comparative studies on GFAP-immunopositive and GFAP-immunonegative entopallia may promote understanding the role of GFAP in neural networks.

Introduction

Glial fibrillary acidic protein (GFAP) is the main intermediate filament protein and a histochemical marker of astroglia. GFAP is supposed to participate in several astrocyte functions: flexibility, motility, connection to the extracellular matrix, regulation of water and ion

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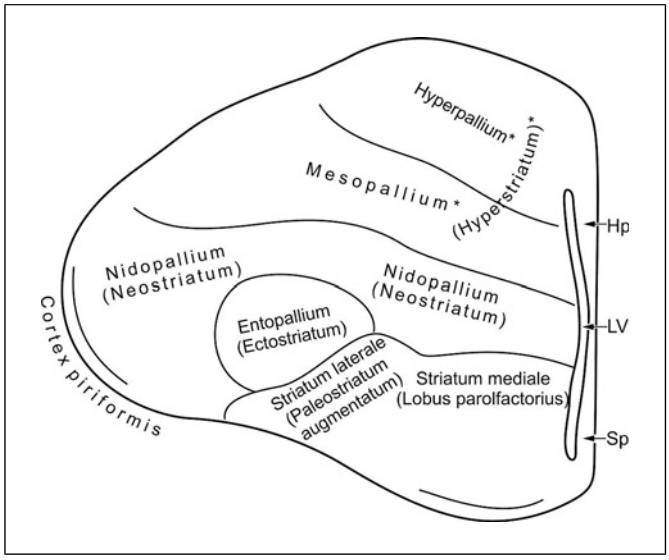


Fig. 1. Sketch of an anterior section of chicken telencephalon. The entopallium and the surrounding pallial areas are redrawn after Reiner et al. [26]. Several details not important for the study are not shown. The lines represent the separating medullary laminae. Names without parentheses: Reiner et al. [26]; names in parentheses: the former terminology; see, e.g., Kuenzel and Masson [36]. Hp, hippocampus; LV, lateral ventricle; Sp, septum. *The former "hypostriatum" comprised the areas called presently hyperpallium and mesopallium, and their subdivisions, which are irrelevant in this study, are not marked.

distribution, axon growth, and post-lesion glial reaction, but all of its functions have not yet been clarified (for reviews of the role of GFAP, see, e.g., [1–3]). Early studies [4–6] demonstrated that GFAP is present in the brains of different vertebrate classes (cartilaginous and ray-finned fishes, reptiles, birds, and mammals), and the antibodies raised against mammalian GFAPs react with the GFAPs of the other vertebrates.

The present study is a continuation of our studies on the distribution of GFAP in representatives of different vertebrate taxa. Both our and independent studies have described that there are brain areas, in which the astrocytes are poor in GFAP, or even lack it, e.g., the caudate-putamen and the tectum in rat (*Rattus norvegicus* albino [7–10]). In the domestic chicken (*Gallus gallus domesticus*), Linser [11] published GFAP immunonegativity in the tectum, and Roeling and Feirabend [12] in the molecular layer of cerebellum. Besides these regions, Kálmán et al. [13, 14] found extended GFAP-immunonegative areas in the telencephalon as well, the striatum, hyperpallium, mesopallium, and nidopallium (Fig. 1). Similar results were obtained in Japanese quail (*Coturnix japonica*) by Cameron-Curry et al. [15]. However, no areas were devoid of GFAP immunopositivity in turtles (*Pseudemys* – presently *Trachemys – scripta elegans* [16], *Mauremys leprosa* [17], *Trionyx sinensis* [18]) and crocodilians (*Caiman crocodilus* [19], *Paleosuchus cuvieri* [20]). In the absence of GFAP, astrocytes were detected with immunohistochemical reaction against glutamine synthetase or S100 protein [7, 11]. However, the GFAP expression is not lost, only suppressed and facultative [1, 21]; for details of the regulation of expression, see e.g., [22, 23]; it is inducible by proper stimuli, e.g., lesion (in rat striatum [24], in chicken cerebellum [25]).

However, early investigations were confined to single species representing a class. It was held that the GFAP distribution found in single species is characteristic of the entire class (e.g., in the case of birds: finch, *Uroloncha* – presently *Lonchura – striata* [5]; Japanese quail, *C. japonica* [15]; domestic chicken, *G. gallus domesticus* [13, 14]). Further studies in representatives of different avian orders are needed to understand whether the same distribution of the GFAP-immunopositive astroglia occurs in all birds.

In accordance with this, we performed an immunostaining against GFAP in zebra finch (*Taeniopygia guttata*), a representative of a group (Passeri suborder, songbirds) phylogenetically distant from Galliformes, the group containing quails and chicken. Surprisingly, we found the entopallium to be GFAP-immunonegative (unpublished observation). The entopallium (formerly "ectostriatum" [26]) is a telencephalic region in birds (Fig. 1), an important visual center, which is held to be analogous with the mammalian extrastriatal cortex, the terminal of the tectofugal visual pathway (for recent reviews on the entopallium, see [27–29]). This region was previously found to be intensely GFAP-immunopositive in Japanese quail (*C. japonica* [15]) and domestic chicken (*G. gallus domesticus* [13, 14]). On the other hand, on a finch species (*Uroloncha* – presently *Lonchura – striata*), Onteniente et al. [5] commented "GFAP immunopositivity... none in the central nuclei" of telencephalon.

