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The Lysosomal Cysteine Protease, Cathepsin S, Is Increased in Alzheimer's Disease and Down Syndrome Brain

An Immunocytochemical Study

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Expression of cathepsin (cat) S, a lysosomal cysteine protease, has recently been shown to cause an increase in production of amyloid β-peptides in transfected human cells. In this study, we examined the presence and localization of cat S by immunocytochemistry in 21 control, 24 Alzheimer's disease (AD), and 10 Down syndrome (DS) postmortem brains. An antiserum to a human cat S fusion protein was affinity purified and its specificity confirmed by abolition of immunoreactivity after adsorption with cat S but not cat L fusion protein. A small minority of control cases showed light, focal staining of scattered cortical neurons. Many control cases, as well as most AD and DS cases, showed prominent staining of vascular smooth muscle cells, particularly in leptomeningeal vessels. Both AD and DS brain tissue showed increased immunoreactivity in a subset of neocortical and hippocampal neurons and glia. Cat S immunoreactivity occurred in a granular, cytoplasmic pattern in some neurons or in a more dense staining pattern in certain neurofibrillary tangle-bearing neurons. Cat S-positive neurons were also present in amygdala and basal forebrain in AD brains. A subset of astrocytes were immunoreactive with the cat S antibody in AD and DS but not in control brains. In rare AD cases, cat S immunostaining was observed in astrocytes in the periphery of amyloid-β-containing plaques. These results suggest that cat S is up-regulated in AD and DS brain. The association of cat S immunoreactivity with tangle-bearing neurons, astrocytes, and rare senile plaques implies a role for altered cat S activity in the pathogenesis of AD. (Am J Pathol 1995, 146:848–860)

Alzheimer's disease (AD) is an age-related dementia involving progressive loss of memory and cognitive function. Its neuropathological hallmarks include extracellular amyloid plaques, intraneuronal neurofibrillary tangles, dystrophic neurites, and vascular amyloid.[1] Amyloid deposited in the parenchyma and blood vessel walls of AD brain is derived by proteolytic cleavage of a 40- to 43-amino-acid peptide, named amyloid-β (Aβ) peptide, from the large β-amyloid precursor protein (βPP). βPP is a single membrane-spanning glycoprotein, and the Aβ fragment spans 28 residues just outside the membrane plus the first 12 to 15 residues of the transmembrane domain.[2] Upon cleavage, Aβ is secreted from the cell.[3–5] βPP is encoded by a gene on human chromosome 21 and is expressed in virtually all mammalian cells. Whereas low numbers of Aβ plaques can occur with aging, patients with Trisomy 21, ie, Down syndrome (DS), as well as AD patients, undergo an excess accumulation of Aβ in the brain.[6–10]

The proteases responsible for generating the secreted derivatives of βPP, including Aβ, are unknown.

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However, two or more distinct proteolytic cleavages are predicted to release Aβ from the membrane. One occurs at the amino terminus of Aβ (residue 672 of $βPP_{770}$) and produces an approximately 12-kd carboxyl-terminal membrane-spanning fragment of βPP. The name β-secretase has been given to the as yet unknown enzyme(s) that cleaves the amino terminus of Aβ. The carboxyl-terminal cleavage of Aβ occurs at residues 711 to 714 and is generated by another unknown protease, designated γ-secretase. Both cleavages are known to occur as normal processing events in cells.[3–5]

Although the proteases responsible for effecting these cleavages *in vivo* (ie, in intact human cells) have not been identified, certain proteases, such as multicatalytic protease,[11] clipsin,[12] a calcium-activated cathepsin (cat) G-like serine protease,[13] metalloendopeptidase 24.15,[14] cat D,[15] cat G,[16] and a thiol-dependent matalloprotease,[17] have the ability to cleave βPP at or near the Aβ amino terminus *in vitro.* Immunocytochemistry of AD brain has demonstrated increases in cat B, D, and L in neurons and senile plaques and cat D, H, and L in reactive astrocytes.[18,19] Cat G has been localized to neutrophils by immunocytochemistry and is not increased in AD brain.[20] Other hydrolases shown to be increased in AD brain by immunocytochemistry include calpain[21] and α-glucosidase.[18]

Here, we examined the distribution and levels of the lysosomal cysteine protease, cat S, the human form of which was recently cloned,[22,23] by immunocytochemistry in AD, DS, and control brains. Human cat S has a 331-residue pre-pro form, a 218-residue mature form, and a 1.7-kb mRNA and shows elastinolytic activity.[22,23] The cat S gene (CTSS; GenBank accession M90696) is on chromosome 1q21 and has highest mRNA expression in lung, heart muscle, and spleen, with lower amounts elsewhere, including brain.[24] Cat S has sequence homology with other cysteine proteases such as cat L (49%), cat H (31%), and cat B (23%) and shares optimal activity at acidic pH (~5.5) but differs from the other enzymes in also having some activity at neutral pH.[22,25,26] Cat S is most similar to cat L in sequence and function.[27] Cat S, L, B, and H are all cysteine proteases belonging to the papain superfamily.[28,29] In addition to its role in protein degradation, this family of proteases has been implicated in the invasion of extracellular matrices by tumor cells in malignant cancer.[30,31] Cat S is variably detected as a 24- or 28-kd band by Western blotting.[22,26] Whereas cat B, L, and H are widely expressed, cat S has a restricted expression in rat tissues by Northern blot analysis.[32] Cat S mRNA is widely distributed throughout gray and white matter in the rat brain and is localized mostly to microglial cells.[33] Cat S mRNA is expressed differently from cat L and B during development and is highly upregulated, unlike cat B and L, after rat entorhinal lesions.[34] Aβ production has recently been found to increase approximately twofold in human kidney 293 cells stably co-transfected with $βPP_{695}$ cDNA and full-length cat S cDNA (J.S. Munger, C. Haass, C.A. Lemere, G-P. Shi, W.S.F. Wong, D.B. Teplow, D.J. Selkoe, H.A. Chapman, submitted).

Materials and Methods

Patient Groups

Autopsied brains from 24 patients with a clinical diagnosis and typical neuropathological findings of AD were examined by immunocytochemistry. Brains from 10 DS patients with neuropathological findings of AD and 21 control patients without a neuropathological diagnosis of AD were also examined. The age ranges and gender distribution for each group are listed in Table 1. Postmortem intervals were available for 31 of 55 total cases; AD, 13.5 (range, 3.5 to 24) hours; DS, 22.4 (range, 6 to 35) hours; and controls, 14.9 (range, 3 to 48) hours.

