American Journal of Pathology, Vol. 137, No. 5, November 1990

Early Accumulation of Heparan Sulfate in Neurons and in the Beta-amyloid Protein-containing Lesions of Alzheimer's Disease and Down's Syndrome

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A monoclonal antibody (HK-249) that recognizes a glucosamine sulfate alpha 1 → 4 glucuronic acid-containing determinant in heparan sulfate (HS) chains of a basement membrane-derived heparan sulfate proteoglycan identified and immunolocalized HS specifically to the amyloid deposits in neuritic plaques (NPs), congophilic angiopathy (CA), as well as in neurofibrillary tangles (NFTs) and non-tangle-bearing neurons in the brains of Alzheimer's and Down's syndrome (DS) patients. Ultrastructural immunohistochemistry demonstrated that HS within neurons of Alzheimer's disease (AD) brain was localized to lipofuscin granules, an aging pigment previously shown also to contain beta-amyloid protein (BAP). Heparan sulfate also was localized to neurite-containing, nonfibrillar 'primitive' plaques that also demonstrated positive BAP immunoreactivity in both AD and DS brains. Antibodies to laminin, fibronectin, and a chondroitin sulfate proteoglycan failed to show positive immunostaining of the HS-containing sites described above. Analysis of DS patients at different ages revealed that HS accumulated within neurons of the hippocampus and amygdala as early as 1 day after birth. Young age-matched controls did not demonstrate similar positive HS immunoreactivity in neurons, whereas positive immunostaining for HS was observed in other regions thought to normally contain HS. The earliest deposition of BAP was first observed as 'amorphous' or 'diffuse' cortical deposits in DS brain in patients aged 18 and 24 years before the accumulation of fibrillar amyloid (observed in DS patients who are 35 years and older). These cortical deposits also contained positive HS immunoreactivity, implying that HS accumulation in conjunction with the BAP is an early event that ultimately may contribute to the early age-related accumulation (ie, as early as 35 years of age in DS) of NPs, NFTs, and/or CA. Furthermore the colocalization of HS and BAP in a number of specific locales in AD and DS brain indicates a possible interaction between these two macromolecules that may be important in lesion development in these two diseases. (Am J Pathol 1990, 137:1253–1270)

The characteristic lesions of Alzheimer's disease (AD) consist of the presence of neuritic plaques (NPs), neurofibrillary tangles (NFTs), and cerebrovascular amyloid deposits termed congophilic angiopathy.[1,2] In addition, granulovacular degeneration[3,4] and Hirano bodies[4] also may be considered pathogenetic markers in the brains of AD patients. Although some of these lesions may be found in the brains of aged individuals, they are present in much lesser amounts, indicating that the diagnosis of AD relies primarily on the quantitative assessment of these lesions.[5]

A number of different components have been identified and localized to the characteristic lesions of both AD and Down's syndrome (DS). The amyloid deposits within NPs and congophilic angiopathy contain the beta-amyloid[6,7] or A4[8] protein, and the serine protease inhibitor, alpha 1-antichymotrypsin.[9] Recent studies also demonstrate that the neuritic component of NPs in the AD brain contain protease nexin II (thought to represent the secreted version of the beta-amyloid protein precursor containing the Kunitz inhibitor domain)[10,11] as well as binding sites for protease nexin I.[12]

A number of different components also have been identified and localized within the substructures of NFTs,

Supported by the Alzheimer's Disease Research Program of the American Health Assistance Foundation and by NIH grant P50 AG05136. Koji Kimata received financial support from the Life Science Research Project of Institute of Physical and Chemical Research (Riken).

Accepted for publication July 9, 1990.

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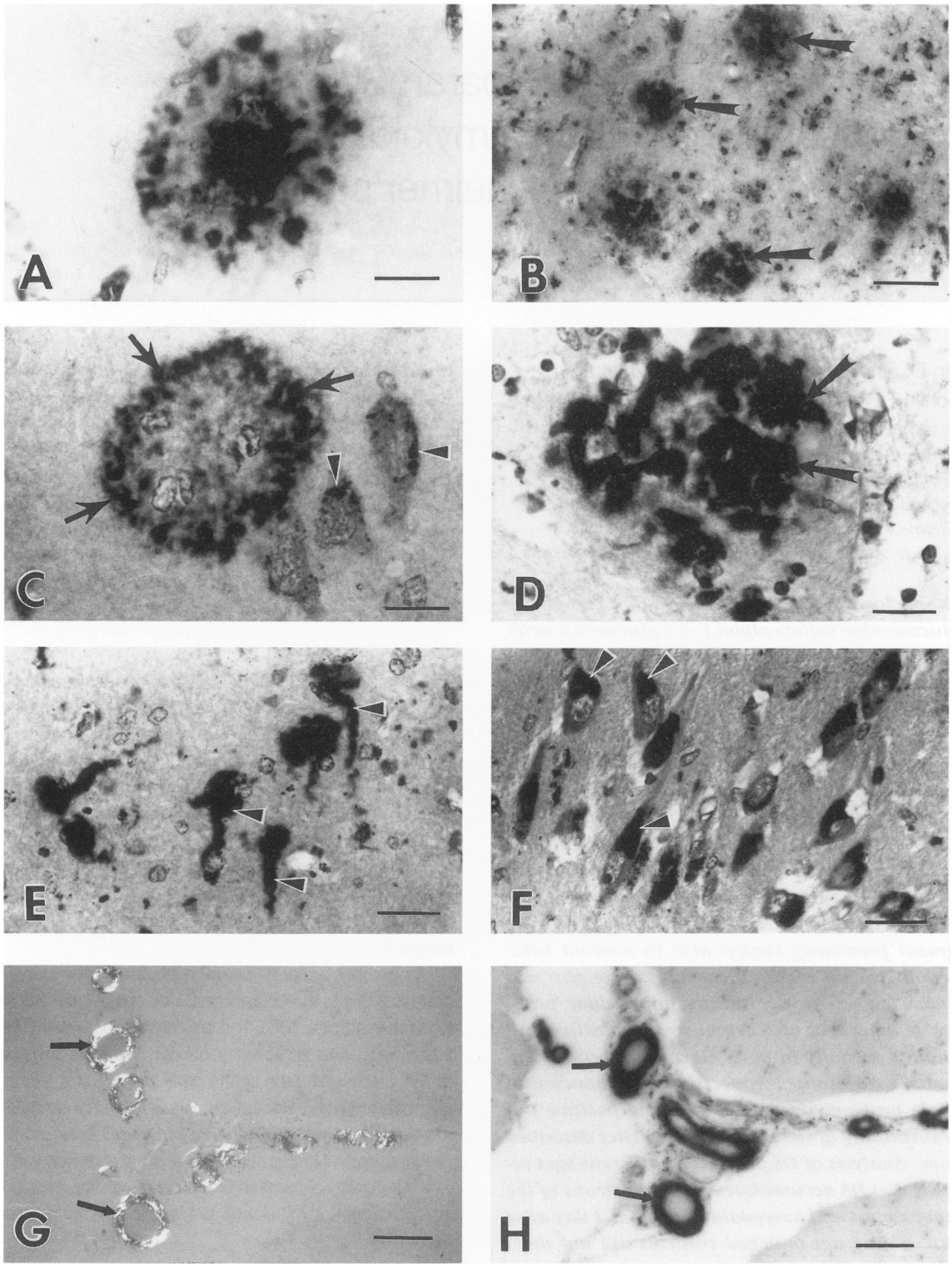

Figure 1. *Immunolocalization of heparan sulfate glycosaminoglycans in neurons and in the characteristic lesions of Alzheimer's disease.* **A:** *Immunostaining of an amyloid plaque in the hippocampus of a 90-year-old woman with AD (5.5 hours after death) using the HS monoclonal antibody (HK-249, undiluted supernatant) recognizing HS GAG chains.*[31] *Note positive immunostaining of central amyloid core and periphery. Formic acid pretreatment; hematoxylin counterstain. Bar, 21 μm.* **B:** *Immunostaining of primitive plaques (arrows) in the amygdala of a 64-year-old man with AD (4 hours after death) using HK-249 (undiluted supernatant). Formic acid pretreatment; hematoxylin counterstain. Bar, 80 μm.* **C:** *Heparan sulfate GAG immunostaining (HK-249, 1:5 dilution) in the periphery (arrows) of a primitive plaque in the hippocampus of a 90-year-old woman with AD (5 hours after death). Note HS immunostaining also in the cell bodies of adjacent neurons (arrowheads). Formic acid pretreatment; hematoxylin counterstain. Bar, 20 μm.* **D:** *Strong immunostaining of a primitive plaque using HK-249 (undiluted) in the hippocampus of a 67-year-old man with AD (5.5 hours after death). Note immunostaining of neurites in plaque (arrows). Formic acid pretreatment; hematoxylin counterstain. Bar, 20 μm.* **E:** *Heparan sulfate immunostaining (HK-249, 1:5) of NFTs (arrowheads) in the amygdala of a 64-year-old man with AD (4 hours after death). Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.* **F:** *Strong*

such as microtubule-associated phosphoproteins, including the 'tau protein,'[13–15] neurofilaments,[16–18] and ubiquitin.[19,20]

