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Glial Fibrillary Acidic Protein and Vimentin Immunoreactivity of Astroglial Cells in the Central Nervous System of the African Lungfish, *Protopterus annectens* (Dipnoi: Lepidosirenidae)

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ABSTRACT The distribution of glial intermediate filament molecular markers, glial fibrillary acidic protein (GFAP), and vimentin, in the brain and spinal cord of the African lungfish, *Protopterus annectens*, was examined by light microscopy immunoperoxidase cytochemistry. Glial fibrillary acidic protein immunoreactivity is clear and is evident in a radial glial system. It consists of fibers of different lengths and thicknesses that are arranged in a regular radial pattern throughout the central nervous system (CNS). They emerge from generally immunopositive radial ependymoglia (tanycytes), lining the ventricular surface, and are directed from the ventricular wall to the meningeal surface. These fibers give rise to endfeet that are apposed to the subpial surface and to blood vessel walls forming the *glia limitans externa* and the perivascular glial layer, respectively. GFAP-immunopositive star-shaped astrocytes were not found in *P. annectens* CNS. In the gray matter of the spinal cord, cell bodies of immunopositive radial glia are displaced from the ependymal layer. Vimentin-immunopositive structures are represented by thin fibers mostly localized in the peripheral zones of the brain and the spinal cord. While a few stained fibers appear in the gray matter, the ependymal layer shows no antivimentin immunostaining. In *P. annectens* the immunocytochemical response of the astroglial intermediate filaments is typical of a mature astroglia cell lineage, since they primarily express GFAP immunoreactivity. This immunocytochemical study shows that the glial pattern of the African lungfish resembles that found in tetrapods such as urodeles and reptiles. The glial pattern of lungfishes is comparable to that of urodeles and reptiles but is not as complex as that of teleosts, birds, and mammals. J. Morphol. 262:741–749, 2004.

KEY WORDS: GFAP; vimentin; immunoreactivity; astroglial cells; CNS; lungfish; *Protopterus*

Radial glia include pear- or spindle-shaped cells with bodies located in the ependymal or periependymal layer constituting ependymal or periependymal radial glia, respectively. They are characterized by long cytoplasmic processes radially oriented to the meningeal surface and the vascular wall, where they terminate as endfeet forming the *membrana gliae limitans externa* and the perivascular glial layer, respectively (Elmquist et al., 1994; Lazzari et al., 1997; Lazzari and Franceschini, 2001). Radial glia not only represent the most phylogenetically primitive form of glia (Onteniente et al., 1983; Miller and Liuzzi, 1986), but also an ontogenetically immature type of glia since they are the first to appear during ontogeny of vertebrates that show a complex glial organization (Levitt and Rakic, 1980; Monzon-Mayor et al., 1990). Moreover, in mammals radial glia become progressively reduced as development proceeds and therefore are virtually absent in adults (Pixley and De Vellis, 1984; Elmquist et al., 1994). In the other vertebrates radial glia are retained in the adult (Ebner and Colonnier, 1975; Lazzari et al., 1997; Lazzari and Franceschini, 2001).

Glial fibrillary acidic protein (GFAP) is the main component of gliofilaments that belong to the intermediate filament class and it is present in mature cells of the astroglial lineage. It is widely considered a reliable molecular marker for this cellular type (Dahl and Bignami, 1985). This protein is expressed in gliofilaments of all astrocyte types: star-shaped fibrous and protoplasmic astrocytes, Bergmann glia, periependymal radial glia, and ependymal radial glia or tanycytes (see Wasowicz et al., 1994; Wicht et al., 1994; Naujoks-Manteuffel and Meyer, 1996, for reviews).

Glial fibrillary acidic protein shows considerable stability in its molecular and antigenic characteristics across vertebrate phylogeny, as indicated by the observation that in each vertebrate group GFAP shows cross-reactivity to antimammalian GFAP antibodies (Onteniente et al., 1983; Dahl et al., 1985;

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Bodega et al., 1994). Nevertheless, the presence of GFAP in the different astroglial cell types, but also the relative proportion of these astrocytic subtypes and their regional distribution in the central nervous system (CNS) of different vertebrates, have phylogenetic and ontogenetic implications (Elmquist et al., 1994; Monzon-Mayor et al., 1998; Lazzari and Franceschini, 2001).