Antibodies/Markers

A polyclonal antibody against cat S was produced by immunizing rabbits with a cat S-maltose binding protein (MBP) fusion protein (J.S. Munger, C. Haass, C.A. Lemere, G-P. Shi, W.S.F. Wong, D.B. Teplow, D.J. Selkoe, H.A. Chapman, submitted). The antiserum was purified by adsorption to cat S-MBP blotted onto nitrocellulose after its separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by elution of bound antibody at low pH, as described.[35] Specificity of the resulting affinity-purified antibodies (designated AP anti-cat S) was determined by their adsorption to nitrocellulose blots containing either cat S-MBP or a distinct but closely related cathepsin, cat L, as a cat L-MBP fusion protein.

Table 1. *Clinical Data*

Diagnosis	No. of cases	Age ranges	Gender
AD	24	62–94	15F 9M
DS	10	36–73	6F 4M
Controls 21			
Young	2	36, 54	1F 1M
Aged	11	60–90	5F 6M
Demented (non-AD)	8	60–92	2F 6M

Preimmune sera and deletion of the primary antibody were used as additional controls.

Two high titer polyclonal antibodies against synthetic peptide $A\beta_{1-40}$, R1280, and R1282, were used to detect Aβ deposition in brain tissue.[3,36] A monoclonal antibody to human fetal tau, 5E2 (gift of Kenneth Kosik),[37] was used to detect neurofibrillary tangles in AD brain. A monoclonal antibody to the high molecular weight neurofilament protein, RT97, was used to detect a subset of tangles as well as the neuritic components of plaques.[38] A polyclonal antibody to glial fibrillary acid protein (GFAP; gift of C. Dahl), served as a marker for astrocytes. Biotinylated *Ricinus communis* agglutinin-1 (Vector Laboratories, Burlingame, CA) was used as a marker for activated microglia.

Western Blotting of Cell Lysates

Slab gels of 12% polyacrylamide were used to separate samples under reducing conditions as described.[24] Samples were prepared by collecting 10^6 cells, washing once with phosphate-buffered saline (PBS), and lysing cells directly in 80 µl of reducing sample buffer. To facilitate loading, DNA was sheared by passing samples 5 to 10 times through a 25-gauge needle. Samples were then placed in boiling water for 5 minutes and loaded. Separated proteins were transferred to nitrocellulose with a Pharmacia Novablot semi-dry blotting system operated according to the manufacturer's recommendations. Blots were washed briefly with water, then blocked for 1 hour in PBS containing 5% nonfat dry milk (milk/PBS). All steps were done at room temperature. Blots were incubated for 1 hour in milk/PBS with rabbit antiserum to cat S at 1:500 dilution. After three 10-minute washes with milk/PBS, goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added in milk/PBS at 1:100 dilution for 1 hour. Blots were washed three times in milk/PBS and once in PBS, followed by development with a peroxidase substrate system (TMB membrane system, Kirkegaard and Perry Laboratories) according to the manufacturer's recommendations. In some cases, gels were developed with ^{125}I-labeled secondary antibody (DuPont-NEN, Boston, MA) at 1:1000 dilution, followed by washing as above and autoradiography.

Active site radiolabeling of cysteine proteases was used to confirm the size and presence of cat S in cat S-transfected human kidney 293 cells (American Type Culture Collection CRL 1573, Rockville, MD) and human alveolar macrophages obtained by lung lavage of apparently healthy volunteers with a history of smoking. Aliquots of 10^5 cells were lysed and permeabilized with 1% Triton X-100 at pH 5. To keep the enzyme active, 3 mmol/L cysteine was added as a reducing agent. Samples were incubated with an iodinated analogue of the lysosomal cysteine protease inhibitor, E64d, at 37 C for 30 minutes. After adding 2X reducing sample buffer, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 12% gel. Radiolabeled bands were visualized by autoradiography after 3 days.

Tissue Processing and Immunocytochemistry

Small blocks from several regions of cerebral cortex, including frontal, temporal, parietal, and occipital lobes, as well as from hippocampus, amygdala, basal forebrain, and cerebellum, were briefly fixed for 1 hour in 10% neutral-buffered formalin and then transferred to Tris-buffered saline (150 mmol/L sodium chloride and 50 mmol/L Tris-HCl, pH 7.6) at 4 C until paraffin embedding. Eight-micron serial sections were prepared and baked at 58 C for 1 hour. For immunostaining, sections were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA) and rehydrated. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 20 minutes. Sections were washed in water and pretreated with 80% formic acid for 10 minutes at room temperature in all staining runs except one. Formic acid pretreatment was found to increase AP anti-cat S immunoreactivity. After rinsing in water, the sections were incubated in a blocking solution of 10% goat serum (for rabbit antibodies) or horse serum (for mouse antibodies) in Tris-buffered saline for 20 minutes. Primary antibodies were applied for either 1 hour at room temperature or overnight at 4 C. The horseradish peroxidase avidin-biotin complex system (rabbit or mouse Elite ABC kit, Vector Laboratories) and diaminobenzidine were used to visualize bound antibody. Sections were counterstained with hematoxylin, dehydrated, cleared in Histoclear, and coverslipped with Permount. Frozen sections from two AD and one control case were prepared and fixed in methanol containing 0.3% hydrogen peroxide for 15 minutes. After rinsing in water, these sections were incubated in blocking solution and were subjected to the same protocol as the briefly fixed, paraffin-embedded sections. Occasionally, primary antibody was omitted from sections during immunostaining, yielding consistently negative results.

Results

In a previous study, we demonstrated that cat S, when stably transfected into human 293 kidney cells, could cleave Aβ from βPP and thus enhance the secretion of the amyloidogenic peptide (J.S. Munger, C. Haass, C.A. Lemere, G–P. Shi, W.S.F. Wong, D.B. Teplow, D.J. Selkoe, H.A. Chapman, submitted). In view of this evidence that cat S could be involved in part in the genesis of AD brain lesions, we carried out the current immunocytochemical study to analyze the presence and cellular localization of cat S immunoreactivity among AD, DS, and control brain tissues.