Previous studies[21–23] demonstrated that the amyloid deposits within NPs and congophilic angiopathy also contain a specific class of complex carbohydrates known as proteoglycans. Immunocytochemical studies recently identified heparan sulfate proteoglycan (HSPG) core protein localized to the amyloid deposits in these lesions.[24] However it was not known whether HSPG accumulation is an early or late event in the accumulation of BAP. Because similar, if not identical, pathologic changes to those of AD are observed in the brains of nearly all patients with DS who live longer than approximately 35 years, DS has been considered a useful model for studying the sequence of changes that occur in AD.[25–29] In the present study, in addition to the identification and localization of heparan sulfate (HS) glycosaminoglycan (GAG) chains in the AD brain, we examined the brains of DS patients at various ages for evidence of early changes that precede the formation and accumulation of fibrillar amyloid.

Materials and Methods

Autopsy Material

Brain tissue (hippocampus, amygdala, and cerebellum) obtained within 5 hours after death from seven cases of AD (confirmed at autopsy) was used in the present study. The brain tissue was obtained from a 90-year-old woman, a 74-year-old man , two 72-year-old men, a 67-year-old man, a 66-year-old woman, and a 64-year-old man. These tissues were obtained from cases of rapid autopsy at the University of Washington Alzheimer's Disease Research Center. Brain tissue (including hippocampus, amygdala and cerebellum) also was obtained from 12 cases of DS, acquired either from the Department of Neuropathology at the University of Washington or from Children's Hospital in Seattle (courtesy of Dr. J. Sibert). Eight of these DS cases were younger than 25 years old (and included ages of 1 day, 4 months, and 2, 7, 15, 16, 18, and 24 years), whereas 4 of these cases were older than 35 years (and included ages of 36, 37, 47, and 51 years). In addition, brain tissue (including hippocampus, amygdala, and cerebellum) was obtained at autopsy from an 11-year-old child with congenital heart disease, a 13-year-old child with Huntington's disease, a 14-year-old child with cystic fibrosis, and a 18-year-old man with cardiomyopathy.

Fixation and Processing of Tissue

The AD brain tissues obtained at rapid autopsy were fixed in Carnoy's solution[30] for light microscopy, whereas the brain tissue obtained from cases of DS and age-matched controls were fixed in 10% formalin for 24 to 48 hours. All tissues were processed routinely and embedded in paraffin. From each paraffin block, 6-μ serial sections were cut and placed on gelatin-coated slides. For electron microscopy (two cases of AD were analyzed), small pieces of hippocampus were fixed in a solution of 3% paraformaldehyde and 0.25% glutaraldehyde in 0.05 mol/l (molar) phosphate buffer and processed as previously described.[24]

Antibodies Used for Immunocytochemistry

A monoclonal antibody (known as HK-249), recognizing a specific epitope on the GAG chain of the basement-membrane derived HSPG, was used for immunocytochemical identification and localization of HS in AD, DS, and age-matched control brain tissue. Detailed characterization of this antibody is described elsewhere.[31] Briefly, a HSPG fraction (a mixture of a low-density and a high-density form, designated LD and HD, respectively) was prepared from the mouse Engelbreth-Holm-Swarm (EHS) sarcoma.[32] A mixture of HSPG fractions (400 μg HSPG as protein in 250 μl of phosphate-buffered saline) and an equal volume of Freund's complete adjuvant was injected intraperitoneally into a 5-week-old Wistar rat. The rat received booster injections with Freund's incomplete adjuvant at 2-week intervals. Three days after the sixth injection, the spleen was harvested. According to a conventional method,[33] hybridomas were produced from rat spleen cells and NS-1 mouse myeloma cells. Hybridoma supernatants were tested for reactivity by enzyme-linked immunoassay with the HSPG preparation used as the immunogen. Hybridomas showing a positive reaction were cloned and then subcloned after dilution. One of the cloned hybridomas (designated HK-249) was found to recognize both the intact LD and HD but not after heparitinase treatment. HK-249 was also found to react with

immunostaining in the cell bodies of neurons (arrowheads) using HK-249 (undiluted) in the hippocampus of a 90-year-old woman with AD (5.5 hours after death). These neurons do not contain NFTs because on adjacent serial sections they were negative with Congo red staining (not shown). Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm. **G:** *Positive congo red staining (as viewed under polarized light) of meningeal blood vessels (arrows) in the cerebellum of a 74-year-old man with AD indicating the presence of amyloid in these vessels. Bar, 108 μm.* **H:** *Serial section of tissue shown in* **G** *demonstrates HK-249 immunostaining (undiluted supernatant) in meningeal blood vessels containing amyloid deposits (arrows). Bar, 108 μm.*