After these data, the need arose for a study to understand whether in the entopallium GFAP occurs in all birds, or it is absent in some orders, e.g., songbirds. The present study compares birds (Table 1) representing groups in different phylogenetic positions (Fig. 2): (i) Galloanserae (Muscovy duck, *Cairina moschata domestica*, domestic chicken, *G. gallus domesticus*, and Japanese and king quails, *C. japonica* and *Excalfactoria chinensis*); and lineages of Neoaves, which emerged in evolution (ii) either rather early, i.e., Columbiformes (domestic pigeon, *Columba livia domestica*), (iii) or more recently, i.e., the Passeri suborder of Passeriformes (songbirds: zebra and Gouldian finches, *T. guttata* and *Erythrura gouldiae,* and

Table 1. The species investigated (every species was represented by 2 animals, except Gouldian finch and cockatiel, where further 2–2 animals were lesioned)

Order/suborder	Family/subfamily	Species	English name	Description
Galliformes	Phasianidae: Gallini	*Gallus gallus domesticus*	Domestic chicken	Linnaeus, 1758
	Phasianidae: Tetraogallini	*Excalfactoria chinensis*	King quail	Linnaeus, 1766
		Coturnix japonica	Japanese quail	Temminck and Schlegel, 1849
Anseriformes	Anatidae	*Cairina moschata domestica*	Muscovy duck	Fleming, 1922
Columbiformes	Columbidae	*Columba livia domestica*	Domestic pigeon	Gmelin, 1789
Passeriformes, Passeri	Estrildidae	*Taeniopygia guttata*	Zebra finch	Vieillot, 1817
		Erythrura gouldiae	Gouldian finch	Gould, 1844
	Corvidae	*Pica pica*	Eurasian magpie	Linnaeus, 1758
Psittaciformes	Psittacidae	*Melopsittacus undulatus*	Budgerigar	Shaw, 1805
	Cacatuidae	*Nymphicus hollandicus*	Cockatiel	Kerr, 1792

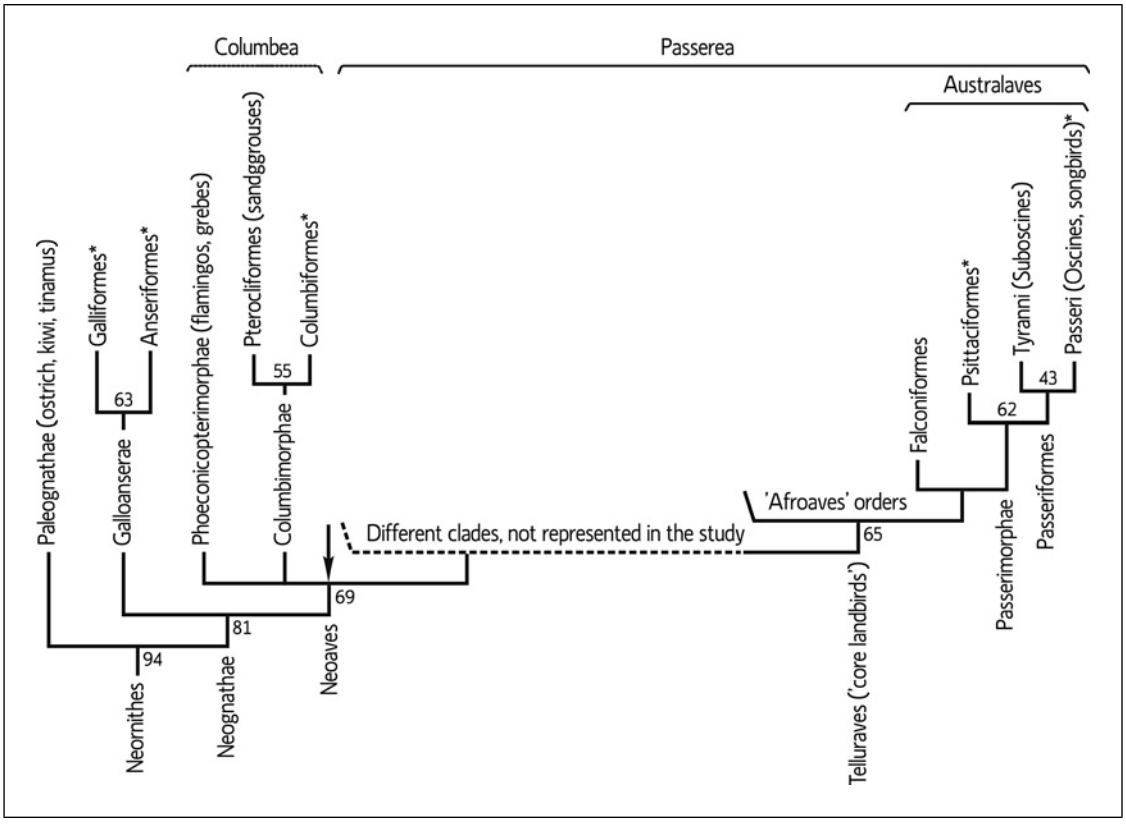


Fig. 2. A simplified phylogenetic tree of birds. Asterisks: these groups are represented in the study. Arrow: the bifurcation, which separates the clade containing Columbiformes from the majority of Neoaves. Numbers: the estimated ages of division in million years (after [34]). This tree is composed on the basis of the trees of [30–34], and the "consensus tree" of Braun et al. [35], which was formed following a critical analysis of the previous ones. The different trees agree in that (i) Galloanserae are sister group of Neoaves; (ii) Columbiformes belong to a clade, which separated before the diversion of the majority of Neoaves; (iii) Passeri is a suborder formed among the latest ones. The diversion of the Neoaves clades is rather different at the different authors and not relevant for our study; therefore, it is not detailed. The category "Columbea" is used only by [30], [33], and [35].

