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Immunohistochemical Demonstration of Proteinase Inhibitor Alpha-1-Antichymotrypsin in Normal Human Central Nervous System

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The presence of alpha-1-antichymotrypsin, a serine proteinase inhibitor with a high affinity for cathepsin G, is demonstrated in the normal human central nervous system (CNS) by immunohistochemical techniques. Paraffin-embedded normal human CNS tissue from five adult, two fetal, one neonatal and three newborn autopsies were stained with monospecific rabbit antibodies to human alpha-1-antichymotrypsin using biotinylated goat anti-rabbit antibodies and an avidin-biotin-peroxidase complex. Positive immunostaining was seen in neurons and glial cells in the cerebral cortex, basal ganglia, hippocampus, cerebellum, brainstem, and spinal cord of the adults. The epithelium of the adult choroid plexus had the most intense staining in apical granular organelles corresponding in position to lysosomes or secretory granules. Ependymal cells, particularly those near the choroid plexus, were immunostained. The fetal CNS had no alpha-1-antichymotrypsin staining. Limited staining of choroid plexus, ependyma, and frontal lobe was found in the newborns. Immunostaining in the neonatal temporal lobe was only found in the choroid-plexus epithelium. These observations establish a widespread distribution of this proteinase inhibitor in the normal human CNS. Developmental regulation of this inhibitor in the human CNS is also indicated.

Key words: alpha-1-antichymotrypsin, proteinase inhibitor, acute-phase reactant, immunostain, central nervous system, neuron, glial cell, choroid plexus

Proteinases perform vital functions in the development and remodeling of tissue structures. Extracellular structures are often protected from untimely degradation by proteinase inhibitors. The liver, which releases massive quantities of serum proteins into the blood circulation, is one of the major sources of inhibitors such as alpha-1-antichymotrypsin (Achy) and alpha-2-macroglobulin [1]. These active inhibitors provide a widespread protection against uncontrolled proteolysis. The biosynthesis of these inhibitors has recently been demonstrated in extrahepatic sites, suggesting that

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they may perform unique functions in specific microenvironments. Such functions may require either selective removal of the inhibitor from the circulation or direct synthesis by cells in these microenvironments. The local production of Achy by human breast epithelial cells under estradiol regulation is an example [2,3]. Achy can also be detected by immunohistochemical techniques in normal human prostatic epithelial cells [4]. This serine proteinase inhibitor is a 66-kDa glycoprotein with a high affinity for cathepsin G, K_a of 5×10^7 $M^{-1}s^{-1}$ [5]. Cathepsin G is a major tissue-degrading enzyme produced by leukocytes that is involved in the hydrolysis of proteoglycans, elastin, collagen, and fibronectin and in the conversion of angiotensin I, C3, and C5 into biologically active molecules. In view of the wide variety of biological functions that cathepsin G exhibits, vital regulatory roles are proposed for its inhibitor, Achy. We report here the finding of Achy by immunohistochemical techniques in the human central nervous system (CNS) and describe its appearance in developing and adult neurons, glial cells, and choroid-plexus epithelium.

MATERIALS AND METHODS

Tissue Specimens

Routinely formaldehyde-fixed, paraffin-embedded tissue sections were obtained from the autopsy files of the Los Angeles County-University of Southern California Medical Center. Deparaffinized sections were cut at 5 μm, washed in water, and rinsed in phosphate-buffered saline (PBS). Normal human CNS autopsy tissue specimens without histopathology were studied from four adults who died of nonneurological diseases. In addition, one neonatal, three newborn, and two midgestational fetal autopsy brain specimens were examined. Sites studied were frontal lobe, basal ganglia, thalamus, temporal lobe including hippocampus, midbrain, medulla, and spinal cord including cervical, thoracic, and lumbar segments. Choroid plexus was studied in the temporal horn of the lateral ventricle and in a few specimens in the third ventricle.