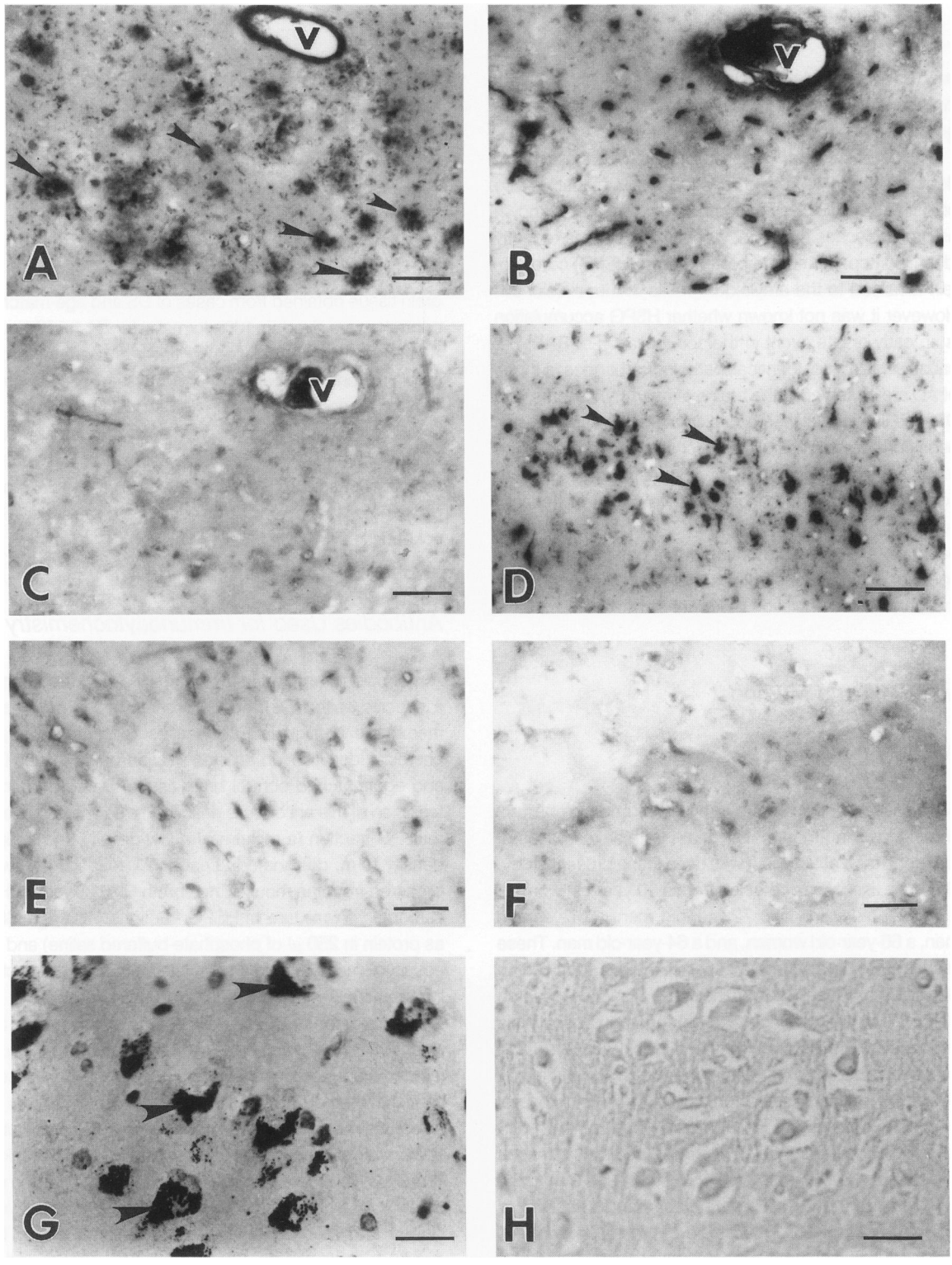

Figure 2. *Specificity of heparan sulfate glycosaminoglycan immunostaining in Alzheimer's brain.* **A:** *Immunostaining of amyloid plaques (arrowheads) using HK-249 (1:10 dilution) in the amygdala of a 64-year-old man with AD. Note immunostaining of vessel (v), which on adjacent section was found to contain amyloid due to positive Congo red staining as viewed under polarized light (not shown). Formic acid pretreatment; hematoxylin counterstain. Bar, 140 μm.* **B:** *Adjacent serial section from* **A** *immunostained with an antibody against fibronectin (dilution 1:10). No positive immunostaining of amyloid plaques (shown in* **A**) *is observed. However positive immunostaining of capillaries in the parenchyma is demonstrated. Formic acid pretreatment; hematoxylin counterstain. Bar, 140 μm.* **C:** *Adjacent serial section from* **C** *immunostained with an antibody against laminin (dilution 1:10). No positive immunostaining of amyloid plaques (shown in* **A**) *is observed. Positive immunostaining of meningeal vessels with the laminin antibody was found (not shown), indicating that the antibody detected laminin in the AD brain. Identical vessel as in* **A** *and* **B** *is marked (v). Formic acid pretreatment; hematoxylin counterstain. Bar, 140 μm.* **D:** *Strong immunostaining of neurons and NFTs (arrowheads) using HK-249 (1:10 dilution) in the amygdala of a 64-year-old patient with AD. Formic acid pretreatment; hematoxylin counterstain. Bar, 108 μm.* **E:** *Adjacent serial section from* **D** *demonstrates lack of positive immunostaining in neurons*

the HS released from the protein core of the HSPG by either proteolysis or alkaline beta elimination.[31] HK-249 also was found to be inactive at the highest concentrations tested (1 mg/ml) with hyaluronic acid, chondroitin sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, heparin, dextran sulfate, polyvinyl sulfate, and DNA. Further characterization of the HK-249 antibody reveals that the epitope it recognizes is a cluster of glucosamine-sulfate glucuronic acid units in intact HS chains, which are particularly rich in the side chains of the EHS proteoglycan.

The HS antibody (medium of the HK-249-producing hybridoma) was used in the present study on deparaffinized sections, either undiluted or at a dilution of 1:5 or 1:10. Other antibodies used in the immunocytochemical studies at the light microscopic level include 1) an affinity-purified polyclonal antibody against fibronectin (Cappel, West Chester, PA; used at a dilution of 1:10 and 1:100), 2) a rat monoclonal antibody that recognizes laminin (ICN Biochemicals, Cost Mesa, CA; used at a dilution of 1:10 and 1:100), 3) two mouse monoclonal antibodies (known as MAB-938 and MAB-941) that recognize epitopes present on the GAG chains of the chondroitin sulfate proteoglycan[34] (used at dilutions of 1:10, 1:50 and 1:100), and 4) a polyclonal antibody against the beta-amyloid or A4 protein (residues 1-42 of the BAP; used at a dilution of 1:100; courtesy of Dr. C. Masters, Australia).

To preclude nonspecific binding and to ensure specificity of the HS monoclonal antibody, sections also were treated with the HS antibody after preincubation (overnight at 4°C) in the presence of excess HSPG antigen (antigen: antibody protein concentration of 7:1). Isolation of the HSPG antigen used for the immunoabsorption experiments was described previously.[32] In addition, TRIS-buffered saline rather than the primary HS antibody also served as a control.

Histochemical and Immunocytochemical Methodology

Congo red staining[35] was used on paraffin sections to detect and localize the NPs, NFTs, and cerebrovascular amyloid deposits in each of the cases. In addition, detection of sites of BAP accumulation was identified by immunostaining with the beta-amyloid or A4 antibody. Adjacent serial sections were immunostained for laminin, fibronectin, or chondroitin sulfate proteoglycan, as described above.

Immunostaining of tissue sections was accomplished using the avidin-biotin-immunoperoxidase method, using the appropriate biotin-labeled secondary antibodies, followed by incubation with avidin-conjugated horseradish peroxidase complex (Vector Labs, Burlingame, CA). Peroxidase activity was detected by treatment with 3,3-diaminobenzidine, as previously described.[24] For immunocytochemical staining, the primary antibody was used initially through a series of dilutions to obtain the best specificity with the least background staining. Only the optimum dilutions of primary antibody are reported. Enhancement of immunodetection of HS and other antigens was achieved by pretreatment of tissue sections for 5 minutes with 88% formic acid before immunostaining.[24,36] The precise mechanism by which formic acid treatment enhances proteoglycan and amyloid antigenicity is not known. In most instances, tissue sections following immunostaining were counterstained with hematoxylin.

Ultrastructural Immunolocalization of Heparan Sulfate in Alzheimer's Brain

Before ultrastructural studies, HK-249 was used to immunolocalize HS in the hippocampus in two cases of AD using ultra-thin (1 μ) sections after removal of epon.[37,38] Briefly, before immunostaining, slides with 1- to 2-μm plastic sections were submerged in a solution of saturated sodium hydroxide in absolute ethanol (sodium ethoxide), diluted 1:1 with fresh absolute ethanol for 15 to 20 minutes at room temperature, and processed for immunocytochemical staining, as previously described.[39]

Heparan sulfate was immunolocalized at the ultrastructural level in the hippocampus in two cases of AD. Thin sections were etched by submersion in a solution of saturated sodium hydroxide in absolute ethanol and diluted 1:1 with fresh absolute ethanol for 1.5 minutes at room temperature. The immunostaining procedure was as follows: 1) grids first were blocked with 10% normal goat serum in 0.05 mol/l TRIS-HCl buffered saline (TBS), pH 7.6 for 10 minutes; 2) four rinses with TBS during a 10-

and NFTs using an antibody directed against fibronectin (1:10 dilution). Only staining of capillaries is observed. Formic acid pretreatment; hematoxylin counterstain. Bar, 108 μm. **F:** *Adjacent serial section from* **E** *demonstrates lack of positive immunostaining in neurons and NFTs using an antibody directed against laminin (1:10 dilution). Formic acid pretreatment; hematoxylin counterstain. Bar, 108 μm.* **G:** *Immunostaining within the cell bodies of pyramidal neurons (arrowheads) using the HS antibody (HK-249, 1:10 dilution) in the hippocampus of an 80-year-old woman with AD. No formic acid pretreatment; no counterstain. Bar, 80 μm.* **H:** *Adjacent serial section from* **G** *demonstrates lack of positive immunostaining in pyramidal neurons using a monoclonal antibody (known as MAb 938) (1:50 dilution) directed against the GAG chains of a chondroitin sulfate proteoglycan. Similar results were obtained with the monoclonal antibody known as MAb 941. The walls of meningeal vessels were found to stain with this antibody (not shown). No formic acid pretreatment; hematoxylin counterstain. Bar, 90 μm.*