Cat S Antibody Specificity

The specificity of the affinity-purified polyclonal antibody to the cat S fusion protein was shown both by Western blotting of cell lysates and by immunocytochemistry on AD brain. Human alveolar macrophages (which express high levels of cat S) and human kidney 293 cells transfected with cat S cDNA were lysed and examined on Western blots (Figure 1). Each lysate contained a single cat S immunoreactive band of approximately 24 kd. Active site labeling with the radioiodinated cysteine protease inhibitor analogue E64d confirmed the identity of the authentic 24-kd cat S band in both cell types (Figure 1). In initial experiments on AD and DS brain sections, the cat S antibody detected a subset of neurons (including some tangle-bearing neurons), astrocytes, and cells in blood vessel walls. Figure 2 demonstrates the specificity of this staining for neurons and for blood vessel cells in adjacent serial sections of AD brain. Staining of selected neurons was obtained with the affinity-purified cat S antibody after preadsorption on a cat L-MBP fusion protein (Figure 2a). Cat L is a cysteine protease that has 49% sequence homology to cat S; adsorbing the cat S antibody on this related but distinct protease did not alter its immunoreactivity. The neuronal staining was greatly diminished when the antibody was adsorbed on a cat S-MBP fusion protein (Figure 2b). Staining of neurofibrillary tangle-bearing neurons was not completely adsorbed by the cat S fusion protein but was significantly diminished (data not shown). Immunostaining of many cells in blood vessel walls that appeared to be smooth muscle cells was routinely observed with the cat S antibody preadsorbed with the cat L-MBP fusion protein (Figure 2c). Complete abolition of this meningocerebral vascular reactivity occurred after adsorption on the cat S-MBP fusion protein (Figure 2d).

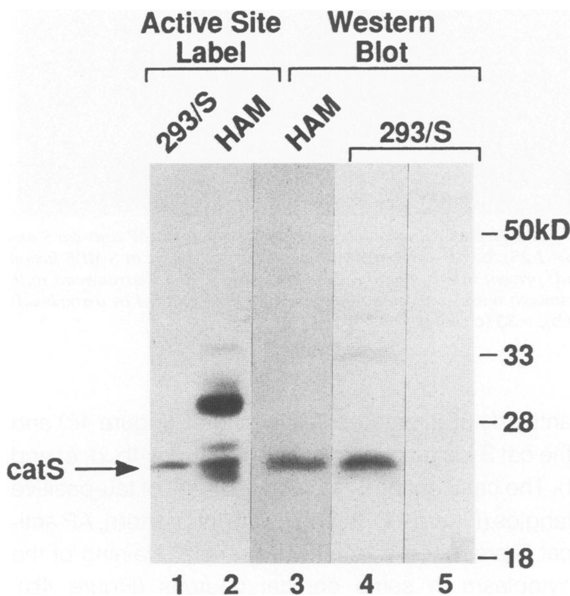


Figure 1. *Cat S antibodies are highly specific for cat S. Human alveolar macraphages (HAM) or human kidney 293 cells expressing recombinant human cat S (293/S) were lysed and either active site labeled with a radioiodinated class-specific, cysteine protease inhibitor (E64d, lanes 1 and 2) or immunoblotted and stained with cat S antibodies (1:500, lanes 3 to 5). Antibodies used to stain the 293/S lysate in lane 5 were preadsorbed with cat S-MBP fusion protein before immunoblotting. The bands above cat S in lane 2 represent cat B (large band) and another unknown cysteine protease in HAM that are also detected by iodinated E64d.*

Characterization of Cat S Immunostaining in Control Human Brain Tissue

Throughout this study, we found that cat S immunoreactivity was much less in control than in AD or DS brain tissue. Figure 3a exemplifies the most common result when cat S antibody is used to immunostain control brains. In this 60-year-old control subject, the hippocampal neurons, including the larger pyramidal cells, are not reactive with the cat S antibody. Such lack of cat S neuronal immunoreactivity was observed in 16 of the 21 control cases examined. However, slight cat S immunoreactivity was observed in a subset of neurons (mostly pyramidal cells) in 5 of the 21 control brains examined. Figure 3b shows an example of such rare immunostaining; a subset of pyramidal cells are lightly stained in this hippocampal section of an 84-year-old control subject. Vascular immunostaining with the cat S antibody was much more common among controls and was observed in 15 of 21 control cases. Immunostaining of the cytoplasm of apparent smooth muscle cells in the leptomeningeal vessels was the most frequently observed pattern, as exemplified in Figure 3c. However, staining of the entire blood vessel wall, including the adventitia, was sometimes observed in smaller vessels in the brain parenchyma. Occasional glial cell staining was observed in only 2 of the 21 controls and appeared to represent astrocytes (data not shown).

