

## Apparent scarcity of glial fibrillary acidic protein expression in the brain of the pygmy shrew *Sorex minutus* as revealed by immunocytochemistry

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### Abstract

We examined astroglial cells in the brain of the pygmy shrew *Sorex minutus* (Insectivora). For that purpose we labeled glial fibrillary acidic protein (GFAP) immunohistochemically in brain sections with a polyclonal antibody. Antigen retrieval experiments were performed to counteract formaldehyde fixation masking of GFAP epitopes. Our results showed remarkable paucity of GFAP-immunoreactive cells and fibers in the cerebral cortex and nuclei, as well as in the majority of the diencephalic and mesencephalic structures. In the forebrain, significant numbers of GFAP-containing astrocytes were found only in the ependyma and subventricular zones, superficial part of layer I of the cerebral cortex, and the majority of white matter structures. In the diencephalon, habenular nuclei were rich in GFAP-immunopositive astrocytes and labeled radial fibers were extended between median eminence and the third ventricle. A considerably higher density of labeled astrocytes was detected in the caudal brainstem and cerebellum. In contrast, in the mouse brain, immunoreactive astrocytes were present in large quantities in various structures. Staining of sections of the shrew brain against glutamine synthetase revealed abundance of immunofluorescent astrocytes in many areas, especially in the shrew cerebral cortex. It seems probable that in the shrew brain only a limited fraction of astroglia expresses GFAP, while other astroglial cells can be detected with different markers. It is possible that the rodent type of astroglial GFAP expression might not be common to insectivores and probably to some other mammalian orders.

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Glial fibrillary acidic protein (GFAP) is one of the markers of astroglia in the nervous system. Its presence has been shown in many vertebrate species and its distribution characterized in brains of a number of them [3,6–8,11]. Recent studies of the astroglial distribution in vertebrate brains revealed considerable variability of the GFAP expression pattern even among the same class [3,7]. On the other hand, in evolutionarily advanced brains, like mammalian brains, there is also variability in the GFAP staining pattern among various brain structures, where the physiological expression of GFAP is down-regulated in some areas, but found at high levels

in others [7]. However, there is not much data concerning comparative aspects of astroglial expression of GFAP among different species. This is also true for mammals, and to the best of our knowledge there are no reports on GFAP expression in insectivores. One family of the order Insectivora comprising shrews (Soricidae) are mammals that have relatively small body size. Some of their species are among the smallest existing mammals [2]. Reduction of their body size resulted in parallel reduction of brain volume. Besides, shrews from the genus *Sorex* are seasonally changing their brain volume as a wintering adaptation [12]. All that could have had profound consequences for shrews' brain structure and functions. For that purpose, and because of the lack of knowledge about the insectivore glia, we wanted to know

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how is the GFAP-positive astroglia organized in the brains of soricid shrews. For our investigations we chose one of the smallest species, the pygmy shrew (*Sorex minutus*).

Seven adult (sexually mature) pygmy shrews and three mice (strain C57BL/6Jx129Ola) were intraperitoneally anesthetized with pentobarbital (100 mg/kg) and perfused transcardially, first with saline (0.9% NaCl), followed by 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Their brains were removed from the skulls, postfixed in the same fixative and soaked in 30% sucrose prior to sectioning. They were cut into 40  $\mu$ m sections on a cryostat and collected in the phosphate buffer. After rinsing in phosphate-buffered saline (PBS), free-floating sections were incubated for 30 min in 1% H<sub>2</sub>O<sub>2</sub> in PBS with 10% methanol, washed with PBS and incubated in 10% normal sheep serum in PBS with 1% BSA and 0.3% Triton X-100 for 1 h. They were subsequently kept overnight in the primary antibody solution (anti-cow GFAP from DAKO, 1:1000 in PBS with 0.3% Triton X-100). After rinsing, sections were incubated for 1.5 h in the solution of the secondary anti-rabbit antibody in PBS (1:200), rinsed again and kept for 1 h in the streptavidin-peroxidase complex diluted in PBS (1:200). Both secondary antibody and streptavidin complex were from The Binding Site, UK. The next step involved visualization of the obtained complex with diaminobenzidine, enhanced with nickel salts (DAB Substrate Kit, Vector). Slices were mounted on slides and coverslipped with DePeX (Serva). Sections of three *S. minutus* brains were taken to compare the staining pattern of anti-GFAP antibody with another astroglial marker. We used rabbit polyclonal anti-glutamine synthetase antibody (Sigma), raised against a synthetic peptide corresponding to the C-terminus of mouse glutamine synthetase (amino acids 357–373 with N-terminally added lysine). The secondary detection was provided by incubation with an anti-rabbit IgG conjugated to Alexa Fluor 488 fluorochrome (Molecular Probes; 1:200 in PBS).