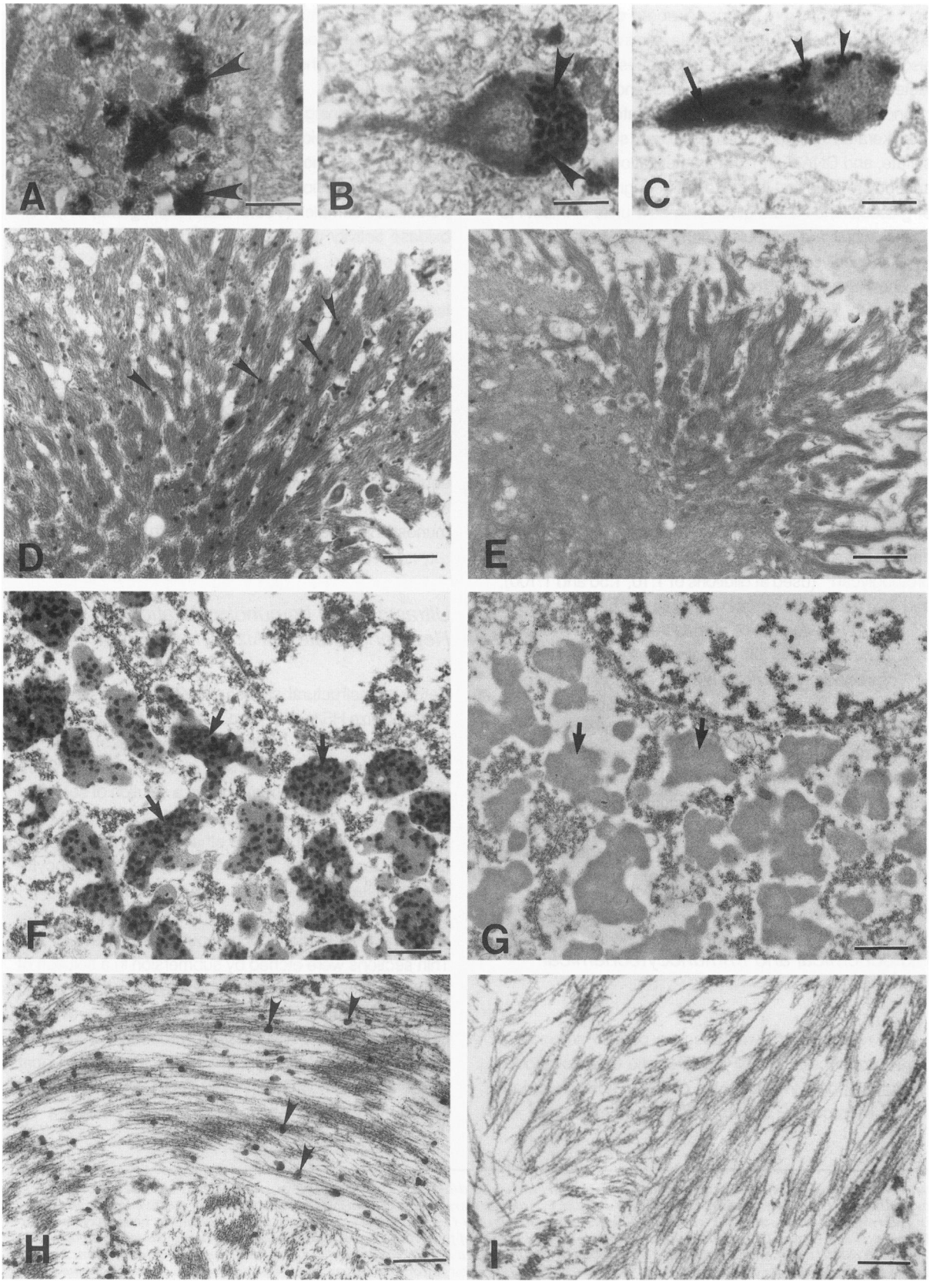
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Figure 3. *Light microscopic and ultrastructural immunolocalization of heparan sulfate in Alzheimer's brain tissue. All tissue sections shown are from the hippocampus of a 72-year-old man with AD (4 hours after death).* **A:** *Heparan sulfate immunostaining (HK-249, 1:10 dilution) of amyloid deposits (arrowheads) in a neuritic plaque. One-micron section immunostained after plastic removal.*[38] *Bar, 13 μm.* **B:** *Heparan sulfate immunostaining (HK-249, 1:10 dilution) of inclusions (arrowheads) within a pyramidal neuron. One-micron-thick section immunostained after plastic removal. Bar, 11 μm.* **C:** *Heparan sulfate immunostaining (HK-249, 1:10 dilution) of neuronal inclusions (believed to be lipofuscin granules) (arrowheads) and filaments (arrow) in a NFT. One-micron-thick section immunostained after plastic removal. Bar, 8 μm.* **D:** *Heparan sulfate immunostaining (HK-249, 1:200 dilution) in a burned-out amyloid plaque at the ultrastructural level. Reaction product (arrowheads) (using 4-chloro-1-naphthol) is specifically localized to the amyloid fibrils. Avidin-biotin-peroxidase method. Bar, 0.78 μm.* **E:** *Serial section from* **D** *showing the identical burned-out plaque, immunostained with HS antibody (HK-249) preabsorbed with excess HSPG antigen. No positive immunostaining of amyloid fibrils is observed. Avidin-biotin-peroxidase method. Bar, 0.78 μm.* **F:** *Heparan sulfate immunostaining (HK-249, dilution of 1:200) in a neuron is specifically localized to lipofuscin granules (arrows) at the ultrastructural level. Avidin-biotin-peroxidase method. Bar, 0.83 μm.* **G:** *Serial section from* **F** *demonstrates lack of positive immunostaining in lipofuscin granules (arrows) in neuron after immunostaining with HK-249 (final dilution of 1:200) preabsorbed with excess antigen. Avidin-biotin-peroxidase method. Bar, 0.83 μm.* **H:** *Heparan sulfate immunostaining (HK-249, 1:200 dilution) of paired helical and straight filaments (arrowheads) in an intraneuronal NFT at the ultrastructural level. This thin section was pretreated with 88% formic acid (1:100 dilution) for 3 minutes before immunostaining. Avidin-biotin-peroxidase method. Bar, 0.40 μm.* **I:** *Serial section from* **H** *demonstrates lack of positive immunostaining of paired helical and straight filaments in an intraneuronal NFT, after immunostaining with HK-249 (final dilution of 1:200) preabsorbed with excess HSPG antigen. Avidin-biotin-peroxidase method. Bar, 0.48 μm*

minute period; 3) primary HS antibody (HK-249) (1:10, 1:50, and 1:200 dilution in TBS) overnight at 4°C; 4) six rinses with TBS during a 15-minute period; 5) incubation with biotinylated goat anti-rat IgG at a dilution of 1:200 for 1 hour at room temperature; 6) seven rinses with TBS during a 30-minute period; 7) incubation with horseradish peroxidase conjugated strepavidin at a dilution of 1:400 for 1 hour at room temperature; 8) seven rinses with TBS during a 30-minute period; 9) incubation with 4-chloro-1-naphthol for 2 minutes; 10) ten rapid rinses with TRIS buffer during a 10-minute period; 11) rinse under a stream of filtered deionized water for 30 seconds; 12) blot dry; and 13) counterstaining with 4% aqueous uranyl acetate and lead citrate. In addition, some thin sections were pretreated with 88% formic acid (diluted 1:100 in distilled water) for 3 minutes and washed in running distilled water for 10 minutes before application of the primary antibody. This procedure was used to determine whether formic acid pretreatment was effective in enhancing HK-249 immunostaining at the ultrastructural level.

Results

Immunolocalization of Heparan Sulfate in Alzheimer's Brain

To determine whether HS was localized to sites of amyloid deposition and/or BAP accumulation, antibodies to HS and BAP were used. Amyloid present in NPs and in the walls of blood vessels and the presence of NFTs in AD brain were identified by positive congo red staining (as viewed under polarized light).[35] Tissue sections immunostained with the BAP antibody not only identified BAP accumulation in fibrillar amyloid-containing NPs and blood vessels but also in neurite-containing 'primitive' plaques. These plaques contained neurites as demonstrated by positive silver staining (not shown) and little or no fibrillar amyloid as shown by congo red staining on serial sections. No positive immunostaining of NFTs was observed with the BAP antibody. Serial sections immunostained with HK-249 clearly demonstrated positive immunostaining of NPs containing a central amyloid core (Figure 1A) and 'primitive' plaques without amyloid cores (Figures 1B to D). Positive immunostaining of 'amorphous' or 'diffuse' plaques, which on adjacent serial sections were congo red negative (suggesting absence of fibrillar amyloid) and BAP positive, also were strongly positive with the HS antibody (not shown).