Vimentin is the component of another intermediate filament type. Even though it 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 found in adult glial cells of teleosts and amphibians (Zamora and Mutin, 1988; Cardone and Roots, 1990; Rubio et al., 1992; Lazzari et al., 1997). The cross-reaction of antibodies produced against mammalian vimentin with the corresponding protein in birds and amphibians (Bennett et al., 1978; Szaro and Gainer, 1988; Zamora and Mutin, 1988; Bodega et al., 1994) suggests that vimentin is highly conserved during phylogeny. Thus, comparative studies on glial cells in the CNS of species belonging to different vertebrate classes might increase knowledge of phylogenetic and ontogenetic history of the different cell types in the glial cell lineage.

The Dipnoi or lungfishes form a monophyletic group of osteichthyan fishes (Schultze and Campbell, 1986) and along with their sister group, the coelacanths (Crossopterygii), are the only extant lobe-finned fish (Moy-Thomas and Miles, 1971). On the basis of morphology, lungfishes appear to be the closest living relatives to the terrestrial vertebrates (Forey, 1986). Moreover in the last decade molecular research, particularly DNA sequencing, supports this hypothesis (Meyer and Wilson, 1990; Meyer and Dolven, 1992; Hedges et al., 1993; Zardoya and Meyer, 1996, 1997).

The aim of the present work is to study the presence and distribution of the two molecular markers specific for glial intermediate filaments, GFAP and vimentin, in the CNS of the African lungfish, *Protopterus annectens*, using immunoperoxidase cytochemistry, and consequently to describe its glial cytoarchitecture.

MATERIALS AND METHODS

Six adult specimens of the African lungfish *Protopterus annectens* (Owen, 1941) were obtained from Euraquarium (Bologna, Italy). The fishes were deeply anesthetized by immersion in 0.1% ethyl-m-aminobenzoate (Sigma, St. Louis, MO). The brains and spinal cords were then rapidly dissected out and fixed by immersion in 3% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C for 4 h. After washing overnight in 0.1 M PB, pH 7.4, at 4°C, specimens were dehydrated in a graded series of ethanol and embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO, melting point 55–57°C). Transverse sections, 10 μm in thickness, were mounted on poly-L-lysine (Sigma) coated slides and dried. In the subsequent processing, all incubation and washing solutions, except those containing primary antibodies, were used at room temperature. Sections were deparaffinized with xylene, hydrated, pretreated with 1% H_2O_2 in 0.05 M PB with 0.15 M NaCl (PBS), pH 7.4, to quench endogenous peroxidase activity and finally preincubated in PBS containing 10% normal goat serum (NGS; Vector, Burlingame, CA), 1% bovine serum albumin (BSA; Sigma) and 0.1% Tween 20 (Merck, Darmstadt, Germany) for 30 min to reduce nonspecific background staining. After H_2O_2 treatment, sections for vimentin immunostaining were pretreated for 15 min in PBS containing 0.1% pronase as the antigen unmasking step. The sections were incubated in a moist chamber on a floating plate overnight at 4°C in either a rabbit polyclonal anticow GFAP antiserum (1:500; Dakopatts, Glostrup, Denmark) or a mouse monoclonal antibovine vimentin antibody (1:5; Cymbus Biotechnology, Chandlers Ford, UK). Antibodies were diluted in PBS containing 3% NGS, 1% BSA, 0.1% Tween 20, and 0.02% sodium azide. After rinsing (three times, 8 min each) in PBS with 0.1% Tween 20, the sections were incubated in the following secondary antibodies for 2 h: HRP-conjugated goat antirabbit IgG (1:200, Vector) for GFAP and HRP-conjugated goat antimouse IgG (1:100, Vector) for vimentin. After rinsing in 0.1% Tween 20 in PBS, the sections were rinsed for 10 min in 0.1 M PB, pH 7.4, and treated with the diaminobenzidine (DAB) method modified by Adams (1981). The sections were then dehydrated in ethanol, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Negative controls were obtained by omission of the primary antibodies, replaced by 3% NGS.