Table 2. The anti-GFAP immunoreagents used in the study

Firm	Code No.	Dilution	Final conc.	RRID
Novocastra*, Newcastle, UK	ga5	1:100	100 µg/mL	AB 563739
Dako**, Glostrup, Denmark	Z0334	1:200	28 µg/mL	AB 10013382

*Monoclonal, mouse, against the GA5 clone. **Polyclonal, rabbit.

Table 3. The specification of chemicals (for the primary anti-GFAP reagents, see Table 2)

Chemical	Firm
3,3-diaminobenzidine (DAB)	Amersham, UK
Avidin-biotinylated horseradish peroxidase complex (ABC)	Vector Labs, Burlingame, UK
Anti-mouse immunoglobulin (goat)	Vector Labs, Burlingame, UK
Anti-rabbit immunoglobulin (goat)	Vector Labs, Burlingame, UK
DePeX	Sigma-Aldrich, St. Luis, MO, USA
Hydrogen peroxide (H_2O_2)	Reanal, Budapest, Hungary
Ketamine (calypsol)	Richter Gedeon Corp., Budapest, Hungary
Normal horse serum	Vector Labs, Burlingame, UK
Paraformaldehyde	Merck, Darmstadt, Germany
PBS	Sigma-Aldrich, St. Luis, MO, USA
Tris-HCl buffer	Sigma-Aldrich, St. Luis, MO, USA
Xylazine (Rompun)	Produlab Pharma B.V., Raamsdonksveer, The Netherlands

magpie, *Pica pica*), and Psittaciformes (parrots: budgerigar, *Melopsittacus undulatus,* and cockatiel, *Nymphicus hollandicus*).

In recent years, several avian phylogenetic trees were composed, applying different genetic analyses [30–34]. Following a critical survey of these, Braun et al. [35] formed a "consensus tree"; all of these were taken into consideration in Figure 2. Despite their differences, the trees agree in that (i) Galloanserae is a sister group of Neoaves; (ii) Columbiformes belong to a clade, which separated before the diversion of the majority of Neoaves; (iii) Passeri is among the groups formed latest. Jarvis et al. [30], Houde et al. [33], and Braun et al. [35] divided Neoaves into two main clades: Columbea and Passerea. Kuhl et al. [34] distinguished "basal landbirds" containing Columbiformes and "higher landbirds" containing Psittaciformes and Passeriformes (besides other orders in both).

Two different anti-GFAP reagents (monoclonal and polyclonal ones) were applied (Table 2) as controls of each other. To check whether astrocytes are capable to produce GFAP of the immunonegative entopallia, we performed stab wounds to provoke astroglial reactions. Besides the entopallium, other areas (the cerebellum and the tectum) were also investigated, to check whether the immunonegativity could be attributed to a general lack of GFAP expression of these species or to the incapability of our reagents to detect their GFAP.