The HS antibody also strongly immunostained tangle-bearing (Figure 1E) and non-tangle-bearing neurons (Figure 1F). Positive HS immunoreactivity in neurons appeared either as small discrete granules in the cell body (Figure 1C) or as large coalescing granules that completely filled the cytoplasm of the neuron (Figure 1F). Most of the pyramidal cell and granular cell neurons of the hippocampus and amygdala demonstrated positive intraneuronal immunostaining with the HS antibody. The HK-249 antibody did not immunostain blood vessels in the brain parenchyma but did immunostain the choroid plexus basement membrane, ependyma, and astrocytes (particularly astrocytes located in the white matter; not shown). These latter locations also demonstrated HS immunoreactivity in the normal non-AD brain (Figures 6 and 7), implying that these areas normally contain HS and are not necessarily related to AD pathology.

Blood vessels that demonstrated accumulation of amyloid deposits (Figure 1G) also were immunostained positively with the HS antibody (Figure 1H). This was true in all regions of brain examined (ie, hippocampus, amygdala, and cerebellum). The immunostaining observed in the brain tissue of AD patients with the HS antibody was consistently similar in all seven cases of AD examined.

To determine if other extracellular matrix molecules were localized to these sites, serial sections stained pos-

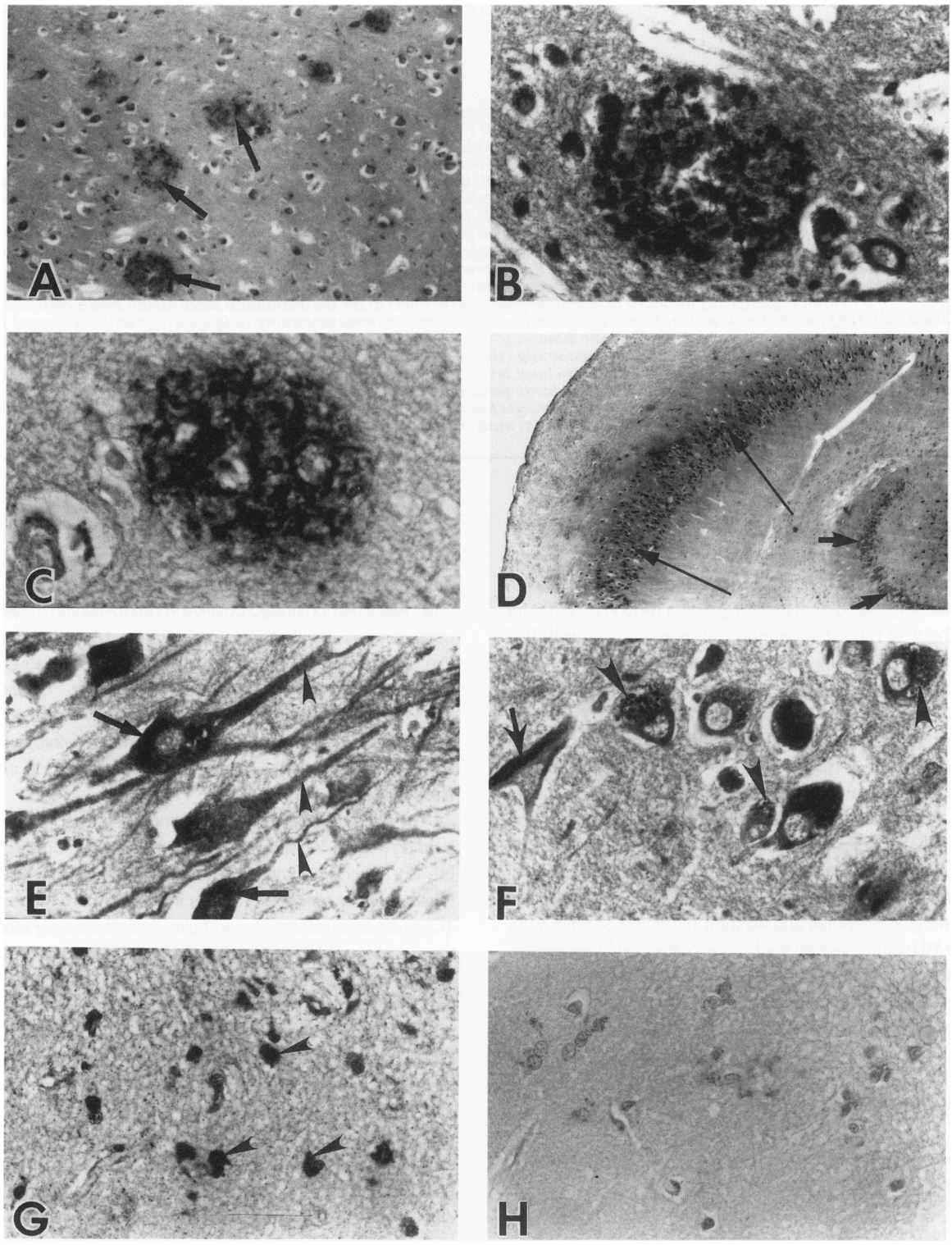

Figure 4. *Immunolocalization of heparan sulfate in older (>35 years) Down's syndrome brain.* **A:** *Heparan sulfate immunostaining (HK-249, undiluted supernatant) of primitive plaques (arrows) in the hippocampus of a 37-year-old woman with Down's syndrome. Bar, 213 μm.* **B:** *Higher magnification of primitive plaque immunostained positively with the HS GAG chain antibody (HK-249, undiluted supernatant). Bar, 30 μm.* **C:** *On the adjacent serial section the primitive plaques (shown to be positive with HK-249 in* **A***) were found to immunostain with a polyclonal antibody (1:100 dilution) directed against the BAP (synthetic peptide to residues 1–42 of the BAP). Bar, 30 μm.* **D:** *Heparan sulfate immunostaining (HK-249, undiluted supernatant) of pyramidal neurons in the hippocampus (long arrows) and the granular cell layer of the dentate gyrus (short arrows) in a 37-year-old woman with Down's syndrome. These neurons did not contain NFTs as shown by negative Congo red staining on the adjacent serial section (not shown). Formic acid pretreatment. Bar, 833 μm.* **E:** *Heparan sulfate immunostaining (HK-249, 1:5 dilution) in pyramidal neurons (arrows) in the hippocampus of a 37-year-old woman with Down's syndrome. Immunostaining for HS was also observed in the apical dendrites of neurons (arrowheads). Bar, 40 μm.* **F:** *Heparan sulfate immunostaining (HK-249, 1:10 dilution) in pyramidal neurons demonstrating granulovacular degeneration (arrowheads) in the hippocampus of a 51-year-old woman with Down's syndrome. Positive HS immunostaining also is demonstrated in an intraneuronal NFT (arrow). Bar, 40 μm.* **G:** *Heparan sulfate immunostaining (HK-249, 1:10 dilution) in the cytoplasm of astrocytes (arrowheads) in the white matter of a 47-year-old woman with Down's syndrome. Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.* **H:** *Adjacent serial section (from* **G***) immunostained with HK-249 (1:10 dilution) preabsorbed in the presence of HSPG antigen. Lack of positive immunostaining of astrocytes demonstrates the specificity of the HS antibody. Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.*

itively for HS were immunostained with antibodies to laminin, fibronectin, and a chondroitin sulfate proteoglycan. No positive immunostaining for these matrix proteins and the chondroitin sulfate proteoglycan was observed in amyloid-containing plaques, 'primitive' plaques, and 'amorphous' plaques (Figures 2A to 2C). Positive immunostaining for laminin was observed in capillaries and arterioles (Figure 2B), whereas positive immunostaining for fibronectin was observed only in large arteries and meningeal vessels (not shown). Tangle-bearing and non–tangle-bearing neurons that immunostained with the HS antibody (Figures 2D and G) were not immunostained with antibodies to fibronectin (Figure 2E), laminin (Figure 2F), or chondroitin sulfate (Figure 2H). Preabsorption of the HS antibody with excess HSPG antigen completely removed positive immunostaining of NPs, NFTs, cerebrovascular amyloid deposits, neurons (not shown for AD cases; see Figure 5 for DS cases).