RESULTS

GFAP-Like Immunoreactivity

GFAP-immunopositive structures were located in both the gray and the white matter throughout the brain and the spinal cord. They were represented by thin long fibers that arose from cell bodies located at the ventricular surface and ran to the meningeal layer. These cells were definitely tanycytic in nature (Fig. 1A). The apical poles of these cells delimited the cerebral ventricles. Each gave rise to a thick process that ran radially through the gray matter. In the white matter, these processes ramified into fine radially oriented fibers which, in the inner part of the neural wall, were often scarcely visible at low magnification (Fig. 1B). These fibers became thicker as they approached the submeningeal neural surface, where they formed variously extended endfeet that constituted the *membrana gliae limitans externa* of the CNS (Fig. 1B). These ependymal cells showed regional specialization with different immunocytochemical staining intensities of their cell bodies and cytoplasmic processes. Their ovoid-shaped cell bodies showed immunoreaction product in the

Fig. 1. GFAP immunostaining of *Protopterus annectens* brain. **A:** Tanycytes in the hypothalamus extending from the ependymal layer (arrowheads) to the meningeal surface. **B:** Thick GFAP-positive fibers form the *glia limitans externa* layer at the diencephalic meningeal surface. **C:** Cross section of GFAP-positive vessel in the mesencephalon. Immunostained fibers reach the vascular wall. **D:** Longitudinal section of a telencephalic vessel with a GFAP-positive wall. **E:** In the telencephalon GFAP-positive radial elements (arrowheads) appear in the gray matter, particularly at the level of the sulci of the lateral ventricles. **F:** Distribution of GFAP-immunopositive structures in the diencephalon. Scale bars = 50 μm (**A,B**), 20 μm (**C,D**), 250 μm (**E,F**).