Due to the capability of formaldehyde fixation to reduce the propensity of antibodies to bind GFAP epitopes [1], we performed antigen retrieval experiments. For that purpose, prior to immunohistochemical procedures, some sections were subjected to one of the following procedures: (1) incubation in 0.05% pronase solution in PBS for 20 min, (2) microwave heating (750 W) for 5 min in the citrate buffer (0.1 M, pH 6.0) or (3) in 0.5% Triton X-100 solution in PBS. There was no difference in the number and distribution of labeled glia between sections pretreated for antigen retrieval and those labeled without pretreatment. Even when the background level on the pretreated sections was raised due to the procedure of antigen retrieval (and it was particularly high in the white matter), no additional labeling was found.

Examination of the GFAP immunostaining in the mouse brain revealed abundance of GFAP-immunoreactive (GFAP-ir) astrocytes. This was especially evident in the hippocampal formation (Fig. 1A), piriform cortex and olfactory bulb. In the diencephalon, high numbers of GFAP-ir cells were found in the hypothalamus and in the medial habenular nucleus. Mod-

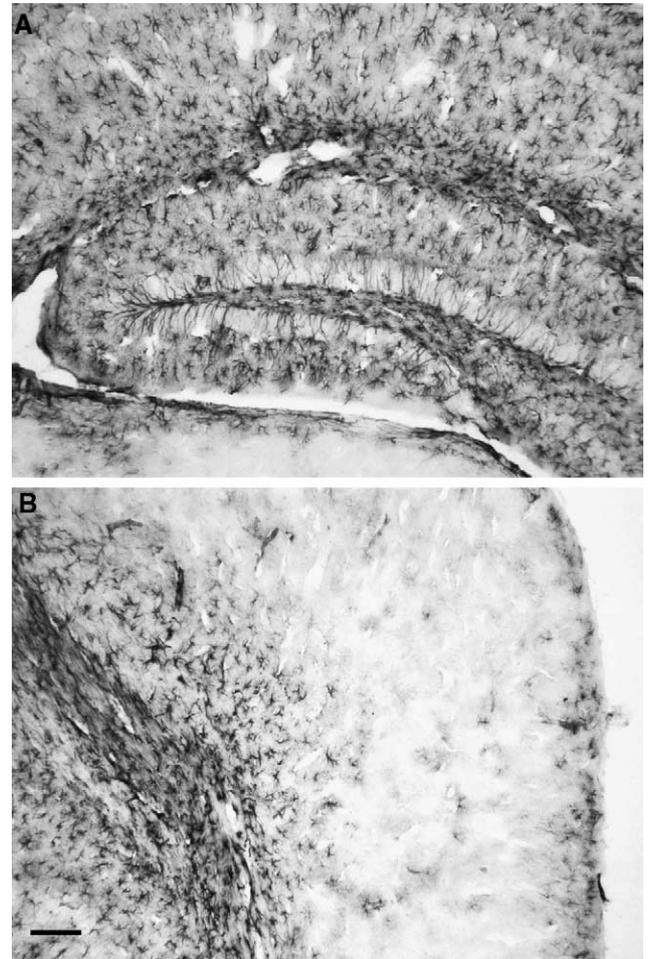


Fig. 1. In the mouse brain large numbers of astrocytes express GFAP. Although relative densities differed among structures, high numbers of GFAP-ir cells were present in the hippocampal formation (A) and lower, but still considerable numbers, in the neocortex, here represented by the retrosplenial cortex (B). Bar: 0.1 mm.

erate numbers of GFAP-ir astrocytes were found in the most superficial and deep layers of the neocortex (Fig. 1B), nucleus accumbens, and several brainstem structures like substantia nigra and interpeduncular nucleus.