Ultrastructural Immunolocalization of Heparan Sulfate in Alzheimer's Brain

Heparan sulfate immunostaining with HK-249 was demonstrated in 1-μ deplasticized sections in AD hippocampus, specifically localized to 1) amyloid fibrils in neuritic plaques (Figure 3A), 2) inclusions in the cell bodies of neurons (Figures 3B and C), 3) both intraneuronal (Figure 3C) and extraneuronal NFTs (not shown), and 4) amyloid-containing blood vessels (not shown). At the ultrastructural level, HS immunostaining was demonstrated in neuritic and 'burned-out' plaques specifically localized to the amyloid fibrils (Figure 3D), and in the amyloid deposits in the walls of some arterioles within the brain parenchyma (not shown). Heparan sulfate immunostaining also was localized within the cell bodies of neurons and oligodendrocytes to lipofuscin granules (Figure 3F). This was apparent in all neurons and oligodendrocytes that contained lipofuscin granules. Some NFTs (10% to 20%) demonstrated immunostaining for HS, which was localized to paired helical and straight filaments, following pretreatment of thin sections with 88% formic acid (1:100 dilution; Figure 3H). Tissue sections treated with the HS antibody, which had been preabsorbed with excess HSPG antigen, demonstrated no positive immunostaining in the regions of brain described above (Figures 3E, G, and I).

Immunolocalization of Heparan Sulfate in the Down's Syndrome Brain

The brains from all four cases of DS, older than 35 years, demonstrated the characteristic lesions (NPs, NFTs, and cerebrovascular amyloid deposits) found in the brains of AD patients. These lesions were identified by positive congo red staining (ie, red-green birefringence as viewed under polarized light),[35] or immunostaining (except in NFTs) with the BAP antibody. Tissue sections immunostained with the HS antibody strongly immunostained amyloid-containing neuritic plaques (not shown) and 'primitive' plaques (Figures 4A and B), which on adjacent serial sections were immunostained with the BAP antibody (Figure 4C). In addition, HS immunostaining was observed in the DS brain (in patients older than 35 years) in the pyramidal neurons of the hippocampus and in the granule cell layer of the dentate gyrus (Figure 4D). Many of the pyramidal neurons demonstrated HS immunoreactivity in both the cell body and apical dendrites (Figure 4E). Also many of the HS-positive neurons contained granulovacular degeneration (Figures 4E and F) or NFTs (Figure 4F). Heparan sulfate immunostaining also was observed in DS brains (in patients older than 35 years) in a number of different sites found to be present in normal brains (Figures 6 and 7), including 1) the cytoplasm of astrocytes, primarily those located in the white matter (Figures 4G and H); 2) the basement membrane of the choroid plexus (not shown); 3) the ependymal lining of the ventricle (not shown); 4) in the walls (primarily media) of meningeal vessels (similar to fibronectin immunostaining of vessels shown in Figure 5H); and 5) in Purkinje cells in the cerebellum (not shown).

The specificity of the HS immunostaining observed in the DS brain (older than 35 years) was confirmed when HS immunostaining of NPs, NFTs and congophilic angiopathy (not shown), and neurons (Figures 5A to 5D) was abolished when serial sections were either incubated with HS antibody (undiluted) preabsorbed with excess HSPG antigen, or using TRIS-buffered saline instead of the primary HS antibody. Antibodies to laminin (Figure 5F), fibronectin (Figure 5G), or chondroitin sulfate (not shown) did not immunostain the DS neurons or characteristic lesions. Immunostaining of blood vessels (primarily meningeal vessels) was, however, observed with the laminin (not shown), fibronectin (Figure 5H), and chondroitin sulfate (not shown) antibodies within the same tissue sections.

The brains from DS cases younger than 30 years (including ages 1 day, 4 months, and 2, 7, 15, 16, 18, and 24 years) did not contain NPs, NFTs, or cerebrovascular amyloid deposits in the brain (hippocampus, amygdala, and cerebellum) as indicated by 1) negative congo red staining as viewed under polarized light and 2) negative BAP immunostaining for these lesions. However the 18- and 24-year-old DS cases demonstrated 'amorphous' or 'diffuse' cortical deposits immunostained with the BAP antibody in some areas of the hippocampus and amygdala (Figure 6A). These regions represented one of the earliest detectable changes observed in the DS brain before the

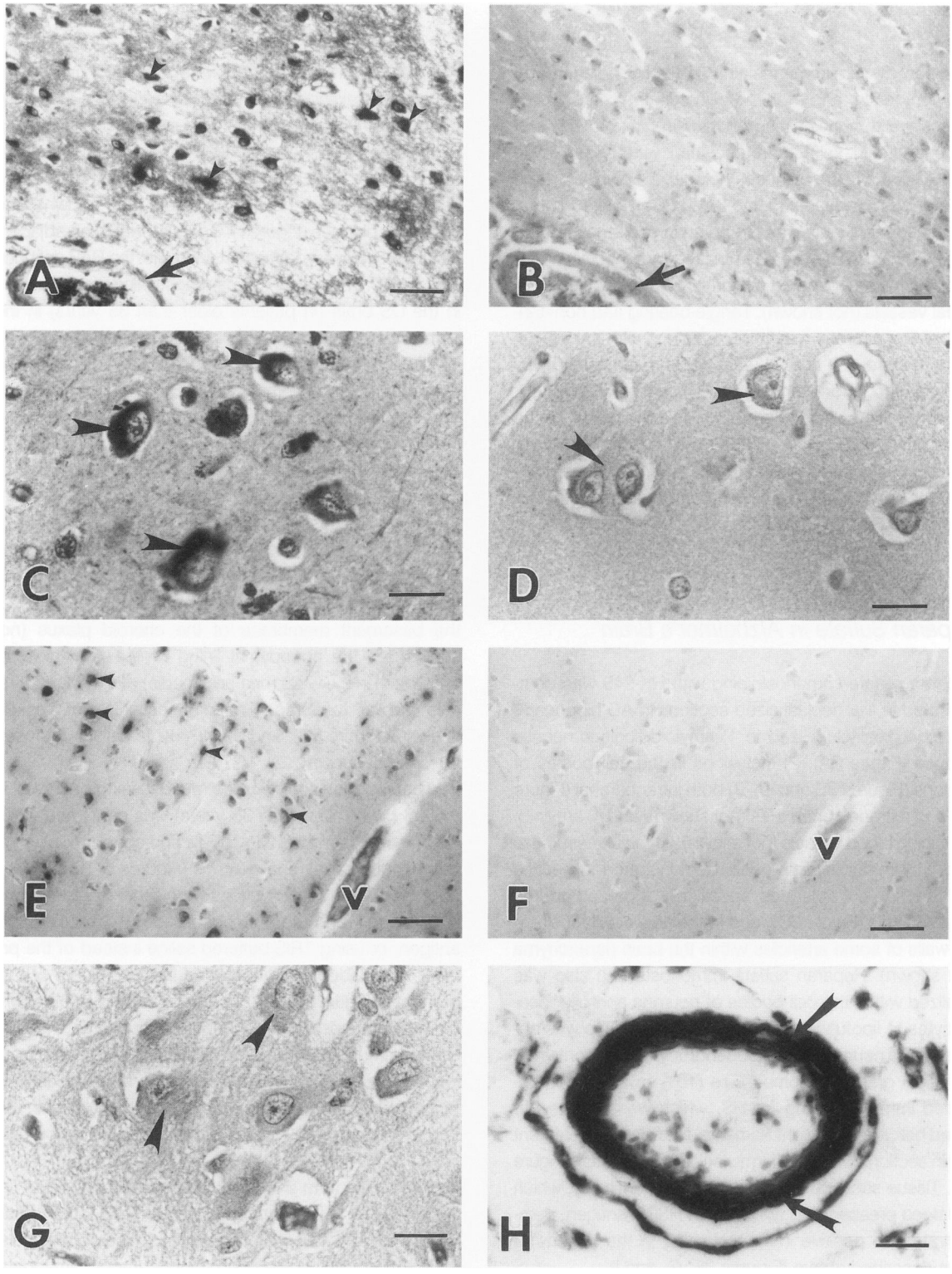

Figure 5. *Specificity of heparan sulfate immunostaining in older (>35 years) Down's syndrome brain.* **A:** *Heparan sulfate (HK-249, undiluted supernatant) immunostaining of neurons (arrowheads) in the hippocampus of a 37-year-old man with Down's syndrome. Nonamyloid containing blood vessel is shown in parenchyma by arrow and is not immunostained with this antibody. Formic acid pretreatment; hematoxylin counterstain. Bar, 80 μm.* **B:** *Adjacent serial section from* **A** *using TRIS-buffered saline instead of the HS antibody. No positive immunostaining of neurons is observed. Arrow marks the same vessel as in* **A**. *Formic acid pretreatment; hematoxylin counterstain. Bar, 80 μm.* **C:** *Heparan sulfate (HK-249, 1:10 dilution) immunostaining within the cell bodies of pyramidal neurons (arrowheads) in the hippocampus of a 47-year-old woman with Down's syndrome. Formic acid pretreatment; hematoxylin counterstain. Bar, 32 μm.* **D:** *Adjacent serial section from* **C** *immunostained with HK-249 (1:10 dilution) preabsorbed with excess HSPG antigen. No positive immunostaining is observed in the cell bodies of pyramidal neurons (arrowheads) indicating the specificity of the HS antibody. Formic acid pretreatment; hematoxylin counterstain. Bar, 32 μm.* **E:** *Heparan sulfate (HK-249, 1:10 dilution) immunostaining of neurons (arrowheads) in the hippocampus of a 47-year-old woman with Down's syndrome.*