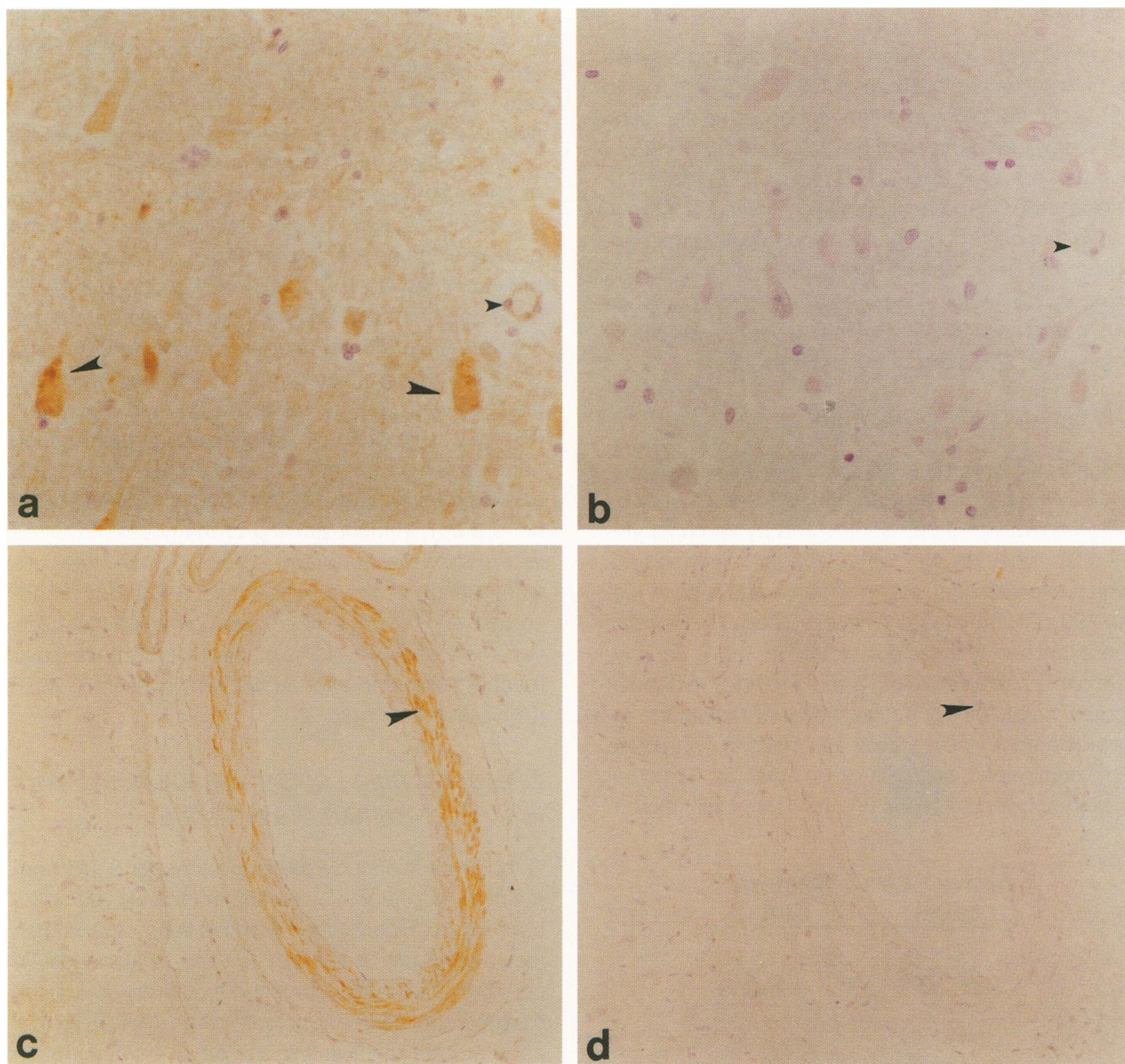


Figure 2. *Specificity of cat S polyclonal antibody on AD hippocampus* (a *and* b) *and leptomeningeal blood vessels* (c *and* d). a: *AP anti-cat S antibody adsorbed on a cat L-MBP fusion protein immobilized on nitrocellulose (1:25).* b: *AP anti-cat S antibody adsorbed on a cat S-MBP fusion protein on nitrocellulose (1:25). Neuronal immunoreactivity (large arrowhead) present in* a *is almost fully abolished in* b. *Small arrowhead indicates the same microvessel in these adjacent 8-μ sections. Similarly, vascular smooth muscle cell immunoreactivity (as exemplified by arrowhead) is apparent in* c *but is completely abolished in* d. *Magnification,* ×*80* (a *and* b); ×*33* (c *and* d).

Characterization of Cat S Immunostaining in AD and DS Brain Tissue

Neuronal, vascular, glial, and rare plaque-like immunoreactivities for cat S were observed in most of the AD and DS brains. Varying degrees of neuronal immunoreactivity in hippocampus and neocortex were seen in all 24 AD cases and in 9 of 10 DS cases. Examples of the three patterns of neuronal staining we observed are shown in Figure 4. Serial sections through AD hippocampus were immunostained with antibody R1280 for Aβ-positive plaques (Figure 4a), antibody 5E2 for tau-positive tangles (Figure 4c) and the cat S fusion protein antibody (Figure 4b, d, e, and f). The cat S antibody stained a subset of tau-positive tangles (Figure 4e). In addition to this pattern, AP anti-cat S produced fine, diffuse granular staining of the cytoplasm in some cortical neurons (Figure 4b). Large pyramidal cells, as well as smaller neurons and some astrocytes, showed such fine cytoplasmic staining. Finally, occasional pyramidal neurons showed a more intense and focal immunoreactivity of coarse cytoplasmic granules, suggestive of a lysosomal distribution of cat S in some hippocampal and

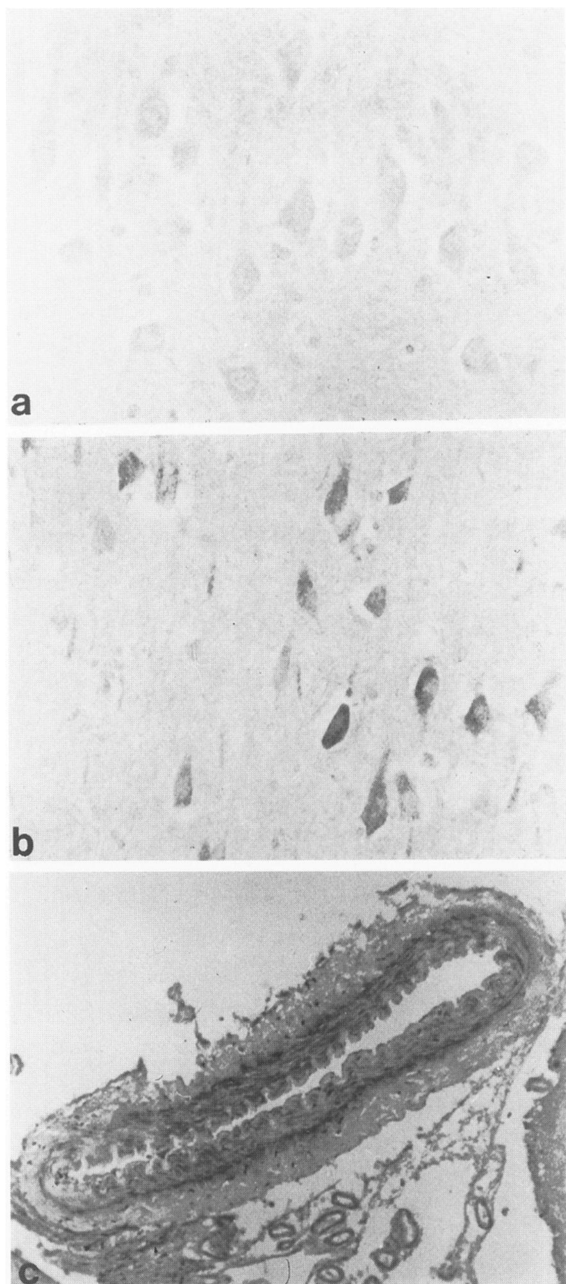


Figure 3. *Cat S immunostaining in control human brain tissue.* **a:** *Lack of neuronal immunoreactivity in the hippocampus (and in cerebral cortex, not shown) of a 60-year-old woman represents the usual finding among control cases.* **b:** *Rare neuronal immunoreactivity of scattered neurons in the hippocampus (and in cerebral cortex, not shown) of an 84-year-old man represents a pattern seen much less frequently.* **c:** *Strong immunostaining in the media of a leptomeningeal blood vessel in a 36-year-old woman represents a common pattern in controls. Magnification,* ×*100* (**a**)*;* ×*80* (**b**)*;* ×*25* (**c**).

cortical neurons in AD brain. The cat S neuronal immunoreactivity in AD and DS brains occurred preferentially in neurons in cortical layers III and V and in pyramidal neurons in CA3, CA2, CA1, and subiculum in the hippocampus. Large pyramidal neurons were the most frequent neuronal type stained, but other morphological types of neurons also reacted. Overall, a small subset of the neuronal populations in AD and DS hippocampal and neocortical sections was reactive with the cat S antibody. When we directly compared the density of cat S-positive neurons in 3 AD brains with that in 3 of the controls that showed cat S staining, neuronal counting revealed an approximately fourfold higher density in the AD cases.