In the pygmy shrews several distinct features of GFAP immunolabeling were visible, in particular the general scarcity of the labeled cells. Perivascular astrocytes (Fig. 2A) were frequently found in the whole brain, also in regions completely devoid of free astrocytes and radial fibers. Many GFAP-ir stellate-shaped cells were present within the ependyma (Fig. 2B). The most striking was almost total absence of any GFAP-ir glial cells in the neocortex (Fig. 2C). Only a few labeled astrocytes were present in the superficial part of layer I, from where they extended their processes into deeper parts of layer I, what was most frequent in the cingulate gyrus. It is also interesting that allocortical areas were almost devoid of GFAP-ir cells. This was particularly evident in the hippocampal formation, where single astrocytes were only sporadically seen in the subgranular layer of the dentate gyrus (Fig. 2D). Olfactory bulb, anterior olfactory nucleus

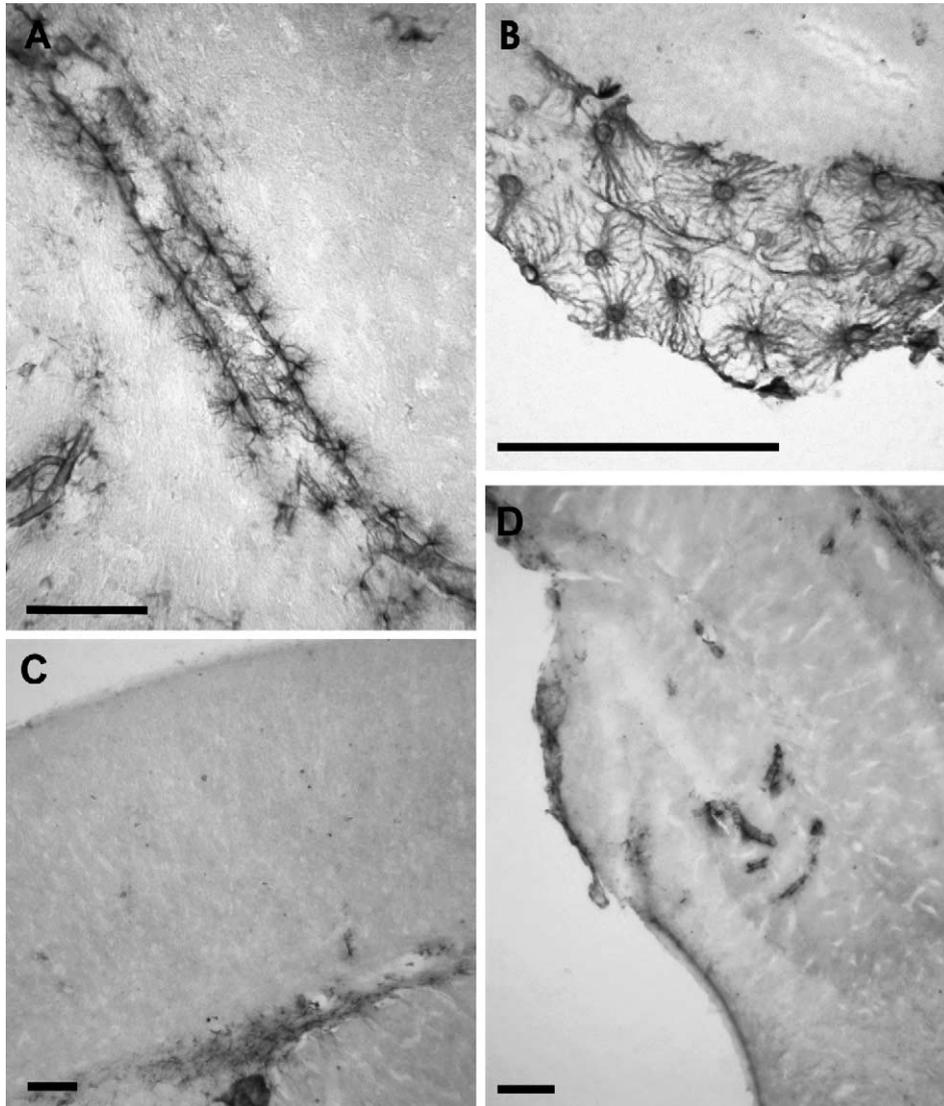


Fig. 2. The most common type of GFAP-immunopositive astroglia in the pygmy shrew brain were perivascular astrocytes, most often found around large blood vessels (A), and cells located in the ependyma (B). There was almost complete lack of GFAP-ir cells across all layers of the neocortex (C, note GFAP-ir cells in the white matter), as well as in the hippocampal formation, except for the perivascular glia and a few labeled cells in the subgranular layer (D). Bar: 0.1 mm.