Antisera

Specific rabbit antiserum to human Achy was obtained from Dako Corp. (Santa Barbara, CA) (lot No. 113A). The antiserum was monospecific for Achy as established by three criteria. In Ouchterlony immunodiffusion and in immunoelectrophoretic assays, only a single precipitin arc was observed against normal human serum. Furthermore, when the immunoglobulin fraction of this antiserum was used for affinity chromatography, a single glycoprotein was specifically adsorbed, which was pure Achy as determined by two-dimensional gel electrophoresis [6]. Rabbit antiserum to human alpha-1-proteinase inhibitor was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Goat anti-rabbit antibodies labeled with biotin and avidin-biotin-peroxidase complexes were obtained from Vector Laboratories (Burlingame, CA).

Immunohistological Staining

The avidin-biotin-peroxidase method was used to stain tissue sections [7]. The sections were treated with 0.6% hydrogen peroxide in methanol to abolish endogenous peroxidase activity and then incubated with normal goat serum (diluted 1:20 with PBS) for 20 min to reduce background staining that was due to subsequent

Fc-receptor-mediated binding in later steps. The primary antiserum (1:50 to 1:800) was added for 60 min. Controls included the use of PBS without a primary antiserum in it, use of diluted normal rabbit serum in place of the primary antiserum, and absorption of antiserum by purified Achy [8]. Biotinylated goat anti-rabbit antibodies were added for 30 min (1:400) followed by avidin-biotin-peroxidase complexes for 30 min. Amino-ethylcarbazole was used as the chromogen for a 10-min incubation to localize the sites of peroxidase activity. Most sections were briefly counterstained with Mayer's hematoxylin. The sections were mounted in glycerin and evaluated by three independent viewers. A Wratten #47 blue filter was used, except as indicated, to completely filter out the hematoxylin counterstain while taking the photomicrographs.

RESULTS

Conditions for immunoperoxidase staining were optimized in order to minimize nonspecific binding of antibodies. All tissue sections were first stained with antibody dilutions of 1:100. Primary antibodies were then used at higher dilutions on sections in which Achy was a prominent component. Control specimens that were reacted with PBS, normal rabbit serum, or antibodies to Achy absorbed with purified human serum Achy were uniformly and consistently negative (Fig. 1).

Adult Cerebrum

Immunostaining in the adult frontal lobe neocortex showed Achy in neurons and glial cells with substantial variations in staining intensities. There was no specific

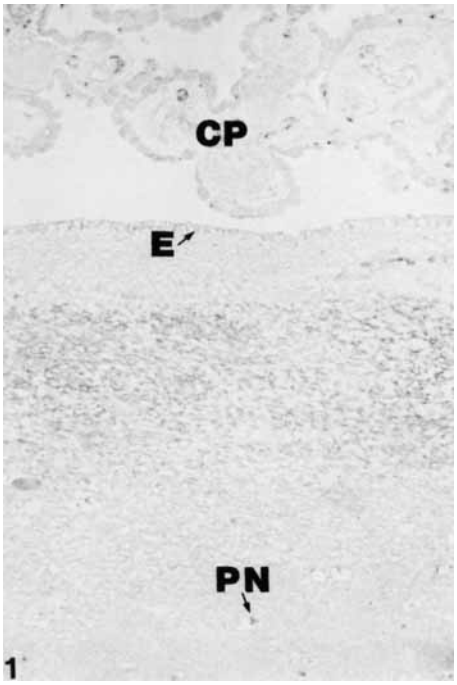


Fig. 1. Photomicrograph of temporal lobe with Achy immunostaining in the presence of purified Achy. No immunolabeling is seen in the choroid plexus (CP), ependyma (E), or pyramidal neurons (PN) of the hippocampus. Achy immunostain (1:100). ×200.