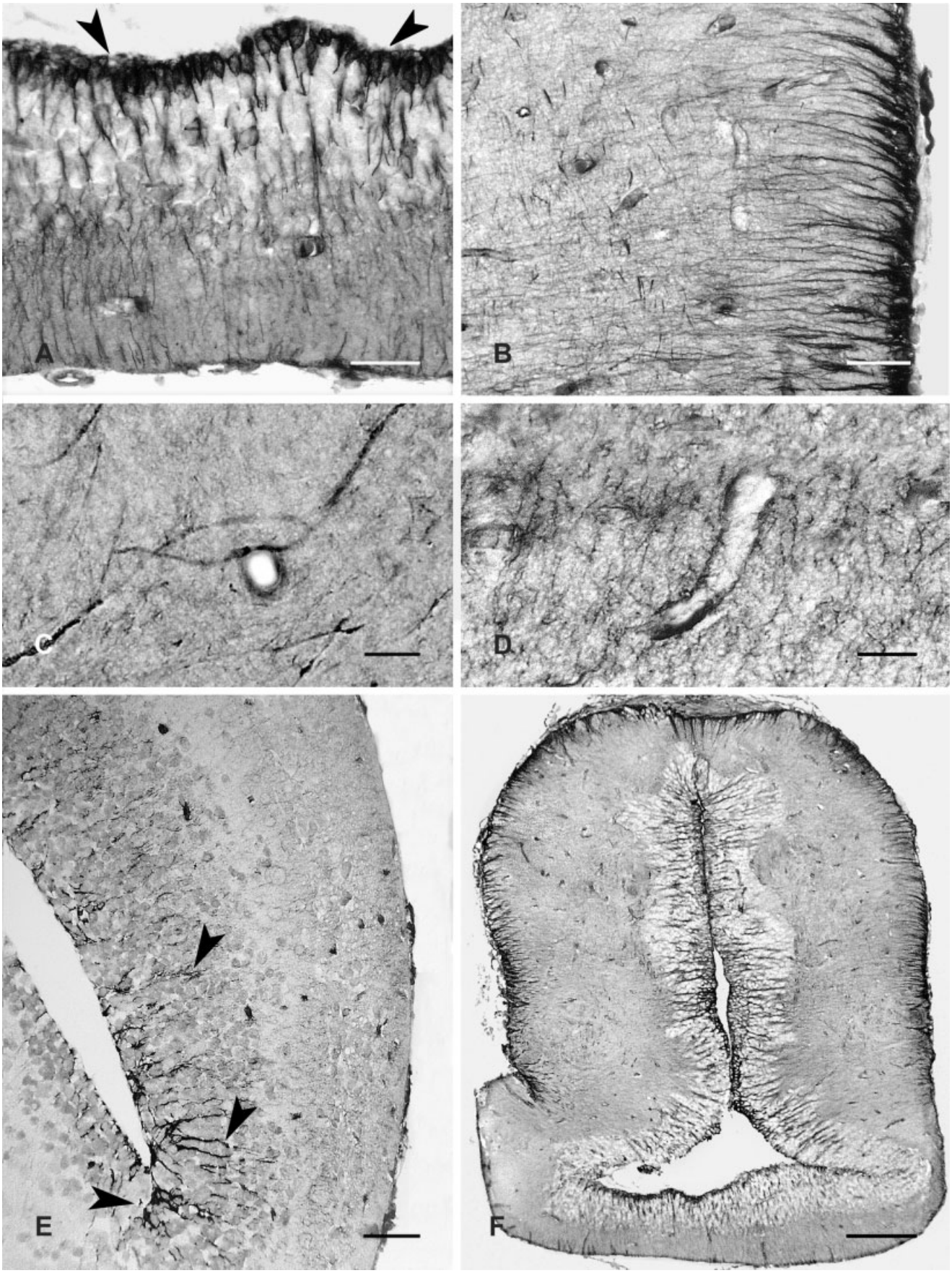

Figure 1

thin perinuclear cytoplasmic region or in the apical pole (Fig. 1A). Therefore, the ependymal reaction did not have the same intensity in all brain regions and it sometimes appeared reduced in the telencephalon, in the posterior part of the mesencephalic tectum, and in the body of the cerebellum. Fine immunopositive fibers converged on blood vessel walls and formed endfeet apposed to the outer surfaces of the vessels, thus forming the perivascular glial coating (Fig. 1C,D).

In the telencephalon, the ependymal cell bodies and their corresponding radial processes showed intense staining in the sulci of the lateral ventricles (Fig. 1E), whereas in the zones between sulci, the radial ependymoglia had smaller cell bodies with thin processes lightly stained and not easily distinguishable.

In the diencephalon and mesencephalon, three zones could be distinguished: a rather regularly radially oriented deep zone, corresponding to the gray matter, a superficial (submeningeal) zone and an irregular middle zone with a fine texture (Figs. 1F, 2A). In the mesencephalon, the ependyma was intensely GFAP-positive anteriorly, while the posteroventral part displayed reduced labeling (Fig. 2B). In the diencephalon, the narrow third ventricle was clearly outlined by an intense GFAP-immunopositive ependymal layer that gave rise to radial processes directed toward the outer surface.

In the medulla oblongata, the raphe was clearly immunostained. The ependymal cells and the *glia limitans externa* appeared moderately immunopositive (Fig. 2C).

The body of the cerebellum showed a lightly stained ependymal layer and a clearly immunopositive *glia limitans externa*, especially at the sulcus between the mesencephalic tectum and the cerebellum (Fig. 2C).

The transversely sectioned spinal cord clearly appeared GFAP-immunopositive with thin radially oriented immunopositive fibers evident throughout the sections (Fig. 2D). Radial astrocytes were detected in the central part of the spinal cord gray matter, with cell bodies displaced from the ependymal layer into a periependymal position giving rise to radial processes directed toward the outer surface of the spinal cord (Fig. 2E,F). The ependymal cells of the spinal cord exhibited clear GFAP immunopositivity.

Star-shaped astrocytes were not detected in the CNS of *Protopterus annectens*.

No specific immunoreaction was seen in CNS sections after exposure to incubation medium in which the primary anti-GFAP antibody was replaced by NGS.

Vimentin-Like Immunoreactivity

In the brain of *Protopterus annectens*, a distinct antivimentin immunopositivity was detectable only in the peripheral zone. Thick positive fibers reached the submeningeal layer but did not constitute a positive *glia limitans externa* (Fig. 3A). In the middle part of the white matter, a few very thin, radially oriented fibers showed a faint immunoreaction (Fig. 3B). In the gray matter, some weakly positive fibers were detected among the cell bodies and sometimes were gathered into fascicles (Fig. 3C). In the brain of *P. annectens*, ependymal cells were vimentin-immunonegative. In the spinal cord, the reaction was weak but visible in the white matter of the ventral region, where radially oriented positive fibers emerged from the ventral horns (Fig. 3D). These faint immunopositive radial fibers did not constitute a vimentin-positive *glia limitans externa* as they do in the brain. The ependymal layer and the surrounding gray matter of the spinal cord did not show appreciable vimentin-immunopositivity.