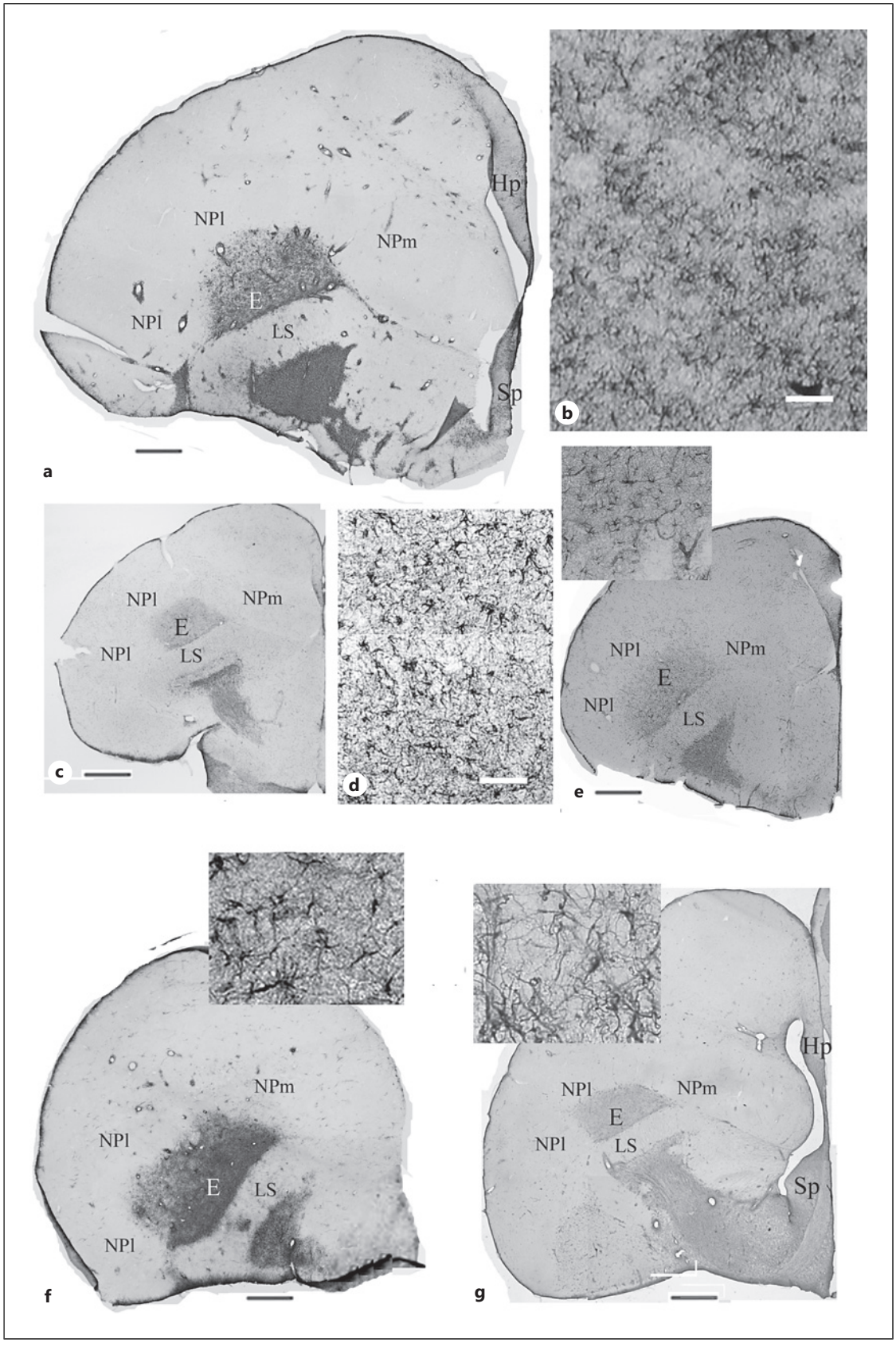
Materials and Methods

Animals

The species (Table 1) were elected to represent Galloanserae (Muscovy duck, *C. moschata domestica*, domestic chicken, *G. gallus domesticus*, and Japanese and king quails, *C. japonica* and *E. chinensis*) and Columbiformes (domestic pigeon, *C. livia domestica*), and lineages, which emerged more recently (Fig. 2): Passeriformes (songbirds: zebra and Gouldian finches, *T. guttata* and *E. gouldiae*, and magpie, *P. pica*), and Psittaciformes (parrots: budgerigar, *M. undulatus* and cockatiel, *N. hollandicus*). The animals were young adults (a little beyond the sexual maturation), in pairs, hatched at breeders, and obtained in pet shops or in the market. Housing lasted for 1–2 days before experiment, at seasonal (summer) natural light, 14–15 h/day. They were supplied ad libitum with tap water and food recommended in the shops: special food mixtures for the different birds from Versele-Laga (Deinze, Belgium); the diet of the magpie was completed with mealworms. Experiments were performed in accordance with the guidelines of European Union Directive (EU Directive 2010/63/EU) and the Committee on the Care and Use of Laboratory Animals at the Semmelweis University of Budapest, Hungary (22.1/3491/003/2008), approval number: KA-1928/2016.

Fixation and Sectioning

The animals were anesthetized with injections into the pectoral muscle of a combination of ketamine and xylazine (10 and 1 mg/kg body weight, respectively). The effect of anesthesia was checked with leg withdrawal and palpebral reflexes, and by the lack of holding head. The animals were intracardially cannuled, exsanguinated with a perfusion of physiologic saline (0.9% NaCl), and then transcardially perfused with 4% paraformaldehyde solution in phosphate-buffered saline (PBS, 0.1 M, pH 7.4; for the



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specification of chemicals, see Tables 2 and 3). Following perfusion, the brains were removed carefully and immersed in the same fixative for further 24 h. Then, they were embedded in agarose and cut into serial sections (thickness 50 μm) transversal to the brain axis using a Vibratome vibration microtome (Intracel, Shepreth, Royston, Herts, UK). The sections were washed in PBS overnight and then processed for immunohistochemical staining.

Immunohistochemistry

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

Two anti-GFAP reagents were applied separately, in parallel experiments, in every animal. Their specifications, dilutions, and final concentrations are given in Table 2. They were applied for 40 h at 4°C, and then the immunohistochemical reaction was further developed according to the "ABC" method. Biotinylated anti-mouse or anti-rabbit immunoglobulin (according to the origin of the anti-GFAP), and then avidin-biotinylated horseradish peroxidase (ABC) complex (for specifications see Table 3) were applied subsequently, each in a dilution of 1:100, for 1.5 h, at room temperature. The immunocomplex was visualized by the diaminobenzidine reaction: i.e., by incubation with 0.05% 3,3-diaminobenzidine and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.4) at room temperature, under visual control until the appearance of brownish color. The sections were mounted, dried in air, covered with DePeX, and coverslipped. Control sections were incubated by substituting the primary antibody with normal serum. No structure-bound labeling was observed in them.

Photomicrographs

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

Operation

Operation was only applied on two further Gouldian finches (*E. gouldiae*) and two cockatiels (*N. hollandicus*). Anesthesia was similar to that mentioned above. Lesions were performed by a syringe needle (diameter 0.5 mm) through a narrow burr hole. Since finch or parrot atlas was not available, the lesions were oriented according to the chicken atlas of Kuenzel and Masson [36] adapting its coordinates to the sizes of the finch and cockatiel brains. Until arousal, the animals were covered against cooling. The postoperative periods were 7 days on the basis of our former lesion experiments in chicken (*G. gallus domesticus*) [25]. The animals were perfused and processed according to the above-described protocols.