As in control brains, AD and DS brains commonly showed vascular cat S immunoreactivity. At least some cat S-positive vessels were found in 23 of 24 AD and 9 of 10 DS brains. Selective smooth muscle cell staining predominated in leptomeningeal vessels, but reaction of the entire media and adventitia was also seen in some meningeal and smaller parenchymal blood vessels. Some amyloidotic vessels showing typical Aβ-positive outer media and adventitial immunostaining (Figure 5a) were positive for cat S in both smooth muscle cells and adventitia (Figure 5b). However, cat S and Aβ immunoreactivity did not always exist in the same vessel. An example is shown in Figure 5c and d, in which a blood vessel in AD temporal cortex that is negative for Aβ is strongly positive for cat S. Staining of adjacent sections also revealed blood vessels that were Aβ positive but almost completely cat S negative (Figure 5e, inset, and f) but this pattern was infrequent.

Glial immunoreactivity for cat S was demonstrated in 19 of 24 AD and 7 of 10 DS cases. Astrocytes were frequently detected by the cat S antibody. For example, adjacent sections of AD frontal cortex immunostained for the astrocyte marker GFAP and cat S are shown in Figure 5e and f, in which numerous astrocytes are labeled by both antibodies. However, many more astrocytes were labeled with GFAP than with cat S antibodies. Microglia were readily demonstrated by *Ricinus communis* agglutinin-1 in AD and DS cortex and failed to co-localize with cat S immunoreactivity. Instead, a more general pattern of the presence of activated microglia within areas of cat S-positive neurons was observed (data not shown). Activated microglia are often found in clusters in and around sites of injury or inflammation within the brain. Thought to be derived from monocytes, they have a phagocytic function. Thus, the presence of activated microglia near cat S-immunoreactive neurons may indicate a response to neuronal injury or inflammation.

Aβ plaques in AD and DS cortex only rarely showed cat S reactivity. In such cases, the cat S immunoreactivity appeared around the perimeter of the Aβ-positive plaque, as demonstrated in the adjacent sec-

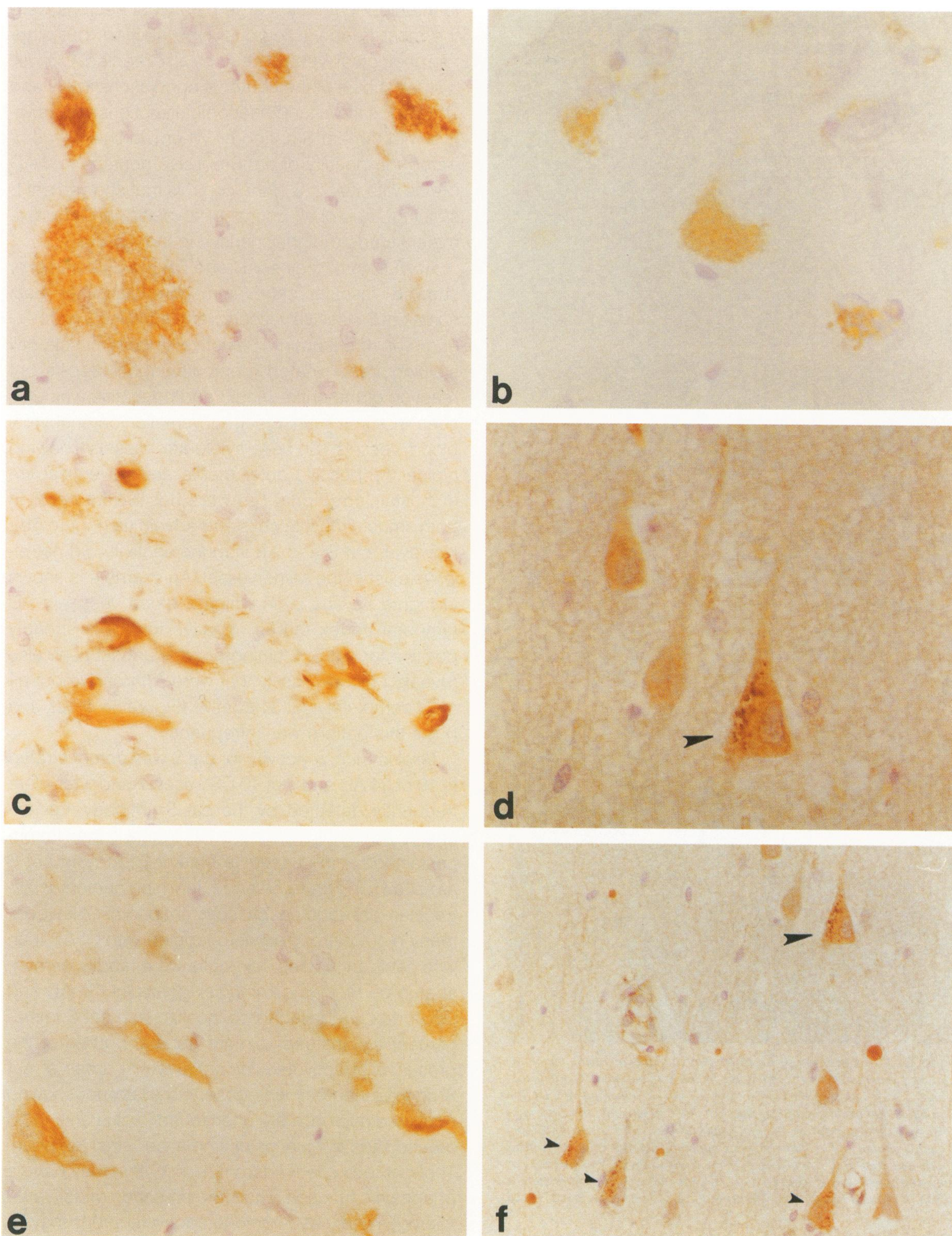


Figure 4. *Cat S immunostaining patterns in AD neurons. Adjacent 8-μ serial sections of AD hippocampus were immunostained with R1280 for Aβ-positive plaques* (a), *5E2 for tau-positive tangles* (c), *and AP anti-cat S, revealing some cat S-positive tangle-bearing neurons* (e). b: *Anti-cat S showed fine, granular cytoplasmic reactivity of selected neurons in AD occipital cortex.* d: *In some hippocampal pyramidal neurons, anti-cat S produced intense immunoreactivity of large granules in the cytoplasm.* f: *Low power view of* d, *showing several pyramidal neurons positive with the cat S antibody. Magnification,* ×*160* (a, c, d, *and* e); ×*200* (b); ×*80* (f).