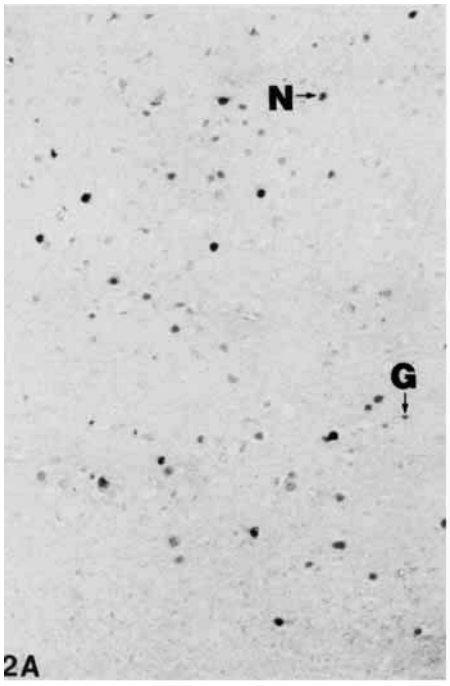
pattern of immunolocalization with regard to neuronal size, neocortical layer, or local groupings of neurons. At least half of the large pyramidal neurons had light to moderate finely granular immunostaining in the perikaryal cytoplasm. A smaller number of neuronal nuclei also were immunostained. Occasional axons and major dendrites were immunostained as they joined the perikaryon. Small neocortical glial cells, which included astrocytes, had light perikaryal immunostaining and sometimes heavy nuclear immunostaining. Subcortical glial cell immunostaining varied, with up to a third of the perikarya showing light to moderate reaction and with occasionally heavy glial nuclear immunostaining (Fig. 2). Some white blood cells within vascular lumina were positive, and the vascular walls were negative (Fig. 3).

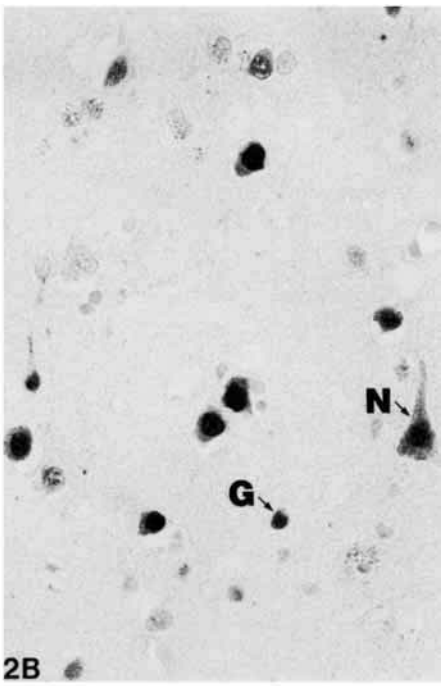
The basal ganglia and hippocampus had Achy immunolocalization in neurons and glial cells, with a higher proportion of cells containing reaction product in a given area in one brain as compared to another. Most neurons and glial cells in the thalamus, hypothalamus, and lenticular nucleus had light to moderate immunostaining that was more often nuclear than cytoplasmic. The heaviest amount of immunostaining was in neuronal nuclei in the thalamus and putamen (Fig. 4). In the hippocampus, some of the large pyramidal neurons and small granule neurons of the dentate gyrus were positive (Fig. 5). Well over half of the subcortical and deep white matter glial cells in the lateral part of the temporal lobe were moderately immunostained. As the temporal horn of the lateral ventricle was approached in the white matter, immunostaining decreased except for moderate immunostaining of the glial cells in the subependymal zone. The ependymal cells varied from negative to moderate with perikaryal immunostaining. The most intense ependymal cell immunoreactivity was located near the choroid plexus (not illustrated).

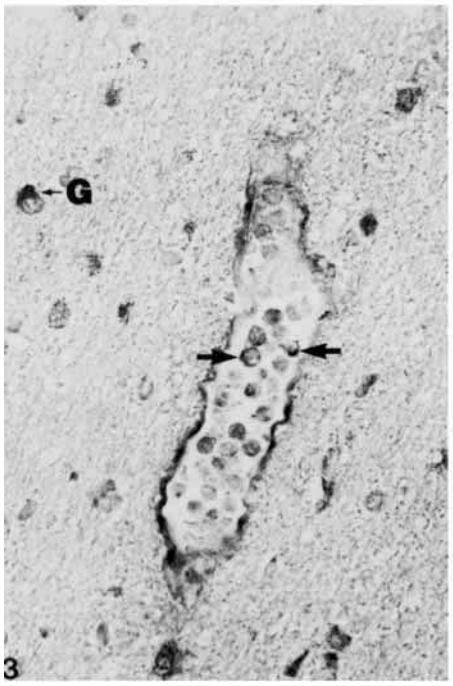
Immunostaining in most of the choroid-plexus epithelial cells in the temporal horn of the lateral ventricle varied from moderate to marked at 1:100 dilution of the antiserum, but the reaction product was generally in larger coarse granules than in neurons or glial cells, and its location was often distinctly apical (Fig. 6). The reaction product in the choroid-plexus epithelium remained moderate to marked at 1:200, even though the immunostaining in the rest of the temporal lobe decreased. Some moderately immunostained nuclei and granules of reaction product in the cytoplasm remained in the choroid-plexus epithelium at dilutions of 1:400 (Fig. 6C), while at 1:800 all of the tissue was negative. The moderately immunostained choroid-plexus epithelium had adjacent stromal immunostaining as well. When the epithelial cells were lightly immunostained, the underlying connective-tissue stroma also had a light reaction (Fig. 6A,B). The stromal immunostaining disappeared with serial dilutions just as the cellular immunostaining did, indicating that reaction product in cells and stroma was due to the same antigen.