Results

GFAP Immunopositivity in Entopallia of Different Species

In chicken (*G. gallus domesticus*; Fig. 3a,b), Japanese and king quails (*C. japonica* and *E. chinensis*; Fig. 3c,d, and 3e), pigeon (*C. livia domestica*; Fig. 3f), and duck (*C. moschata domestica*; Fig. 3g), the astrocytes of the entopallia were highly immunopositive to GFAP. The surrounding areas of telencephalon, the nidopallium (formerly neostriatum), and the lateral striatum (formerly paleostriatum augmentatum) were very poor in GFAP-immunopositive elements. In magpie (*P. pica*; Fig. 4a–c), parrots: budgerigar (*Melopsittacus undulates*; Fig. 4d) and cockatiel (*N. hollandicus*; Fig. 4e), and zebra and Gouldian finches (*T. guttata* and *E. gouldiae*; Fig. 4f–h), entopallia were devoid of GFAP immunopositivity applying either Novocastra or Dako anti-GFAP, except for perivascular glia, which were GFAP-immunopositive here and throughout the telencephalon in every species studied. Dense population of GFAP-immunopositive astrocytes was found in the globus pallidus (Fig. 4a, c).

The Effect of Lesion

Stab wounds induced the appearance of GFAP immunopositivity of astrocytes in the territory of GFAP-immunonegative entopallia (Fig. 4h, i). There was no considerable difference between the results obtained with Novocastra or Dako reagents; the photomicrographs were taken on sections treated with Novocastra.

GFAP Immunopositivity in Other Brain Parts

In the areas investigated besides the entopallium, in the tectum and the cerebellum (Fig. 5, 6) the distribution of GFAP immunopositivity was similar in every species; e.g.,

Fig. 3. Brains with GFAP-immunopositive entopallium. **a** Domestic chicken (*Gallus gallus domesticus*), distribution of GFAP-immunopositive elements (astrocytes). In the entopallium, there is a dense population of GFAP-immunopositive astrocytes. Scale bar: 1 mm. **b** Astrocytes of chicken entopallium. Scale bar: 25 μm. **c** Japanese quail (*Coturnix japonica*). Scale bar: 1 mm. **d** Astrocytes in an enlarged detail of the entopallium of Japanese quail. Scale bar: 25 μm. **e** King quail (*Excalfactoria chinensis*). Inset: astrocytes of entopallium enlarged. Scale bar: 1 mm, for the inset: 25 μm. **f** Domestic pigeon (*Columba livia domestica*). Inset: astrocytes in a detail of entopallium enlarged. Scale bar: 1 mm, for the inset: 10 μm. **g** Muscovy duck (*Cairina moschata domestica*). Inset: astrocytes of entopallium enlarged. Scale bar: 1 mm, for the inset: 20 μm. E, entopallium; Hp, hippocampus, LS, lateral striatum (formerly paleostriatum augmentatum); NPl and NPm, nidopallium (formerly neostriatum) lateral and medial; Sp, septum.