No staining was detected in the control sections.

DISCUSSION

This study has demonstrated the presence in *Protopterus annectens* of only one type of astroglial lineage throughout the brain: ependymal radial glia or tanycytes. Radial glia proper or radial astrocytes with their cell bodies displaced from the ependymal layer were observed only in the spinal cord. This immunocytochemical study also revealed that the staining intensity is not identical for the same cell type. Therefore, as in tetrapods (Monzon-Mayor et al., 1990, 1998; Lazzari and Franceschini, 2001), the present results indicate a certain degree of heterogeneity regarding both the morphological and immunological characteristics of astroglial lineage cells in *P. annectens*.

Studies in mammals have shown that the glial cytoarchitectural pattern changes during development. In fact, while radial glia are present prenatally, they disappear postnatally (Voight, 1989; Elmquist et al., 1994). Conversely, in other tetrapods such as birds (Kalman et al., 1993), reptiles (Monzon-Mayor et al., 1990; Yanes et al., 1990; Lazzari and Franceschini, 2001), and urodels (Zamora and Mutin, 1988; Lazzari

Fig. 2. GFAP immunostaining of *Protopterus annectens* brain and spinal cord. **A:** Distribution of GFAP immunostaining in the wall of the anterior part of the mesencephalon. **B:** In the posterior part of the mesencephalon the GFAP immunostaining is reduced at the ependymal level and in the gray matter. **C:** GFAP-immunopositive elements in the medulla oblongata and cerebellum. Radial fibers are evident in the raphe (arrows) and at the sulcus between mesencephalon and cerebellum (arrowheads). c, cerebellum; m, mesencephalon; mo, medulla oblongata. **D:** In a cross section of spinal cord the GFAP immunostaining clearly appears in both gray and white matter. **E:** Radial astrocyte cell bodies show intense GFAP immunopositivity in the spinal cord gray matter. **F:** Radial fibers form the *membrana gliae limitans externa* in the spinal cord. Scale bars = 200 μm (**A–C**), 250 μm (**D**), 100 μm (**E**), 50 μm (**F**).