Adult Cerebellum

The molecular layer of the folial cortex had a few small, scattered cellular perikarya with light to moderate immunostaining for Achy and an occasional positive nucleus. Small intrinsic neurons and glial cells were both included in this population, and at least some of the positive cells were astrocytes. Long, thin processes that appeared to be parallel fibers from the internal granule cell layer were lightly to moderately immunostained. Most Purkinje cells were negative, yet a few had moderate perikaryal immunostaining with some immunostaining of the dendrite extending a short distance into the molecular layer. Thin, moderately immunostained processes







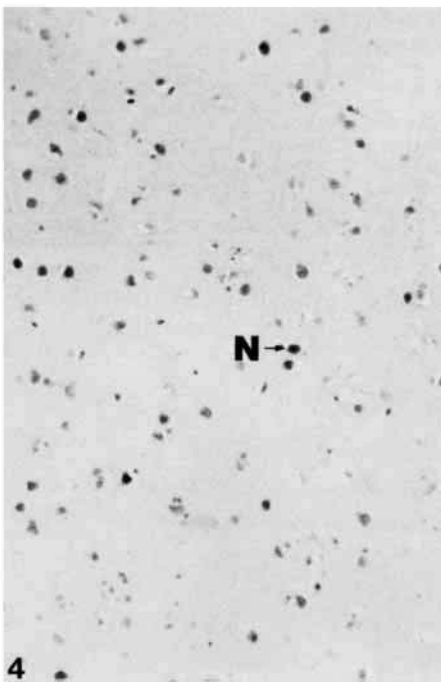
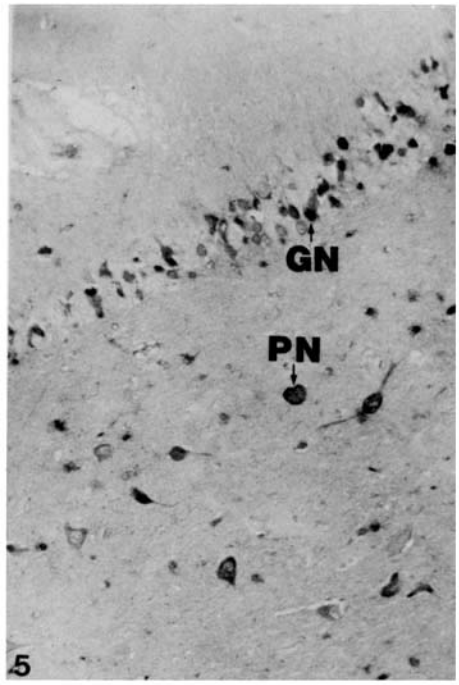
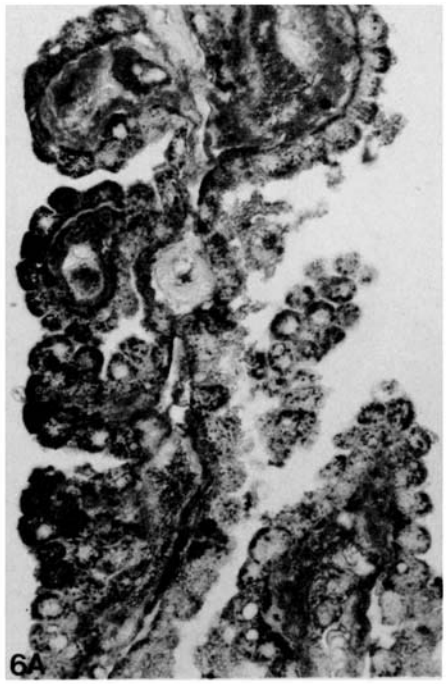