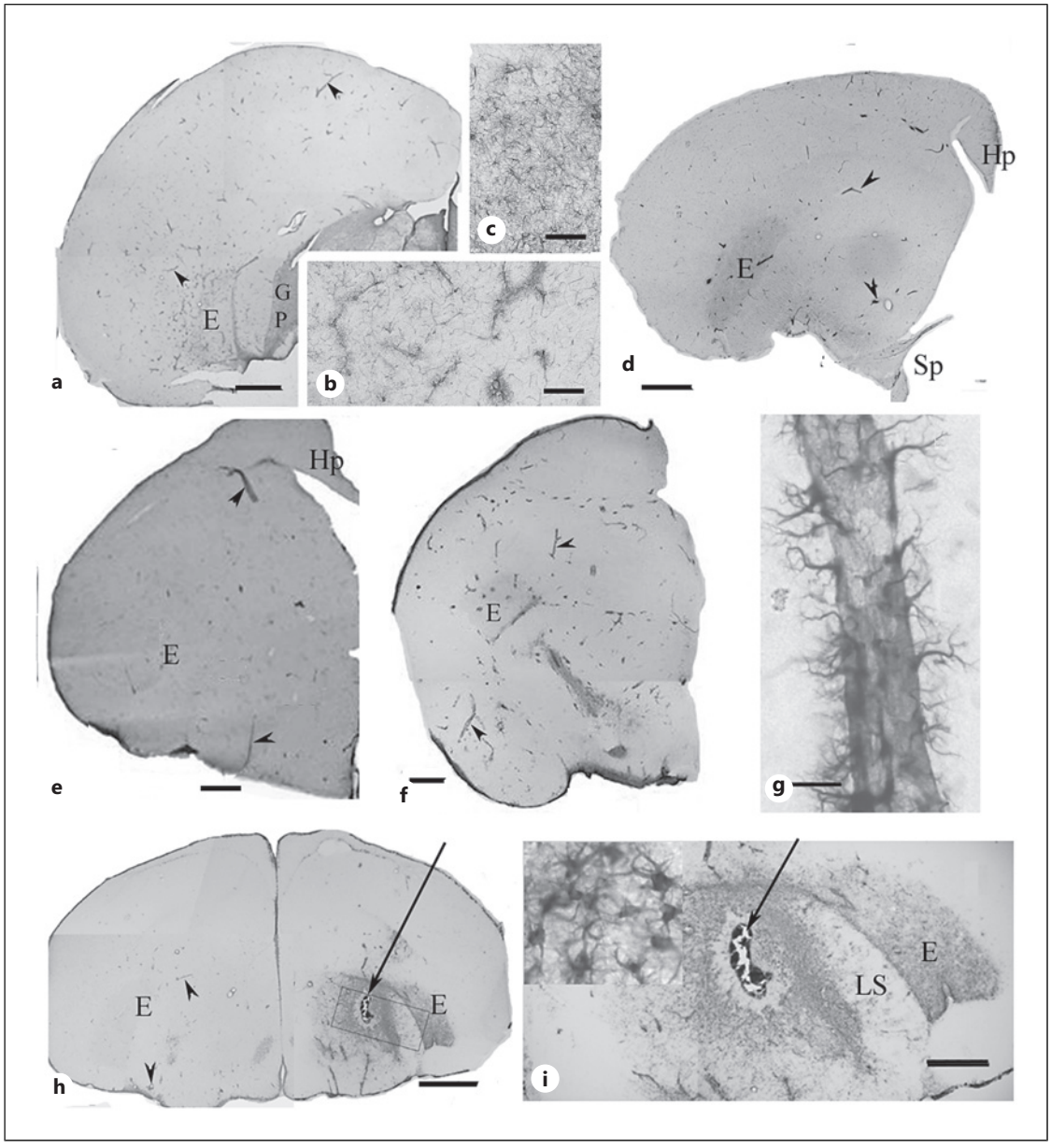

Fig. 4. Brains with GFAP-immunonegative entopallium. Arrowheads: vessels with perivascular glia. **a** Magpie (*Pica pica*). Entopallium is only delineated faintly (see panel **b**), whereas the GP (formerly: paleostriatum primitivum [26]) is intensely GFAP-immunopositive (see also panel **c**). Scale bar: 1.2 mm. **b** In the entopallium shown in panel **a**, the GFAP immunopositivity is confined to perivascular glia. Scale bar: 60 µm. **c** Astrocytes in the globus pallidus shown in panel **a**. Scale bar: 60 µm. **d** Budgerigar (*Melopsittacus undulates*). The territory of entopallium is delineated by weak non-specific staining. Scale bar: 1 mm. **e** Cockatiel (*Nymphicus hollandicus*). Scale bar: 1 mm. **f** Zebra finch (*Taeniopygia guttata*). Scale bar: 1 mm. **g** A vessel enlarged from the telencephalon of zebra finch. Note the perivascular glia. Scale bar: 5 µm. **h** Gouldian finch (*Erythrura gouldiae*). In the left hemisphere, only faint contour marks the position of entopallium (E). GFAP immunopositivity is only represented by perivascular glia (arrowheads). In the right hemisphere, a post-lesion reactive gliosis is visible. Note the dense population of GFAP-immunopositive astrocytes in the vicinity of the wound (arrow) and corresponding to the entopallium. The framed part is enlarged in panel **i**. Scale bar: 1.4 mm. **i** The framed part of panel **h** enlarged. Inset: High magnification of an area beside the lesion (arrow). Note: following lesion GFAP appeared in the entopallium. Scale bar: 250 µm, for the inset: 5 µm. E, entopallium; Hp, hippocampus; Sp, septum; GP, globus pallidus.

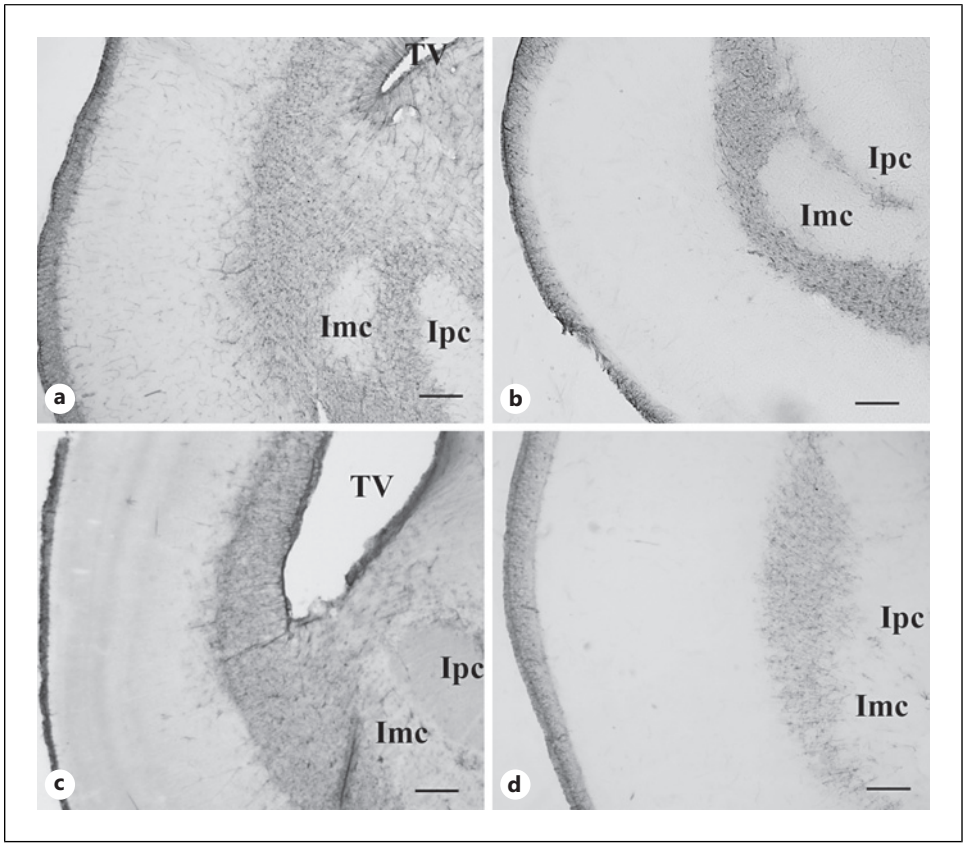

Fig. 5. The characteristic distribution of GFAP immunopositivity is similar in the tecta of different species. The GFAP immunopositivity is confined to the deeper layers, approximately the stratum album central and stratum griseum centrale, as estimated on the basis of [13]. **a** Japanese quail (*Coturnix japonica*). **b** King quail (*Excalfactoria chinensis*). **c** Cockatiel (*Nymphicus hollandicus*). **d** Budgerigar (*Melopsittacus undulatus*). Scale bars: 200 μm. Ipc and Imc, nuclei isthmi partes parvo- and magnocellularis; TV, tectal ventricle.

in the tectum it was confined to a deeper zone, and in the cerebellum to the granular layer. The Bergmann glia were immunonegative or only stained very faintly.