tions of AD temporal cortex shown in Figure 5g and h. The cat S staining of these infrequent plaques was fine, punctate, and not directly on the amyloid component. GFAP also labeled the perimeter of these plaques (not shown), suggesting that cat S was localized in astrocytic processes associated with the outer edge of the plaques.[39]

Regional Specificity of Increased Cat S Immunoreactivity in AD Brain

We examined the regional specificity of cat S reactivity within representative AD brains. Cat S-positive neuronal staining was observed in the cerebral cortex, amygdala, and hippocampus but not in the cerebellum, as exemplified in the AD case shown in Figure 6. This distribution of cat S staining was observed in numerous AD and DS brains; ie, cat S-positive neurons, astrocytes, and blood vessels were present in the cortex of all four cerebral lobes as well as in amygdala and hippocampus. Although cat S staining of leptomeningeal blood vessels (primarily of smooth muscle cells) was frequently observed in cerebellar sections, neuronal reactivity was much less common here. Thus, the regional distribution of the increased cat S reactivity in AD brains paralleled in part the principal sites of senile plaque formation and neuronal and glial cytopathology.

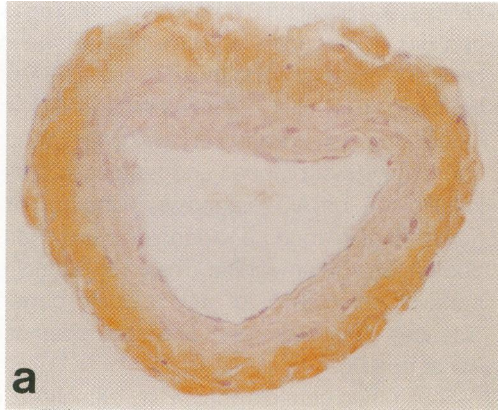
Discussion

We have used affinity-purified antibodies to human cat S to demonstrate a highly consistent increase in immunoreactivity of selected neurons and astrocytes in AD and DS brains as compared with age-matched control brains. Cat S reactivity was also present at the periphery of a few Aβ-positive plaques in some AD and DS brains. Immunostaining of smooth muscle cells and sometimes adventitia was observed in large leptomeningeal blood vessels and some smaller intraparenchymal blood vessels in AD, DS, and control brain tissue.

Whereas vascular smooth muscle immunostaining was commonly seen in control brains (15 of 21 cases), only a small subset of the controls (5 of 21 cases) showed any neuronal immunostaining. In all 5 of these cases, a diffuse, cytoplasmic staining pattern was observed in focal populations of pyramidal neurons in hippocampus and/or cerebral cortex. Of the 5 control subjects who showed such positive neuronal staining with the cat S antibody, 4 were clinically demented at the time of death. Neuropathological examination revealed substantial hippocampal and cortical neuronal loss and gliosis in 3 demented cases (ages 60, 81, and 84). A diagnosis of Pick's disease without Pick bodies was assigned to these cases. The fourth control case was mildly demented (age 92) and was found to have mild neuronal loss in the hippocampus. The numbers of plaques and tangles in cortex were insufficient for AD diagnosis, although abundant numbers of plaques and tangles were seen in hippocampus and amygdala. The case did not achieve pathological criteria for any specific degenerative diseases. This patient's mother was diagnosed with AD at age 95.

Four other controls that were demented did not show any neuronal cat S immunoreactivity. Two of these cases (ages 70 and 77) had no neuropathological changes. The other two cases (ages 80 and 82) had rare plaques and tangles, accompanied in one case by neuronal loss and gliosis in the substantia nigra without Lewy bodies and in the other case by Lewy bodies without significant substantia nigra degeneration. The fifth control that showed positive neuronal staining was a 36-year-old woman who died of breast cancer after undergoing extensive chemotherapy and radiotherapy. Therefore, it appears that cat S protein levels can be increased or its epitope unveiled in the presence of some kinds of neuronal or glial damage.