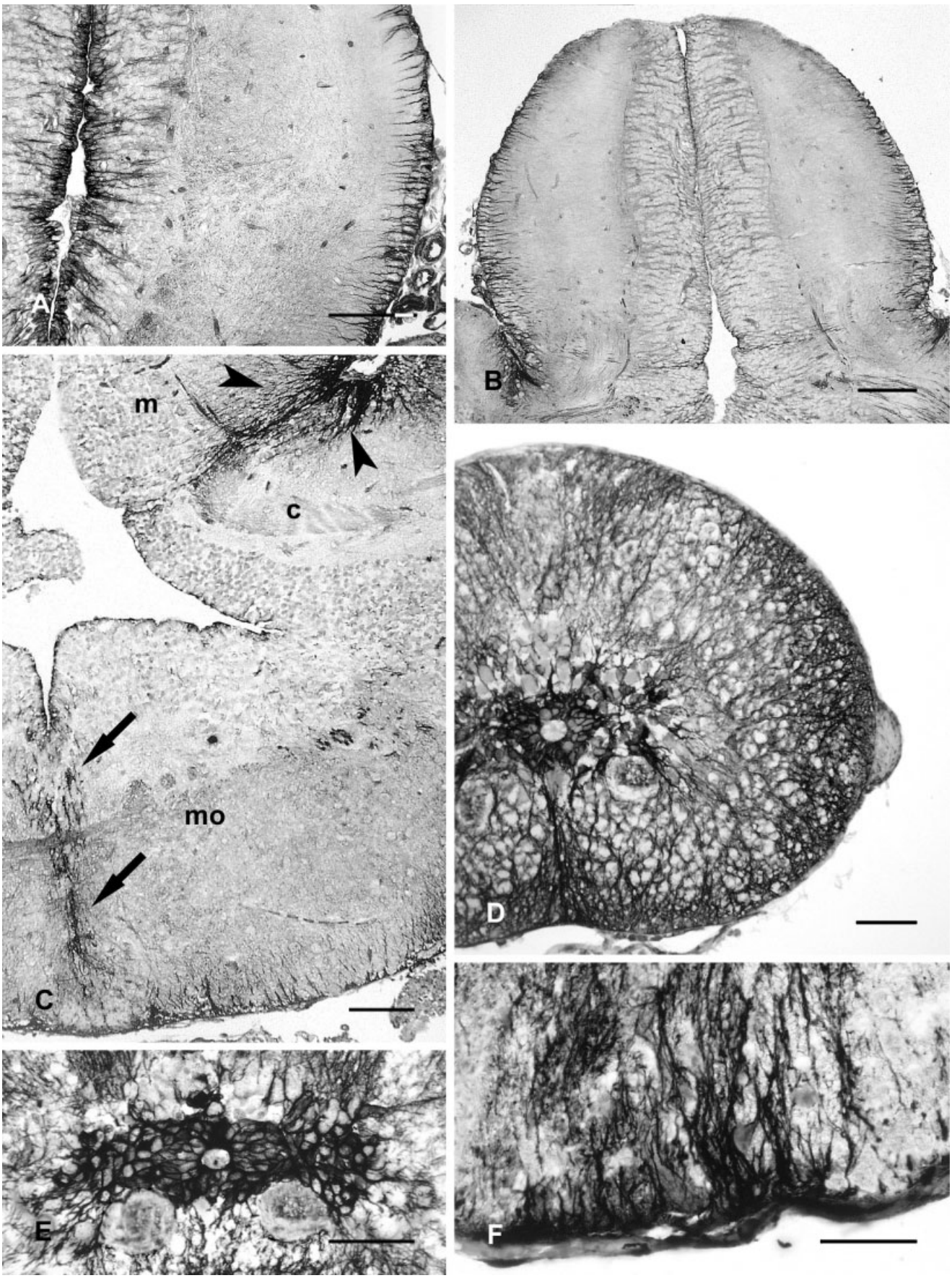

Figure 2

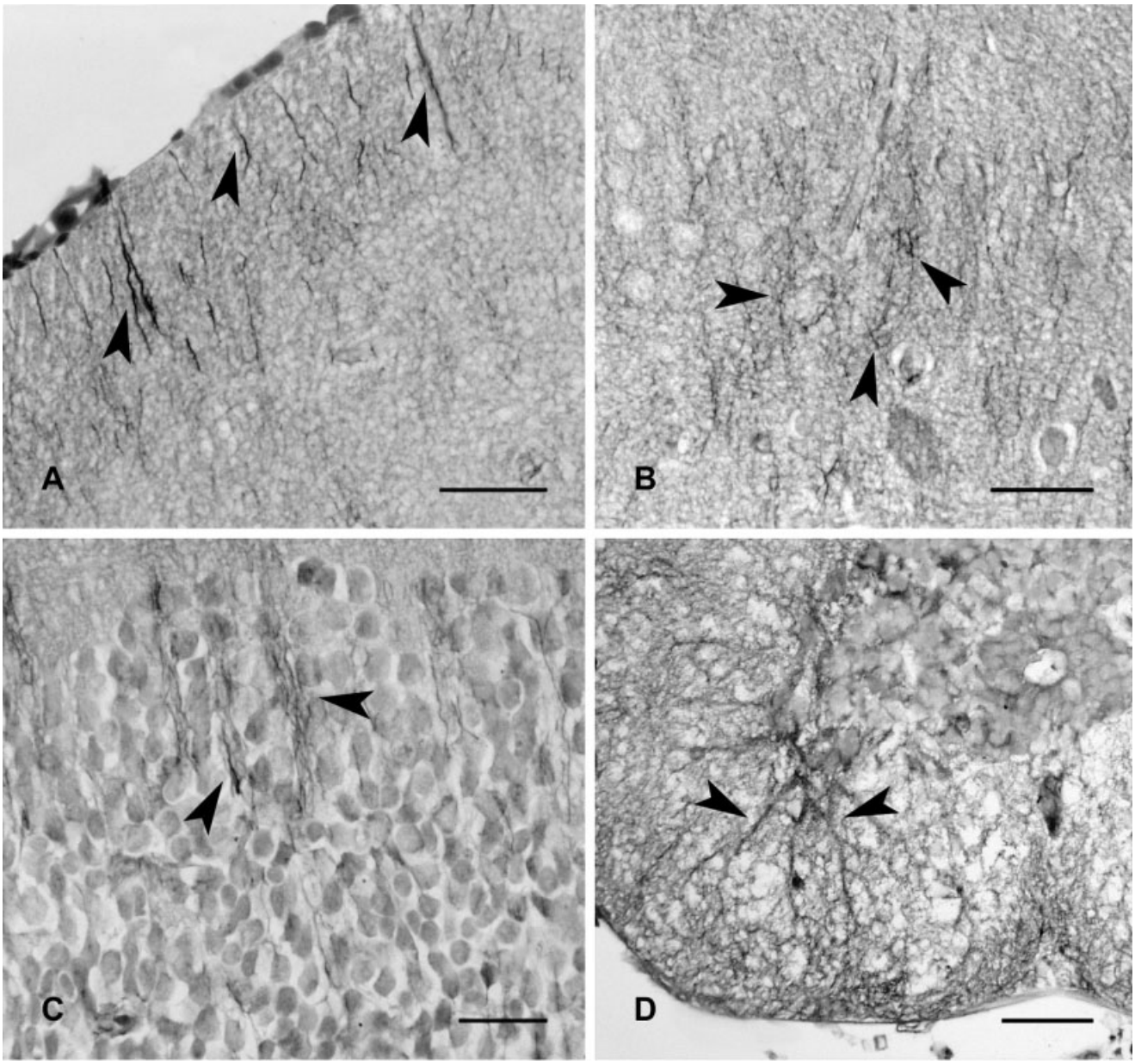

Fig. 3. Vimentin immunostaining of *Protopterus annectens* CNS. **A:** Some vimentin-positive radial fibers (arrowheads) reach the meningeal surface in the mesencephalic optic tectum. **B:** Some fibers (arrowheads) show slight vimentin immunopositivity in the central part of the diencephalic white matter. **C:** Slightly immunopositive radial fibers (arrowheads) in the mesencephalic gray matter. **D:** Immunopositive fibers (arrowheads) in the ventral white matter of the spinal cord. Scale bars = 50 μm (**A–C**), 100 μm (**D**).

et al., 1997), radial glial elements are still present in adults, even if the molecular composition of the intermediate filaments changes during development. In fact, during CNS formation a 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 and Marani, 1991; Elmquist et al., 1994). However, vimentin-immunoreactive glial structures do not completely disappear in adult tetrapods. Vimentin is still expressed in some glial elements in different regions of the adult CNS in urodeles (Zamora and Mutin, 1988; Lauro et al., 1991; Lazzari et al., 1997), reptiles (Monzon-Mayor et al., 1990, Lauro et al., 1991; Lazzari and Franceschini, 2001), birds (Alvarez-Buylla et al., 1987; Kalman et al., 1998), mammals (Chouaf et al., 1989; Oudega and Marani, 1991; Yamada et al., 1992), and also in teleosts (Cardone and Roots, 1990; Rubio et al., 1992; Bodega et al., 1993). In adult *Protopterus annectens*, CNS antivimentin immunostaining is scarce, with the exception of the thin glial fibers of the submeningeal zone.

According to Lauro et al. (1991), the presence of a mesenchymal molecular marker in cells of neuroec-

todermic origin could indicate a degree of histogenetic indetermination. If vimentin expression is related to the mesenchymal character of cells, the expectation would be that groups with greater regenerative ability would show a more intense vimentin expression, but since the more intense vimentin immunoreactivity was found in mammals, questions arise regarding the biological significance of the vimentin-positive structures present in the adult CNS. The answer is still unknown; therefore, further studies are required concerning vimentin expression in the adult CNS.

Another problem concerns the presence of true astrocytes (i.e., stellate-shaped cells) in fishes. These cells, which are absent in the CNS of *Protopterus annectens*, are present in small numbers in teleosts, as evidenced by immunocytochemistry, classical impregnation methods, and electron microscopy (see Kalman, 1998). Wicht et al. (1994) hypothesized parallel evolution of star-shaped astrocytes and radial glia in the early vertebrates, assuming that in evolution the thickness of the brain wall would determine the ratio of these astrocyte types.