appearance of NPs, NFTs, and congophilic angiopathy.[40,41] On adjacent serial sections, these BAP-positive cortical deposits also were immunostained with the HK-249 antibody (Figure 6B). All young DS cases (less than 35 years old) demonstrated HS immunostaining in the 1) cell bodies of pyramidal (Figures 6C to E) and granule cell neurons (Figures 7E and F) in the hippocampus; 2) astrocytes, particularly those located in the white matter (Figures 7G and H); 3) the choroid plexus basement membrane (not shown); 4) the ependymal lining of the ventricles (not shown); 5) the walls of meningeal vessels (not shown); and 6) in the cell bodies of Purkinje cells in the cerebellum (Figure 7B). The young non-DS brains did not demonstrate HS immunostaining in the cell bodies of neurons (Figure 7C), whereas HS immunoreactivity was observed in all the other tissue sites described above, including the choroid plexus (Figure 6H), the ependymal lining of the ventricles (Figure 7C), the walls of meningeal vessels (Figure 7D), and the astrocytes (not shown). The specificity of HS immunostaining was confirmed when immunoreactivity was abolished after immunostaining with the HS antibody preabsorbed with excess HSPG antigen (Figures 6D and 6F). The HS immunostaining observed in young DS brains in the areas of brain described above was found in all cases, regardless of age. Heparan sulfate immunostaining in the neurons of the hippocampus, amygdala, and cerebellum (ie, Purkinje cells) was observed even in the 1-day- and 4-month-old DS brains (Figures 7A and B). In comparison, immunostaining with the HS antibody in young controls (including an 11-year-old child with congenital heart disease, a 13-year-old child with Huntington's disease, a 14-year-old child with cystic fibrosis, and an 18-year-old with cardiomyopathy) demonstrated weak to no HS immunostaining of pyramidal and granule cell neurons in the hippocampus and amygdala (Figures 6G and 7C). However HS immunostaining was demonstrated in non-DS cases in 1) astrocytes in the white matter (not shown), 2) the choroid plexus basement membrane (Figure 6H), 3) the ependymal lining of the ventricles (not shown), 4) the walls of meningeal vessels (Figure 7D), and 5) in the cell bodies of Purkinje cells in the cerebellum (not shown), indicating that the lack of immunostaining of the neurons was not due to preparation artifact. These observations suggest that HS accumulation in neurons of the hippocampus and amygdala is amplified in DS brain at an early age (even at 1 day) and is not observed to the same extent in non-DS patients.

Discussion

The specific localization of HS GAG chains to the BAP-containing amyloid deposits in NPs and congophilic angiopathy in AD and DS brain correlates with the previous demonstration of a basement membrane HSPG core protein in these amyloid sites in the AD brain.[24] These results now indicate that both the protein and carbohydrate components of a basement membrane-derived HSPG are present in amyloid deposits, either separately as degradation products or together as part of an intact HSPG. Although HS was localized to amyloid deposits, NFTs and neurons in AD and DS brains, other basement membrane components such as laminin and fibronectin, or a chondroitin sulfate proteoglycan, were not present at these sites. This correlates with previous studies demonstrating the lack of immunoreactivity for laminin and fibronectin at these locales.[24,42]

Heparan sulfate accumulation in both the AD and DS brains also was observed in BAP-positive neuritic plaques. These 'primitive' plaques[43] and their containing neurites previously have been shown to contain epitopes for BAP (residues 1 to 24 and 1 to 28)[43–46] and BAPP (residues 46 to 62 of the BAPP).[45] The presence of HS in both neurons and astrocytes in AD and DS brains suggest that these cells may serve as a source for the HS found localized to the BAP-containing lesions.

Heparan sulfate GAG immunoreactivity also was observed in tangle-bearing neurons and extraneuronal NFTs (known as 'ghost tangles') in both AD and DS brains. Heparan sulfate immunostaining was localized to the paired helical and straight filaments in some (about 10% to 20%) intraneuronal and extraneuronal NFTs in the AD brain. This represents the first demonstration of a PG component present in NFTs. The finding of HS GAGs as a common component in both NPs and NFTs in the AD brain merely may be incidental, but it is consistent with the hypothesis that plaques and tangles are related.[47] Although HS GAGs were found only in some NFTs, cationic dye studies[22] suggest that PGs and/or GAGs are present within all NFTs, specifically localized to the paired helical

Vessel in brain parenchyma is marked (v). Formic acid pretreatment; hematoxylin counterstain. Bar, 125 μm. **F:** *Adjacent serial section from* **E** *immunostained with an antibody to laminin (1:100 dilution) demonstrates no positive immunostaining of neurons in hippocampus. The antibody did detect laminin localized to meningeal vessels in the same tissue section (not shown). Formic acid pretreatment; hematoxylin counterstain. Bar, 125 μm.* **G:** *Lack of positive immunostaining in pyramidal neurons (arrowheads), using an antibody to fibronectin (1:10 dilution), in the hippocampus of a 47-year-old woman with Down's syndrome. This same antibody demonstrated positive immunostaining of meningeal vessels* **(H)**. *Formic acid pretreatment; hematoxylin counterstain. Bar, 27 μm.* **H:** *Immunostaining in the wall (arrows) of a meningeal vessel in the hippocampus of a 47-year-old woman with Down's syndrome using an antibody to fibronectin (1:10 dilution). This same antibody did not immunostain neurons in the same section (see* **G**). *Formic acid pretreatment; hematoxylin counterstain. Bar, 98 μm.*