Fig. 2. Frontal lobe. **A**:Inner half of neocortex (upper half of field) and subcortical white matter (lower field) showing immunostaining of Achy in many neurons (N) and in a few glial cells (G). **B:**Achy immunostaining in some neuronal nuclei and perikarya (N) and in glial cells (G), with many cells negative in this area of neocortex. Achy immunostain (1:100). A, ×200; B, ×400.

Fig. 3. Cerebral white matter immunostaining. White blood cells (arrows) have some immunostained cytoplasmic granules in a porsmortem clot within a small venule. Some glial cells (G) have granular cytoplasmic immunostaining. The density of the venular wall is due to photographic contrast, but there is no immunostaining at that site. Achy immunostain (1:100). ×400.

Fig. 4. The nuclei of many neurons (N) in the putamen are heavily immunostained. Achy immunostain (1:100). ×200.





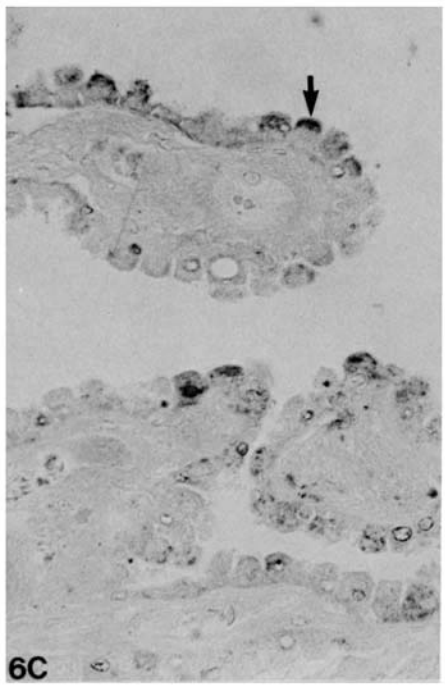


Fig. 5. Granule neurons (GN) and pyramidal neurons (PN) in the hippocampus have nuclear and cytoplasmic immunostaining. Achy immunostain (1:100). ×200.

Fig. 6. Choroid-plexus immunostaining for Achy varies from area to area and with dilution of the primary antiserum. At 1:100, heavy reaction product is seen in the cytoplasm of the epithelial cells in **A**, while in **B** there is less reaction product; but in both areas the immunostaining is coarsely granular. **C**: At 1:400, the immunostaining is less, but coarse apical granules of reaction product are still seen in the epithelial cells (arrow). Achy immunostain. ×400.

corresponding in location to basket cell axons were observed by the Purkinje cell somata. A small proportion of Bergmann astrocytes had moderate perikaryal reaction product. The internal granule cell layer had relatively extensive and moderate immunostaining with about a third of the small cells being positive. The widest regions of the internal granule cell layer had the highest concentration of immunostaining located externally, while the thinner regions of this layer had evenly dispersed cellular reaction product. Most of the glial cells in the folial white matter had light to moderate immunostaining (Fig. 7).

Adult Brainstem

Most neurons had some immunostaining. The most prominent immunostaining was in the pigmented neurons of the substantia nigra and in the neurons of the inferior olivary nuclei, where the cellular nuclei had a moderate to heavy level of staining and the perikarya were negative or lightly stained (Fig. 8). The floor nuclei of the fourth ventricle were heterogeneously stained with no specific pattern discernible. Scattered glial cells had light to moderate immunostaining.

Adult Spinal Cord

Observations were made on cervical, thoracic, and lumbar segments. The large motor neurons were mostly negative, but a few had light perikaryal reaction product or moderate nuclear staining (Fig. 9). The glial cells in the gray matter were immunostained more than those in the white matter, with light to moderate reaction product in many of these cells.