Discussion

Lack of GFAP but Not Lack of Astroglia

The summary of our results is the entopallium was not GFAP-immunopositive in the representatives of some avian lineages that emerged more recently in evolution; under stimulative effect (i.e., lesion), however, GFAP immunopositivity appeared in them. The lack of GFAP expression of a brain area does not mean the lack of astroglia. Astrocytes express GFAP at different levels [1, 21, 37]; some brain areas proved to be devoid of GFAP consistently across studies; e.g., in rats (*R. norvegicus* albino), the II–IV layers of neocortex, the tectum, and the caudate-putamen complex [7–10, 38], and in birds most parts of the pallium (e.g., nidopallium, mesopallium, hyperpallium), the striatum, the molecular layer of cerebellum, and the superficial layers of tectum (domestic chicken, *G. gallus domesticus* [11–14]; quail, *C. japonica* [15]). In these areas, the astrocytes were visualized using other markers (S100: [38] rat cortex, [39] quail pallium; glutamine synthetase: [7, 11]; vimentin: [12]). Especially in the description of the entopallium of zebra finch (*T. guttata*), astrocytes were also mentioned (Nissl-staining [40]; aromatase immunohistochemistry [41, 42]).

The results of our lesion studies (Fig. 4h) also proved the presence of astrocytes in the entopallium, and that the lack of GFAP immunopositivity is not to be attributed to the lack of their capability of GFAP production. The GFAP production seems to be suppressed of some functional reasons, which needs further investigations. Since both males and females were used, the differences of GFAP expression cannot be attributed to the sexual differences as described in mammals (see, e.g., [43, 44]).

In the areas investigated besides the entopallium (the tectum and the cerebellum), we found no conspicuous difference between the distributions of GFAP immunopositivity of the different species. The distribution of GFAP and the areas of its high and low expression

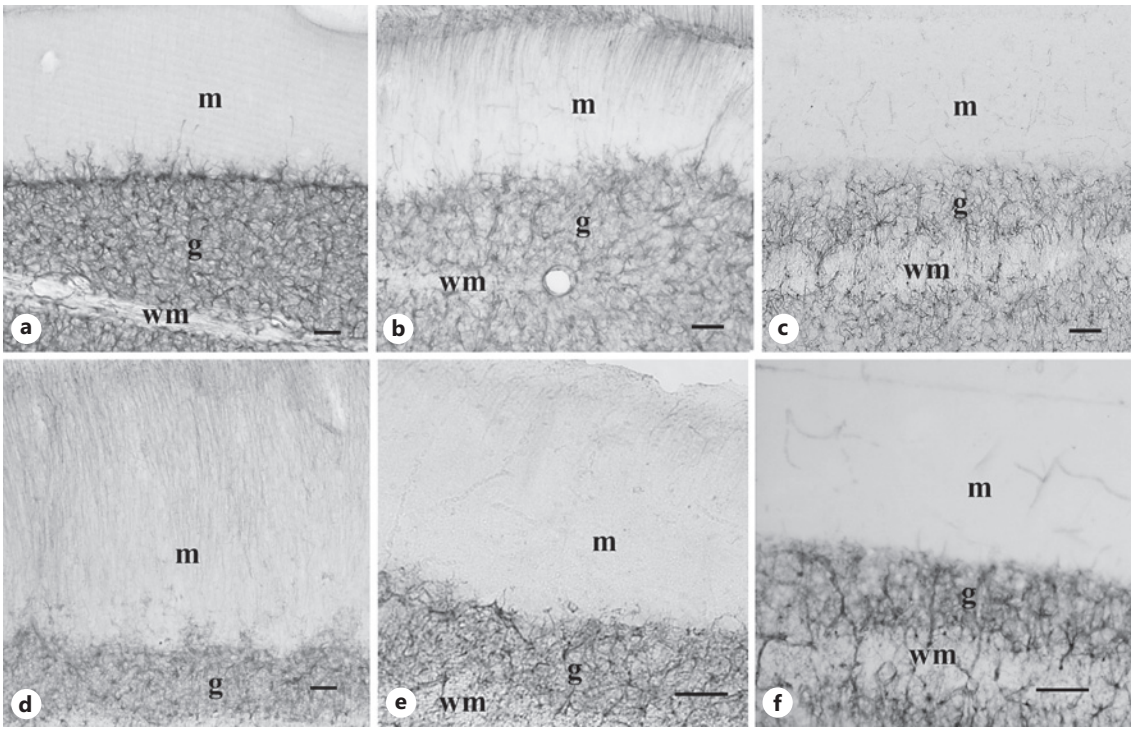

Fig. 6. The characteristic distribution of GFAP immunopositivity is similar in the cerebella of different species. The Bergmann glia are GFAP-immunonegative or stained faintly (see panels **b** and **d**). **a** Chicken (*Gallus gallus domesticus*). **b** Japanese quail (*Coturnix japonica*). **c** King quail (*Excalfactoria chinensis*). **d** Magpie (*Pica pica*). **e** Budgerigar (*Melopsittacus undulatus*). **f** Zebra finch (*Taeniopygia guttata*). Scale bars: 50 μm. m and g, molecular and granular layers; wm, white matter.