and the piriform cortex lacked GFAP-immunopositive astroglia, but such astrocytes were sometimes present in the superficial part of the lateral olfactory tract and some were usually visible around the olfactory ventricle.

Labeled cells were observed around all ventricles of the brain. GFAP-ir astrocytes were positioned just beneath the ventricular ependyma and extended their processes into the brain parenchyma. Many GFAP-ir astrocytes were also present in several major fiber tracts. Highest numbers of such cells were found in the anterior commissure, fimbria/fornix, optic tract (Fig. 3A), mammillothalamic tract, cingulum bundle and at the border between corpus callosum and the external capsule. However, within the corpus callosum itself there were only scattered single astrocytes. Astrocytes were also abundant in the white matter bordering the hippocampal formation and neocortex. GFAP-ir cells were not detected in the basal ganglia and basal forebrain. In the diencephalon,

the most conspicuously labeled structure was a dense plexus of GFAP-ir tancytic fibers, extending between the lining of the third ventricle and the base of the hypothalamus, where the external ependymal layer was also highly immunoreactive. Habenular nuclei were also rich in GFAP-ir astrocytes (Fig. 3B). In the mesencephalon GFAP-ir was scarce, with no labeling in the substantia nigra or colliculi. More caudally in the brainstem, numerous labeled astrocytes were visible along the midline and at its lateral and ventral walls (Fig. 3C), and some groups of GFAP-ir cells were scattered in between. Significant numbers of GFAP-ir cells were found in the sensory root of the trigeminal nerve. Generally, the more caudally in the brainstem, the more GFAP-ir astrocytes were present. At more caudal levels, the astrocyte-rich band appeared at the medial border of the spinal trigeminal nucleus. The cerebellum had variable, but often substantial numbers of GFAP-ir cells, most of them located in the granule cell layer