Fetal CNS

The midgestational fetal brains were negative for Achy (Fig. 10). Several areas of the cerebrum and brainstem that included choroid plexus were tested.

Postnatal CNS

The three newborns with an estimated gestational age of 38–39 weeks had congenital renal disease and were hypoxic. Two died on the day of delivery, and one lived for 5 days. The brains were normal microscopically. The temporal lobe was sampled for immunostaining in two cases. In one of these there was a uniformly moderate cytoplasmic reaction product in the choroid-plexus epithelium with some very heavy apical granular immunostaining (Fig. 11). The ependyma and the remainder of the brain parenchyma were negative. The other brain had light cytoplasmic immunostaining in some of the choroid-plexus epithelial cells and a trace of cytoplasmic immunostaining in some ependymal cells, with the rest of the parenchyma being negative. The frontal lobe was examined in the third newborn. Neocortical neurons were mostly negative, but some had light reaction product or moderately immunostained nuclei. Scattered glial cell nuclei were lightly to moderately immunostained both in the gray and white matter. Slightly enlarged glial perikarya in the white matter were also lightly to moderately immunostained. The ependyma along the body of the lateral ventricle was negative. Occasional subependymal glial cells had light to moderate perikaryal immunostaining, and about half of their nuclei were negative with the other half being moderately immunostained. The fourth child died of congenital heart disease at 1 month of age. The sampled temporal lobe showed

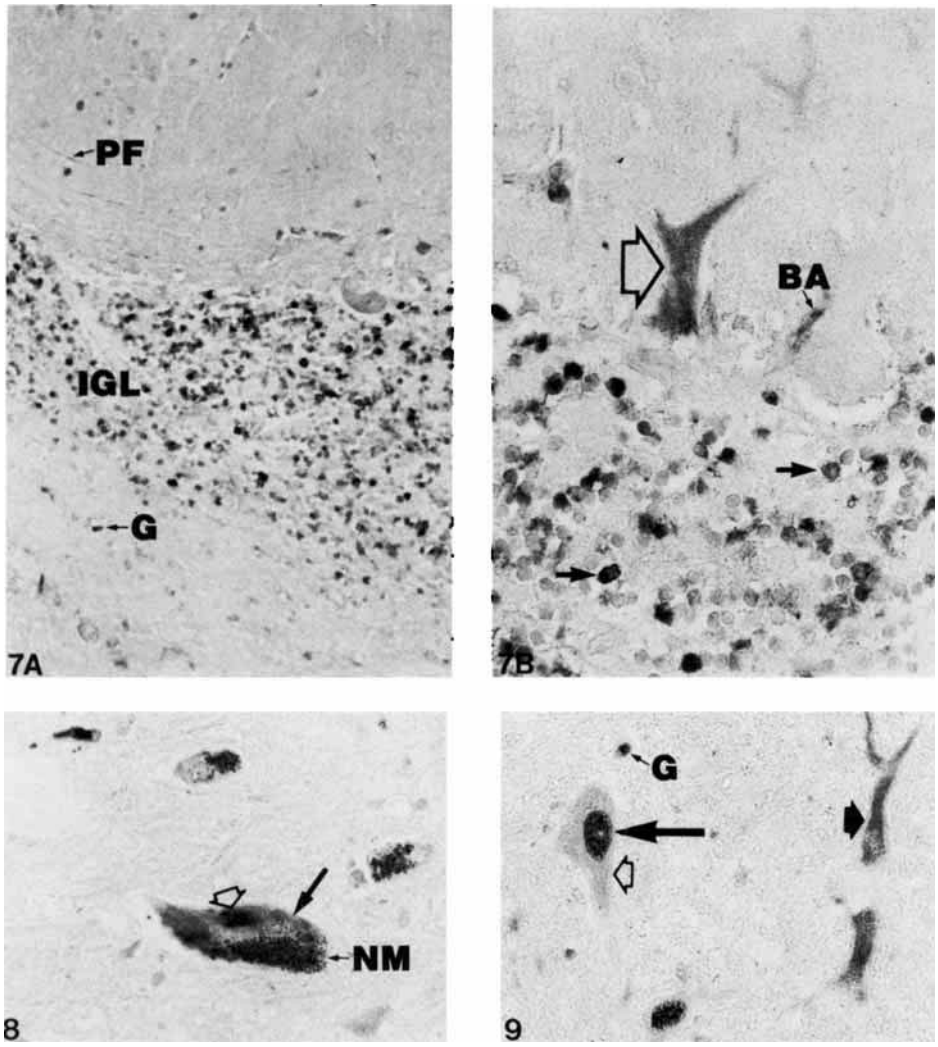
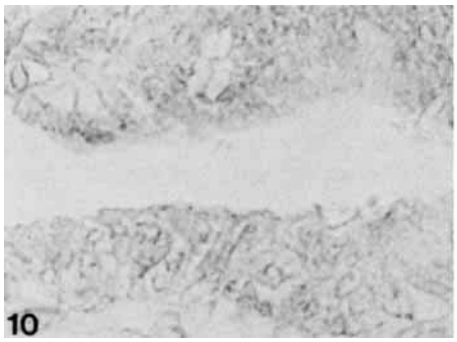