Cat S immunoreactivity in AD and DS brains was consistently and substantially increased over that observed in control brains. The most commonly observed neuronal staining pattern in AD and DS was a diffuse, fine granularity in the perikaryal cytoplasm. Less commonly, an intense, coarsely granular pattern was observed. The granular reactivity may represent cat S localized in lysosomes; immunoelectron microscopy on very well preserved AD brain tissue is needed to prove this. Antibodies to other cathepsins (B, D, and L) have also shown granular, lysosomal patterns of neuronal immunostaining.[18,40,41] It is interesting that Cataldo and co-workers[18] found intense, diffuse cytoplasmic cat D and B reactivity of lysosomes and lipofuscin granules, suggestive of accumulation, in the perikarya and proximal dendrites in degenerating neurons only in AD brains and observed punctate, lysosomal-like neuronal staining in both AD and control brains. They reported an increase in the total amount of intracellular neuronal staining and extracellular lysosomal staining within senile plaques by cat D and B antibodies in AD cortex. These authors hypothesized that dying neurons may accumulate and release the contents of their lysosomes. Such a phenomenon could explain the increased cat S reactivity in the cytoplasm of neurons in AD brain observed here.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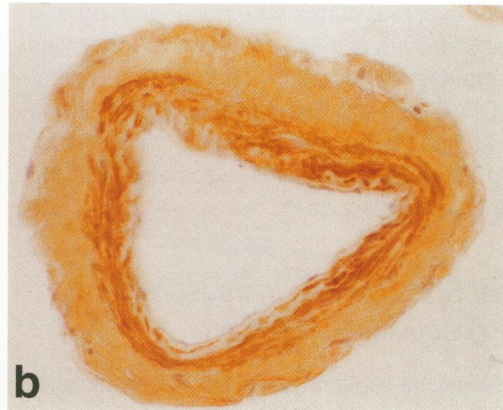
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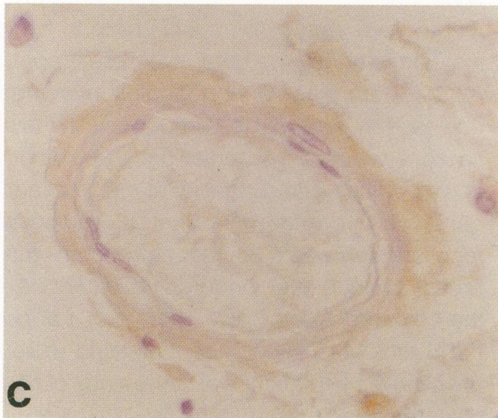
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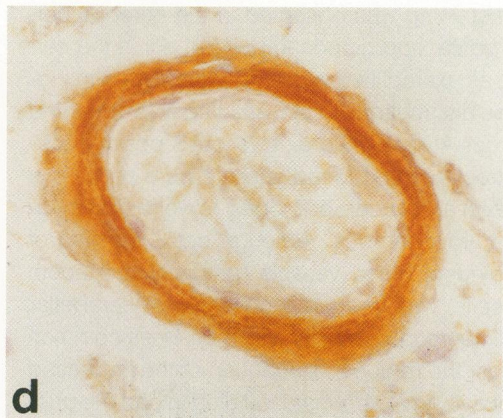
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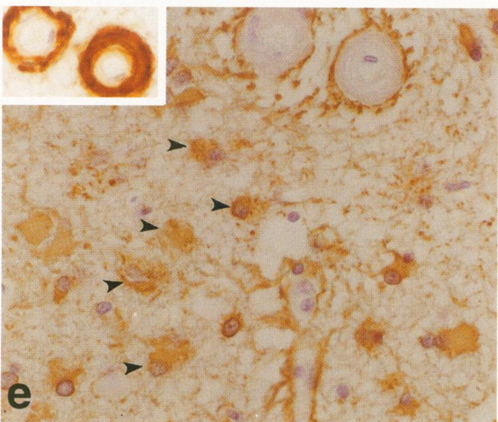
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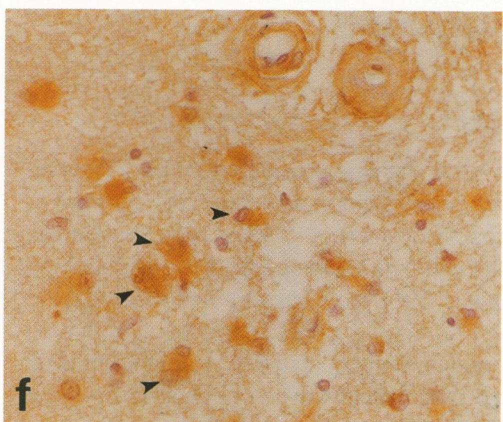
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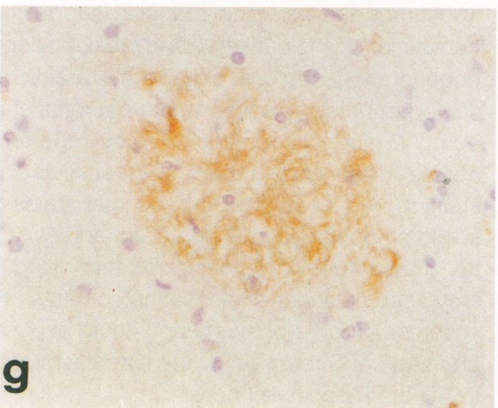
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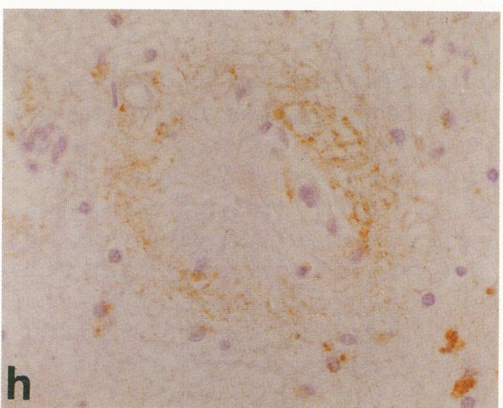
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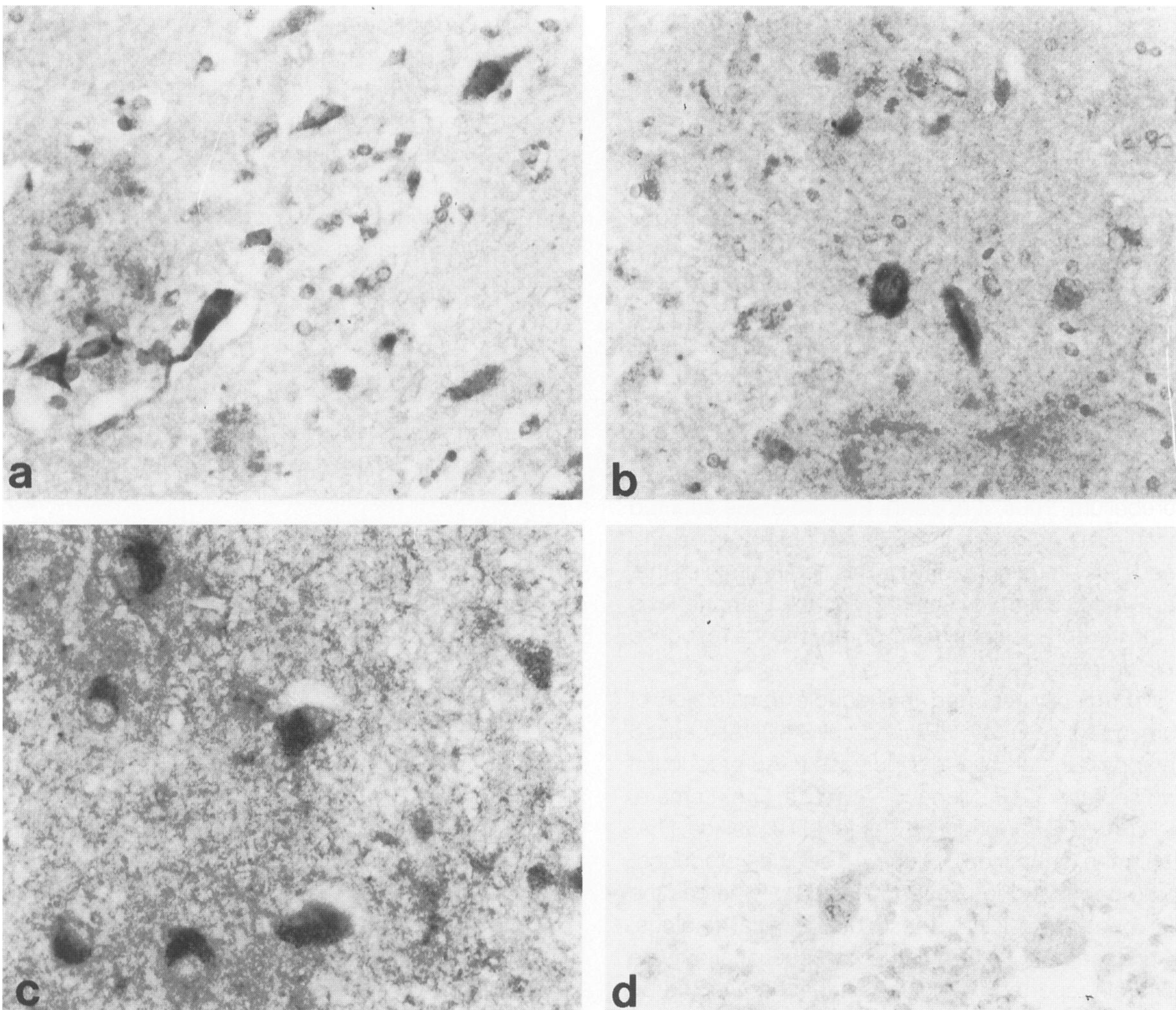