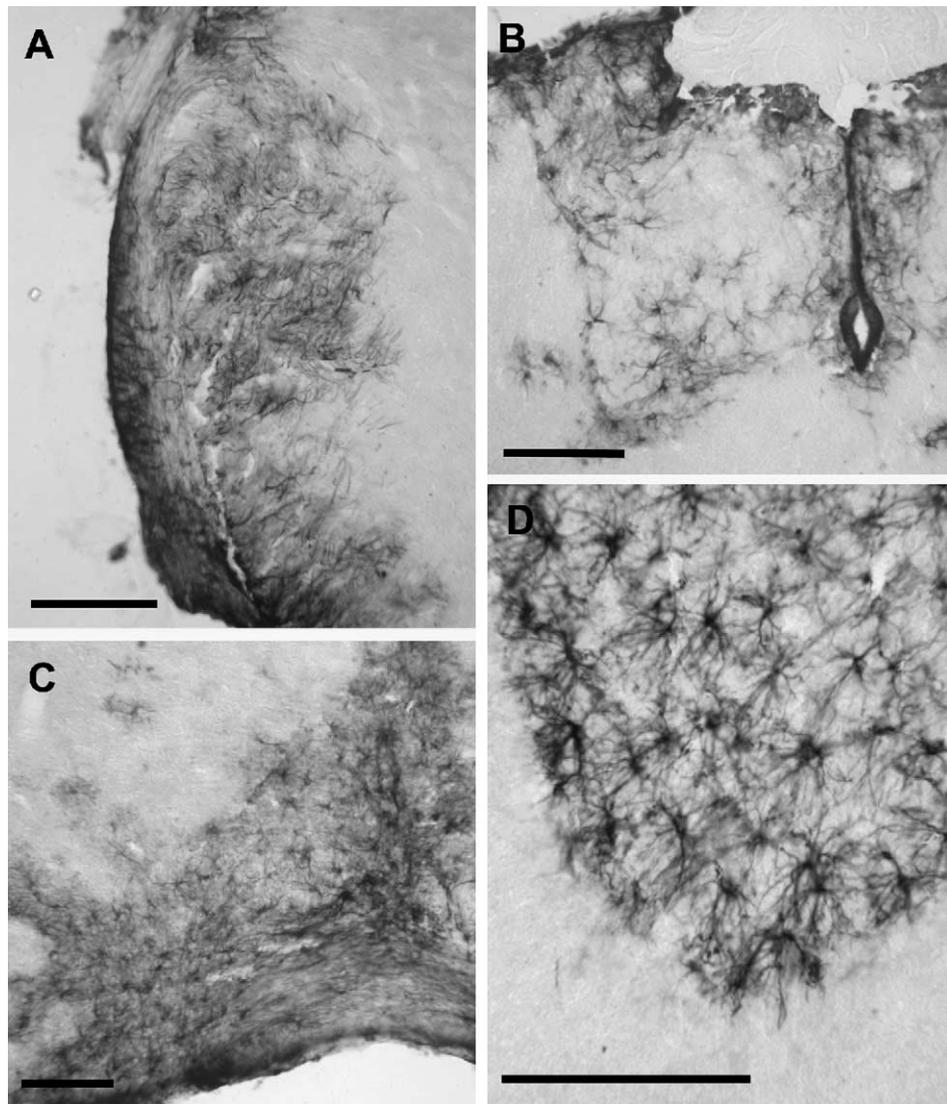


Fig. 3. In the pygmy shrew brain numerous GFAP-ir glial cells were present in several large fiber tracts, including the optic tract (A). Habenular complex possesses conspicuous GFAP-ir astrocytes located primarily in its medial part (B). Numerous labeled astrocytes were found in the brainstem, here shown in the ventral and medial part of the rhombencephalon (C). Dense network of GFAP-ir glia was found in the cerebellar granule cell layer (D). Bar: 0.1 mm.

and in the cerebellar white matter, where they formed dense networks (Fig. 3D). Higher density of these cells was seen in more ventral and lateral parts, especially in the paraflocculi. Sporadically, GFAP-ir Bergmann glia was visible in the molecular layer of the cerebellum.

Glutamine synthetase immunolabeled cells were frequent in the brain of the pygmy shrew. Immunoreactive profiles usually consisted of lightly immunofluorescent round or elongated cell bodies with several extending processes. Such cells were encountered in large numbers in the cerebral cortex, especially in the piriform cortex, hippocampal formation and neocortical areas (Fig. 4). In the subcortical regions numbers of cells immunoreactive to glutamine synthetase were variable, however in some regions, like amygdala, it was very high. There was also a dense network of immunofluorescent processes in the granule cell layer of the cerebellum, but the labeling was absent in the cerebellar molecular layer. Glu-

tamine synthetase-immunolabeling was also infrequent in the fiber tracts, although some labeled cells were visible in the trapezoid body.

The major finding of this first study of the distribution of GFAP-immunoreactive cells in the insectivore brain is the relative paucity of GFAP in many areas of the shrew brain. This is especially evident when we make comparisons with the rodent brain, where GFAP-ir astrocytes are ubiquitous [3,4,6,15]. In the mouse, as we confirmed in this study, numerous GFAP-expressing cells are present in many brain structures. In rodents there are many GFAP-ir astrocytes in the cortex, especially in allocortical structures, including the olfactory bulb, hippocampal formation and other cortical areas placed medially to the rhinal sulcus, as well as in the most superficial and deepest layers of the neocortex. Also some subcortical regions like pallidum and many diencephalic and mesencephalic structures have abundant expression of GFAP