Fig. 7. Cerebellar folial immunostaining. **A**: Parallel fibers (PF) in the molecular layer are lightly immunostained. Most of the small granule cells in the internal granular cell layer (IGL) and a Purkinje cell (PC) are negative, but some of the granule-cell nuclei are heavily immunostained. A few immunostained glial cells (G) are seen in the white matter. **B**: In this area, a Purkinje cell is immunostained (arrowhead), and many of the IGL cells (arrows) are positive. Basket-cell axons (BA) by a Purkinje cell are also positive. Achy immunostain (1:100). A, ×200; b, ×400.

Fig. 8. Midbrain. The nucleus (arrowhead) of a pigmented neuron in the substantia nigra is heavily immunostained. There is moderate cytoplasmic immunostaining (arrow). The very dark perikaryal granules are composed of neuromelanin (NM). Achy immunostain (1:100). ×400.

Fig. 9. Anterior horn of cervical spinal cord. Motor neurons have light (open arrowhead) to moderate cytoplasmic immunostaining (solid arrowhead). One of the few motor neurons with heavy nuclear immunostaining is shown (arrow). Glial-cell immunostain (G) is seen. Achy immunostain (1:100). ×400.



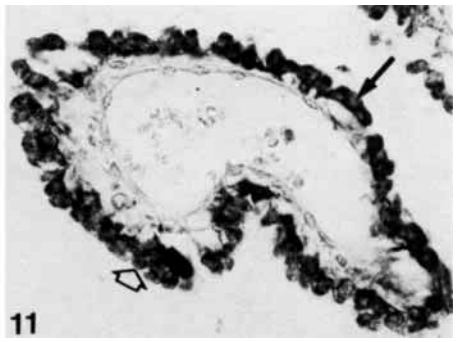


Fig. 10. Fetal choroid plexus. The epithelial cells are negative. Achy immunostain (1:50); light hematoxylin counterstain. ×500.

Fig. 11. Newborn choroid plexus. The epithelial cells have coarsely granular immunostain (arrowhead). Cellular nuclei are negative (arrow). Achy immunostain (1:100). ×300.

immunostaining only in the apical cytoplasm of the choroid-plexus epithelium, where light to moderate granular reaction product was seen (not illustrated).

DISCUSSION

These experiments demonstrate that Achy is present in the structurally intact human CNS in the absence of inflammatory disease. The specificity of immunostaining for the presence of Achy in neurons, glial cells, and the choroid plexus is confirmed by control studies that include the inhibition of its immunolocalization by purified Achy. At high dilutions of the antiserum to Achy (eg, $>1:800$), the inhibitor is known not to be demonstrated in the human CNS parenchyma [9]. However, specific inhibition of its localization by purified Achy at lower dilutions in this study indicates that Achy is present in relatively low amounts. Achy is not found in the midgestational fetus, and it appears in the brain by the newborn period at the time when it begins to be synthesized in the liver. There are general patterns of immunolocalization such as the apical granular stain in choroid-plexus epithelium, heavier neuronal than glial cell immunostain in the basal ganglia and in other regions, heavier subependymal staining near the choroid plexus than at a distance from it.