The brains of teleosts are anatomically complex, with parts that either have no homologs in tetrapods or show a different morphology. In teleosts, the basic organization is represented by radial glia, but this pattern is modified in the more caudal areas of the brain where the radial organization is less regular. This pattern is modified by brain tracts and by lamination in the optic tectum and cerebellum (Kalman, 1998). This tendency is not seen in the CNS of *Protopterus annectens*.

From the present study, the glial cytoarchitecture in the CNS of *Protopterus annectens* appears similar to the condition found in urodeles (Lazzari et al., 1997), but less elaborate than that of reptiles (Lazzari and Franceschini, 2001), and certainly more simple than in teleosts, represented by *Cyprinus carpio* (Kalman, 1998). It must be remembered that in studies on the glial cytoarchitecture the immunological GFAP-staining methods are unable to display the finest branches, which are evident in Golgi-stained material (Kalman, 1998). This technical limitation is in agreement with the study of Connor and Berkowitz (1985), who demonstrated, by electron microscopic immunocytochemistry, that the GFAP contained in the finest branches of the glial cell arborization is insufficient to be detected by light microscopic immunocytochemistry. The African lungfish astroglia showed a satisfactory cross-reaction with antibodies raised against mammalian GFAP, but there has been no study with antibodies raised against dipnoan GFAP that could permit evaluation of the results obtained with commercial antimammalian GFAP regarding the intensity and details of the reaction. This information is available, however, for teleost fishes (see Kalman, 1998) where antibodies raised against mammalian GFAP show a good cross-reaction.

Moreover, as reported by Kalman (1998), the most complex vertebrate brains (avian and mammalian) show a phyletic trend in which the development of glial structure includes not only the prevalence of star-shaped astrocytes, but also the uneven distribution of GFAP-immunopositivity. In fact, in the brains of reptiles, in which radial glia is prevalent, both the glial element density and the GFAP-immunostaining intensity are rather homogeneous (Monzon-Mayor et al., 1990; Yanes et al., 1990; Kalman et al., 1994; Lazzari and Franceschini, 2001). Marked differences in GFAP-staining of adjacent areas are shown in both avian and mammalian brains (Ludwin et al., 1976; Hajos and Kalman, 1989; Kalman and Hajos, 1989; Zilles et al., 1991; Kalman et al., 1993) in which star-shaped astrocytes express GFAP at different levels (Linser, 1985; Patel et al., 1985). Alternating GFAP-rich and GFAP-poor zones, similar to those in avian and mammalian brains, were found in *Cyprinus carpio* by Kalman (1998), but this irregular distribution of GFAP-immunostaining in this species is ascribed to an uneven distribution of astroglia in teleosts, rather than to differences in GFAP-expression as in birds and mammals.

Kalman (1998) concluded that in teleosts the glial cytoarchitecture is not less variable and specialized than that found in some tetrapods (amphibians and reptiles) and that this condition can be ascribed to the fact that teleosts are the bony fishes more advanced in evolution. In teleosts, the different glial structures could have developed by the modification of the basic radial glia system rather than by the acquisition of star-shaped astrocytes, and this may be related to the development of the complex ventricular system of teleost brains.

The lungfish, *Protopterus annectens*, shows a glial cytoarchitecture in which the basic radial glial system is clear and does not show secondary modifications comparable to those found in teleost brain, which has a complex morphology (i.e., several parts either have no homologs in tetrapods or have a very different structure). Like the brains of urodels and reptiles, the glial system of *P. annectens* is formed mainly by radial glia and the density of glial elements and GFAP-staining intensity are rather uniform (Lazzari et al., 1997; Monzon-Mayor et al., 1990; Yanes et al., 1990; Kalman et al., 1994). This lungfish does not show striking differences between the GFAP-immunopositivity of adjacent brain areas, as is found in carps (Kalman, 1998). Only slight differences are seen in *Protopterus* GFAP-immunopositivity and, according to Kalman (1998), may be related to an uneven distribution and/or different size of astroglial elements rather than to differences in GFAP expression, as in mammals and birds.

The CNS of lungfishes displays a glial cytoarchitecture less variable and specialized than that of

teleosts; this condition instead resembles that seen in amphibians and reptiles.

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