Figure 6. *Regional distribution of cat S immunostaining within a brain from an 80-year-old woman with AD. Positive neuronal staining with AP anti-cat S was observed in the occipital cortex* (a), *amygdala* (b), *and hippocampus* (c) *but not in cerebellum* (d). *Magnification,* ×*100.*

A minority of our AD cases with positive cat S neuronal immunostaining also showed cat S-positive neurofibrillary tangle reactivity. It is not clear whether the tangle-like staining represents cat S that has been released from lysosomes and become associated with the abnormal cytoplasmic filaments comprising the tangles or whether it represents cat S in small peri-tangle vesicles. Granular cat B immunoreactivity on and along neurofibrillary tangles in AD neurons was recently reported.[42] In our study, AD and DS brains also showed prominent cat S labeling of some astrocytes. The presence of other proteases, eg, cat D, L, and H,[18] and certain protease inhibitors, eg, α1-antichymotrypsin[43,44] and anti-thrombin III,[45] has been demonstrated in reactive astrocytes in AD brain. These molecules have been hypothesized to play a role in the proteolytic cleavage of βPP and/or the subsequent accumulation of extracellular Aβ peptides. At the mRNA level, cat S message has been demonstrated in microglia in adult rat brain by *in situ* hybridization.[33] Petanceska and co-workers[34] recently found that cat S expression was up-regulated and its message localized to astrocytes and activated microglia after lesioning of the entorhinal cortex in rats.

Figure 5. *Characterization of cat S immunoreactivity in AD blood vessels, astrocytes, and plaques in AD brain.* a *and* b: *Adjacent 8-μ sections of a typical meningeal blood vessel immunoreactive for Aβ in the adventitia* (a) *and cat S in smooth muscle cells as well as adventitia* (b). c *and* d: *Adjacent sections of meningeal blood vessels negative for Aβ* (c) *but positive for cat S* (d). e *and* f: *Adjacent immunostained sections of AD temporal cortex showing co-localization of the astrocytic marker, GFAP* (e), *and cat S* (f). *Arrowheads indicate several of the doubly positive astrocytes. Note that the two arterioles in* e *and* f *are negative with GFAP* (e) *and positive with antibody R1280 for Aβ* (e, inset). *These arterioles are only slightly reactive with the cat S antibody* (f). g *and* h: *Adjacent sections of AD temporal cortex showing a senile plaque stained with R1280 for Aβ* (g) *and its perimeter stained with the cat S Ab. Magnification,* ×*80* (a *and* b); ×*160* (c *and* d); ×*100* (e, f, g, *and* h).

This finding is consistent with the hypothesis that cat S up-regulation may represent a secondary response to neuronal damage.

Although the increase in cat S immunoreactivity in AD brain tissue was usually less extensive than the density of amyloid plaques and neurofibrillary tangles, it followed closely the regions of brain affected pathologically in AD. Often, the hippocampus and temporal cortex contain the highest density of tangles and plaques in AD brain. It was these areas that also showed the greatest numbers of cat S-immunoreactive cells in AD and DS brains. Other areas affected in AD, such as occipital, parietal, and frontal cortex, showed less frequent cat S-positive neurons and reactive astrocytes. In concert with this pattern, little cat S immunoreactivity was found in AD cerebellum. Thus, it appears that the areas affected most in AD are those in which we observed heightened cat S immunoreactivity. This finding implies that cat S may either play some role in the pathogenesis of the cerebral lesions of AD or represent a response to their formation.

We recently reported that double transfection of human 293 cells with βPP and cat S cDNAs caused an approximately two-fold increase in Aβ production and secretion (J.S. Munger, C. Haass, C.A. Lemere, G–P. Shi, W.S.F. Wong, D.B. Teplow, D.J. Selkoe, H.A. Chapman, submitted). Of the Aβ species produced, some began at the usual Asp^{1} residue whereas the rest began at Met^{-1}. These biochemical findings, taken together with the immunocytochemical data on AD brain reported here, suggest that cat S could be involved in part in processing of βPP to Aβ; ie, it may be one of multiple proteases capable of creating the amino terminus of Aβ. Different Aβ-generating proteases may be specific to different cell types. Although the immunoreactivity for cat S in autopsied control brain appears to be quite low, there may be factors during the neurodegenerative process in AD that trigger an up-regulation of cat S, create an altered protein form of cat S, or unmask the cat S epitope. If more cat S protein is made in AD neurons and glia, more Aβ might be generated from βPP. It is now important to determine where cat S mRNA is expressed in human brain and whether the expression levels increase in AD. *In situ* hybridization studies are underway to address these questions.

Our findings underscore the need to determine when and how increases in neuronal and astrocytic cat S contribute to AD pathogenesis. Because the βPP gene is localized to chromosome 21, trisomy 21 may provide cat S with an increase in substrate, perhaps resulting in a secondary increase in cat S protein levels relatively early in the course of DS. In this regard, we are currently examining DS brains of increasing ages beginning with ages (10 to 15 years) at which Aβ plaque formation is just beginning. However, increased cat S immunoreactivity may also represent a secondary event that follows AD-type neuropathological changes as well as some other kinds of neuronal injury and/or gliosis. To address this question, we are now examining cat S immunoreactivity in non-AD neurodegenerative diseased brains and head trauma cases.

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