Immunolocalization studies of alpha-1-antitrypsin indicate that coarsely granular stain can be seen in cells that synthesize the inhibitor, and nuclear or finely granular cytoplasmic stain might be found in cells that take the inhibitor up from the extracellular fluid [10]. Thus, Achy immunolocalization in neurons and glial cells in the normal human CNS does not mean that this inhibitor is necessarily synthesized in the neuroepithelial cells, although this possibility requires further attention. Resident macrophages could introduce Achy into the ventricular system [11–13] or subarachnoid space [14,15]. Proteinase inhibitors, which can be adsorbed or released by cells [2,16–20], may be selectively endocytosed in neural cells from inflammatory cells, as has been noted for serine proteinases in myocytes [21,22]. Alternatively, Achy in neurons and glial cells could originally be sequestered or synthesized by the choroid-plexus epithelium. Achy is produced in an active form in vitro by epithelial cells [2], including a line that is of neuroepithelial origin [23]. Therefore, it is conceivable that

Achy immunolocalized in the CNS could be produced by the neuroepithelium for unknown homeostatic functions.

Choroid-plexus epithelial cells contain apical secretory granules that may be mostly lysosomal in nature [24,25]. These cells regulate the type and amount of protein entering the ventricular fluid [25,26], and they actively regulate the return of molecules from their apical to their basal side [27,28]. Normal cerebrospinal fluid contains a low level of proteinase inhibitors [29–31], and, with immunoreactive Achy concentrated in the apical portion of the choroid-plexus epithelium, it should be expected that Achy is being transported to or from the cerebrospinal fluid. Serine proteinase inhibitors probably have a decreased transfer from serum to cerebrospinal fluid as the blood-cerebrospinal fluid barrier matures early in life [32], yet the immunoreactive Achy in the normal CNS could be derived by slow accrual from this source. The immunolocalization of Achy in ependymal cells close to the choroid plexus may indicate an uptake pathway that includes serum, choroid plexus, cerebrospinal fluid, and, finally, ependymal cells at the highest point of Achy concentration. This hypothesis also needs more investigation.

Glial cells, and to some extent neurons, can protect themselves from environmental changes by selectively endocytosing and degrading foreign substances, including potentially toxic serum proteins [33–38]. Albumin, prealbumin, and transferrin have been immunolocalized in the developing human CNS, perhaps having endocytosed from the cerebrospinal fluid before the blood-cerebrospinal fluid barrier forms [39]. They may be harmful unless sequestered and deactivated. Alternatively, they may play developmental roles in the fetal brain [39]. No functional role is known for serum proteins in the mature brain, where experimentally one mechanism of deactivating destructive serum proteinases is through an excess of a neutral proteinase inhibitor [37,40,41]. Ongoing surveillance in the brain against serum constituents may require an excess of proteinase inhibitors to provide for local damage control.

Proteinases active in synaptic transmission might require various levels of control, both of their own activity and of metabolic intermediates that accumulate [42–47]. Short-lived turnover of accumulating proteins requiring neutral proteinases would in turn require appropriate proteinase inhibitors. Achy-like activity has been shown to control this type of protein degradation in cell culture [48,49]. Glial cells also detoxify neurotransmitters, and they may then possess proteinase-proteinase inhibitor systems for the regulation of this activity [50,51]. The specific localization of Achy in the synapse-rich internal granule cell layer corresponds to the presence of relatively high levels of lysosomal proteinase activity, in which both neutral and acid proteinases may act in concert in regulating synaptic metabolism [24,42].

Cells such as neurons that are postmitotic for most of their existence may particularly require preferential destruction of displaced or altered proteins, as well as protection from adventitious proteolysis [52]. In this regard, a deficiency of proteinase inhibitors could affect the brain, where posttranslational modification and accumulation of cytoskeletal proteins seem to occur in degenerative diseases. Neurofilaments and microtubules, both of which can be degraded by neutral proteinases and protected by proteinase inhibitors [53–55], are particularly affected in Alzheimer disease [56]. Even a small deficit in the control of proteinase activity might provide a devastating effect on a neuron, and the senescent neuron, like aging cells in culture, may be increasingly susceptible to defects in its protein-degrading machinery [52,57].

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