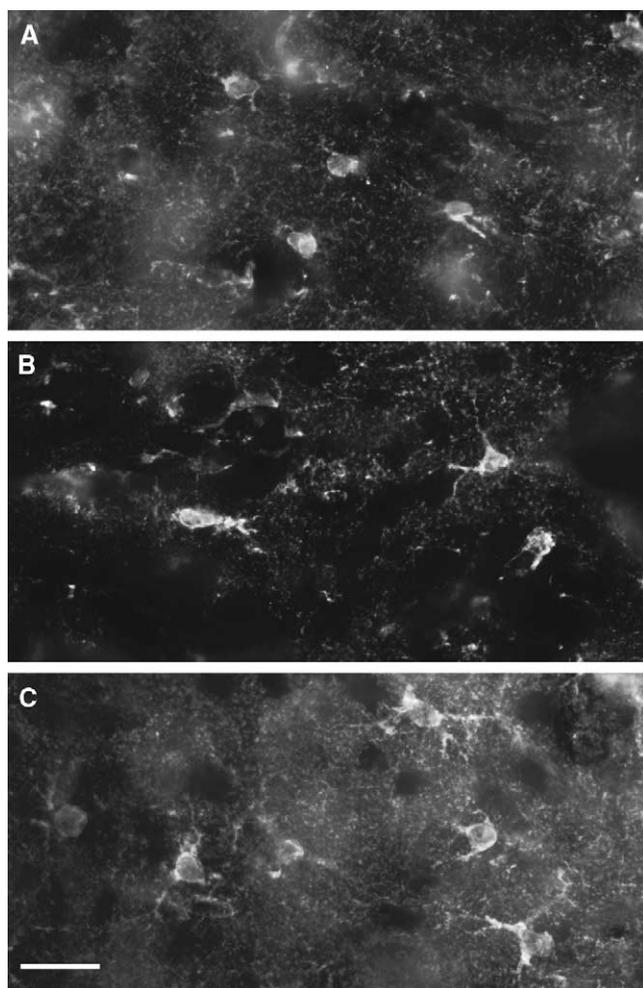


Fig. 4. Immunofluorescent staining against glutamine synthetase in the shrew brain disclosed a large number of cells, presumably astrocytes, located also in regions completely devoid of GFAP-immunostaining like the hilar dentate gyrus (A), neocortical areas (B) or the piriform cortex (C). Please note round cell bodies characteristic for glutamine synthetase-immunopositive astrocytes. Bar: 20  $\mu$ m.

[6,8,15]. This contrasts with the almost complete lack of astroglial cells expressing GFAP in the majority of structures of the shrew forebrain and mesencephalon. The notable exceptions were astrocytes located perivascularly and in the ependyma and subependymal layer, subventricular zones, white matter and several structures in the diencephalon and mesencephalon, like habenulae or hypothalamus. Labeling in the caudal brainstem and cerebellum was similar to that in rodents. As it was noted earlier, GFAP is prone to become undetectable under standard formaldehyde fixation, and the negative results obtained in this study could be attributed to masking of its epitope [1]. However, our antigen retrieval experiments indicated that this was not the case here. What is more, widespread occurrence of perivascular and periventricular astrocytes, as well as of many GFAP-ir cells in several other structures, constitute an internal positive control, that confirms with high probability the absence of the GFAP expression in the unlabeled areas of the brain. Finally, it is im-

portant to emphasize, that this labeling shows only GFAP-ir elements, and not all astroglia. We suppose that in the shrew brain there is a multitude of astroglial cells that do not express this protein. Present results aiming at identifying GFAP-negative population of astrocytes using glutamine synthetase as a marker confirm this hypothesis. Many of the glutamine synthetase-immunopositive cells are supposedly astrocytes, even in the regions devoid of GFAP-immunoreactivity, which was also demonstrated in the rat brain [9,14]. Therefore, the pattern of GFAP expression in the pygmy shrew is significantly different from that of rodents, the latter being commonly assumed to be a general mammalian one [3]. Although there is a paucity of data regarding the distribution of GFAP in brains of other mammals, the assumption that the rodent type is universal is contradicted by our results. Insectivores are usually thought of as conforming to a relatively unspecialized mammalian morphotype, although they are not regarded any more as primitive basal mammals [5]. On the other hand, shrews possess some unique physiological features, e.g. low levels of drug-metabolizing enzymes [10] or the metabolic rate higher than expected for their body mass [13], therefore in the future it should be examined whether or not this particular pattern of GFAP expression is unique to shrews, insectivores, or is present in some other mammalian orders.

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