(Fig. 3a–d, 5a, 6a, b) were congruent with that found previously in quail (*C. japonica* [15]) and chicken (*G. gallus domesticus* [11–14]); see above. It indicates that the GFAP immunonegativity in the entopallium cannot be attributed either to a general lack of GFAP expression or to an incapability of our reagents to detect GFAP in these species.

Possible Functional Correlations of the Presence and Absence of GFAP

In several functions of astrocytes, the role of GFAP has been revealed. Other functions happen without GFAP. As examples, Walz [45] distinguished two electrophysiologically different astrocyte types: one ("passive") is rich in GFAP, and it has strong K+ accumulation; the other ("complex") is poor in GFAP, but it has intense Na-K currents. GFAP influences the swelling of astrocytes, which arise from water influx, usually due to osmotic or toxic effects [46–48]. Glutamate/glutamine transformation appears to correlate inversely with the GFAP content [7, 49, 50]. The composition of extracellular matrix produced by astrocytes also seems to correlate with the expression of GFAP [51]. The absence of GFAP in the astrocytes increases vulnerability of the white matter to mechanical effects [52]. The functional significance of GFAP, however, has not been clarified from every aspect [1–3]. It remained unclear, what is the functional significance of GFAP in astrocytes for the surrounding neural network.

The lack of GFAP of some brain areas [53] suggests that the presence of GFAP in astrocytes is not a condition "sine qua non" of neural functions. The entopallium operates in the presence of GFAP in some species of birds and also does in the absence of GFAP in other species. A comparative functional study on different species with GFAP-immunopositive or GFAP-immunonegative entopallia may promote the understanding of the role of GFAP in neural networks.

GFAP-Free Areas: A Common Evolutionary Phenomenon in Different Vertebrate Lineages

Mammalian: albino rat (*R. norvegicus* [8–10]) and avian: quail (*C. japonica* [15]) and chicken (*G. gallus domesticus* [13, 14]) brains contain extended areas almost free of GFAP immunopositivity. However, similar areas were not found in the brains of turtles (*Pseudemys* – presently *Trachemys – scripta elegans* [16]; *M. leprosa* [17]; *T. sinensis* [18]) and crocodilians (*C. crocodilus* [19], *P. cuvieri* [20]). Avian homologues of some GFAP-rich turtle or crocodilian brain areas were frequently almost free of GFAP (e.g., the molecular layer of cerebellum, the superficial layers of tectum, striatum, most of the dorsal ventricular ridge [53, 54]). It indicates that a lack of GFAP expression has evolved in different brain regions in vertebrate evolution, typically in lineages that emerged more recently in evolution. and the areas free of GFAP immunopositivity may be regarded as advanced, apomorphic features. The present observations are in accordance with this proposal: the entopallium was found to be GFAP-immunonegative in the representatives (Fig. 2) of avian lineages that emerged more recently – the Passeri suborder of Passeriformes and Psittaciformes, but not in the representatives of the other groups studied, Galliformes, Anseriformes, and Columbiformes.

The expression of GFAP seems to correlate with the extracellular matrix components produced by astrocytes [51]. Via these components, astroglia may influence axonal growth. The decrease of GFAP expression may promote both the neurite outgrowth [55–59] and the synaptic plasticity [57, 60, 61]. Free of GFAP, the process systems of astrocytes are more versatile and adaptable [48]. These correlations support the suggestion that the lack of GFAP of some brain areas may be an evolutionary advantage. Notably, the capability of GFAP expression is not lost, it just becomes facultative, as the lesion studies prove it.

It is noteworthy that the lack of GFAP of the entopallium is only a further addition to the several apomorphic features of the brains of Passeri (songbirds, including corvids) and Psittaciformes. They are usually considered as the most intelligent birds, and some of them match apes regarding body-related brain size [62–66], density of neurons [67, 68], and task-solving capability [62, 66, 67, 69].

In conclusion, the absence of GFAP immunopositivity in the entopallia of parrots and songbirds is congruent with our former observations that a lack of GFAP expression has evolved in different brain regions in vertebrate evolution, typically in lineages that emerged more recently. It supports our former proposal [53] that it is a common phenomenon, a "trend" in the brain evolution of different vertebrate lineages. Furthermore, comparative functional studies of areas, which contain GFAP in some species but not in others, e.g., entopallium, may help understand the functional role of GFAP.

Acknowledgments

Technical assistance of A. Őz and S. Deák, graphic work of E. Nemcsics, and photographic work of E. Oszwald, and consultation on the text corrections with Prof. A. Csillag are highly appreciated.

Statement of Ethics

This article does not contain any studies with human participants. All procedures in the study involving animals were performed in accordance with the European Union Directive (EU Directive 2010/63/EU) and the Committee on the Care and Use of Laboratory Animals at the Semmelweis University of Budapest, Hungary (22.1/3491/003/2008), approval number: KA-1928/2016.

Conflict of Interest Statement

The authors declare that there is no any actual or potential conflict of interest, including any financial, personal, or other relationships with other people or organizations.

Funding Sources

The work was supported by the Excellence Program for Higher Education of Hungary (FIKP-2018).

Author Contributions

Olivér Marcell Sebők: operation and histochemistry; Mihály Kálmán: experimental design and manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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