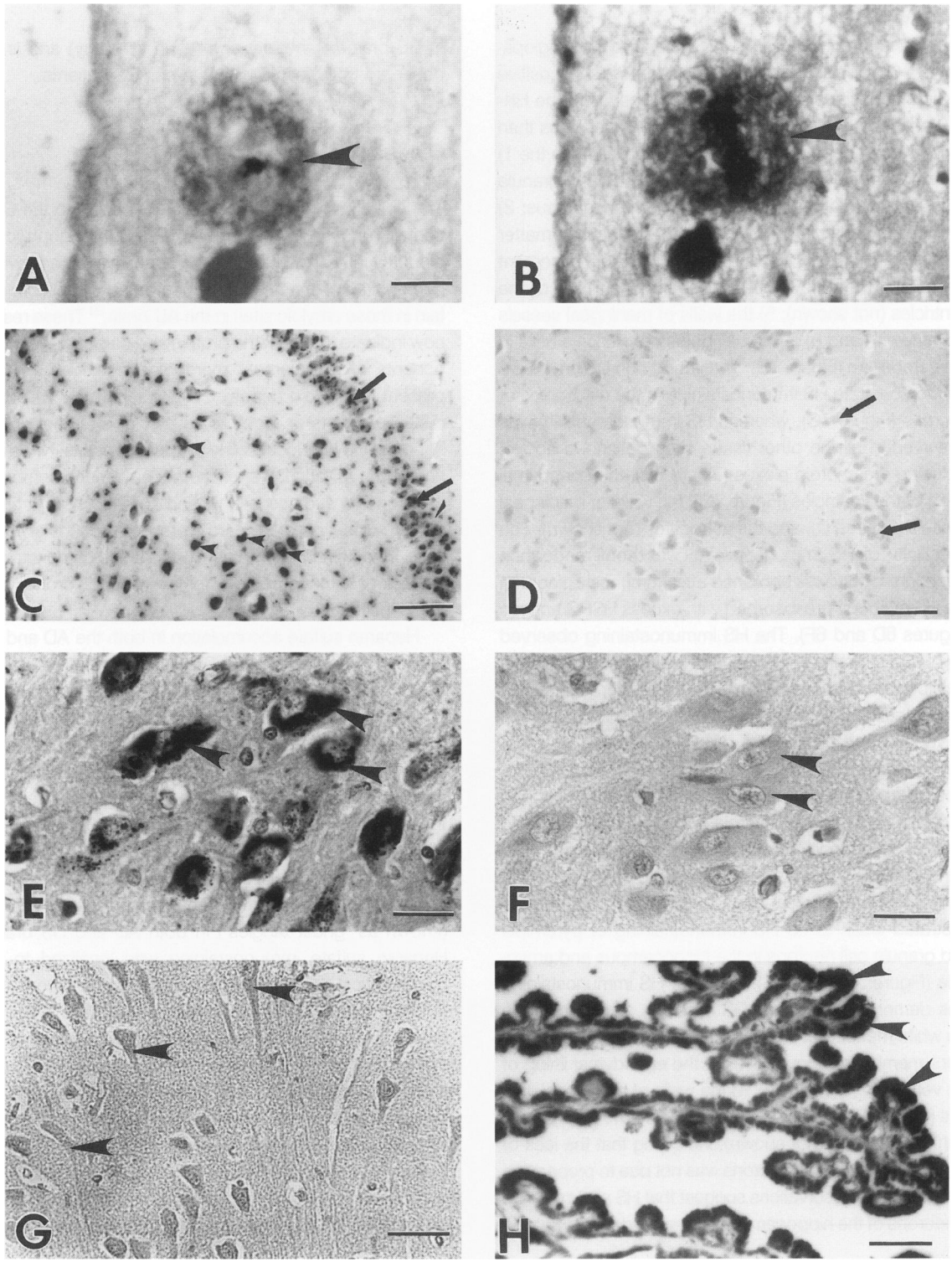

Figure 6. *Immunolocalization of heparan sulfate in young (<30 years) Down's syndrome and control brains.* **A:** *A fine diffuse area of positive BAP immunoreactivity (arrow) in the hippocampus of a 24-year-old woman with Down's syndrome detected using a polyclonal antibody (1:100 dilution) to the BAP (residues 1–42 of the BAP). These localized cortical deposits were found to be Congo red negative, suggesting the absence of fibrillar amyloid. Formic acid pretreatment; no counterstain. Bar, 40 μm.* **B:** *Adjacent serial section from* **A** *demonstrating the same cortical deposit (arrow) immunostained positively with the monoclonal antibody to HS (HK-249, 1:5 dilution). This particular patient did not have any NPs or NFTs in the hippocampus. Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.* **C:** *Strong immunostaining of pyramidal (arrowheads) and granule cell (arrows) neurons with the HS antibody (HK-249, 1:10 dilution) in the hippocampus of a 16-year-old girl with Down's syndrome. This patient did not have NPs, NFTs or primitive plaques as detected by negative congo red staining and negative immunostaining with the BAP antibody. Formic acid pretreatment; hematoxylin counterstain. Bar, 160 μm.* **D:** *Adjacent serial section of* **C** *immunostained with HK-249 (1:10 dilution) preabsorbed with excess HSPG antigen. No positive immunostaining of pyramidal neurons or granule cell*

filaments and straight filaments. These latter observations suggest that NFTs may contain other PGs that have not been identified. Preliminary evidence (Snow, Kresse, and Wight, unpublished data) suggests that dermatan sulfate PG core protein is present also in intraneuronal and extraneuronal NFTs.

Heparan sulfate immunoreactivity also was localized to intracellular structures within neurons in both AD and DS brains. Analysis of the AD brain at the ultrastructural level revealed a specific localization of HS GAG chains to neuronal lipofuscin granules and represented the first demonstration of a GAG in lipofuscin granules. Recent immunocytochemical studies using monoclonal antibodies to regions of the BAP clearly show that BAP is localized also to lipofuscin granules in aging neurons in AD and normal brains.[48–50] This represents another site within the AD brain that demonstrates the colocalization of HS and BAP and further supports the hypothesis that there may be an interaction between these two macromolecules.

Lipofuscin granules are believed to be age-related intracellular inclusions, derived from lysosomes,[51,52] consisting mainly of polymeric lipids and phospholipid structures.[53] Previous studies have demonstrated that the average amount of lipofuscin increases in neurons with age, at the same rate in AD, DS, and normal age-matched controls,[40,41,54,55] suggesting that lipofuscin accumulation in AD neurons is an age-related phenomenon not associated with the pathology of AD. Although lipofuscin accumulation does not appear to be related to the pathology of AD, it is nevertheless an aging phenomenon that should not be regarded as a normal process. A number of studies demonstrated a correlation between the accumulation of lipofuscin in neurons and interference with normal metabolic activity, loss of dendrites, decrease in total cellular RNA, and cell death.[41,56,57]

The significance of the colocalization of HS and BAP in intraneuronal lipofuscin granules is not clear. It may be that the intraneuronal accumulation of the BAP in lipofuscin granules is due to its binding with HS. Previous studies demonstrated that highly sulfated GAGs, such as heparin and HS, can inhibit lysosomal enzyme activity.[58,59] Such an interaction may prevent BAPP processing, causing BAPP/BAP to accumulate within cells. The accumulation of BAPP/BAP and HS in the lysosome may be a prerequisite for the eventual change in neuronal function, ultimately leading to neuronal death, a morphologic hallmark of AD and DS.[60] In addition, both BAPP/BAP and HS may be exocytosed, leading to their accumulation as extracellular cortical deposits such as those observed in young DS patients. With time, some of these cortical deposits eventually may develop into fibrillar amyloid.

It is also possible that HS accumulation in neurons may be due to a lysosomal defect in an enzyme that normally degrades HS. It is interesting to note that the neuronal accumulation of HS appears morphologically similar to the accumulation of HS in the mucopolysaccharidoses, including Hunter's, Hurler's, and Sanfilippo syndromes.[61–63] In these diseases, specific lysosomal enzymes that normally degrade HS are deficient and lead to the accumulation of HS in lysosomes in a number of different tissues, including brain. Some of these patients also demonstrate dementia at a young age, thus mimicking one of the clinical hallmarks of AD.

In the present study, HS accumulation in neurons of the hippocampus and amygdala was most prominent in young DS brains, as early as 1 day after birth, whereas age-matched controls (ages 11, 13, 14, and 18 years) demonstrated weak or no positive HS immunoreactivity in neurons (compare Figures 4D and 7C). The lack of HS immunoreactivity in neurons of young age-matched control patients was apparently not due to fixation or preparation artifacts because positive HS immunoreactivity was observed in other areas in the same tissue sections (ie, meningeal blood vessels, choroid plexus) in which HS is normally found. It is not clear whether the early intraneuronal HS accumulation in young DS patients occurs in lipofuscin granules or in other sites (ie, Golgi region) within the neuronal cell body. Previous studies suggest that lipofuscin granules are first observed ultrastructurally in neurons at about 9 years of age.[64,65] Because lipofuscin accumulation in neurons of DS patients are no different than observed in controls of the same age,[54] the HS accumulation that we observed in the neurons of DS cases at ages 1 day, 4 months, and 7 years may occur before the formation of lipofuscin granules. These results suggest that HS accumulation in neurons is an early event in DS, which may

neurons (arrows) is observed indicating the specificity of the HS antibody. Formic acid pretreatment; hematoxylin counterstain. Bar, 160 μm. **E**: *Strong immunostaining within the cell bodies of neurons (arrowheads) with HK-249 (1:10 dilution) in the hippocampus of an 18-year-old man with Down's syndrome. These neurons did not contain NFTs (congo red negative) nor demonstrate any positive immunoreactivity with the polyclonal BAP antibody (not shown). Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.* **F**: *Adjacent serial section of* **E** *immunostained with HK-249 (1:10 dilution) preabsorbed with excess HSPG antigen. Lack of positive immunostaining is observed in neurons (arrowheads) indicating the specificity of the HS antibody. Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.* **G**: *Lack of positive immunostaining in pyramidal neurons (arrowheads) using the HS antibody (HK-249, undiluted supernatant) in the hippocampus of a 13-year-old boy with Huntington's disease. In the same section, the HS antibody did immunostain the basement membrane of the choroid plexus (see* **H**). *Formic acid pretreatment; hematoxylin counterstain. Bar, 70 μm.* **H**: *Immunostaining of choroid plexus basement membrane (arrowheads) with HK-249 (undiluted supernatant) in the hippocampus of a 13-year-old boy with Huntington's disease. This antibody did not immunostain neurons present in the hippocampus (see* **G**). *Formic acid pretreatment, hematoxylin counterstain. Bar, 64 μm.*

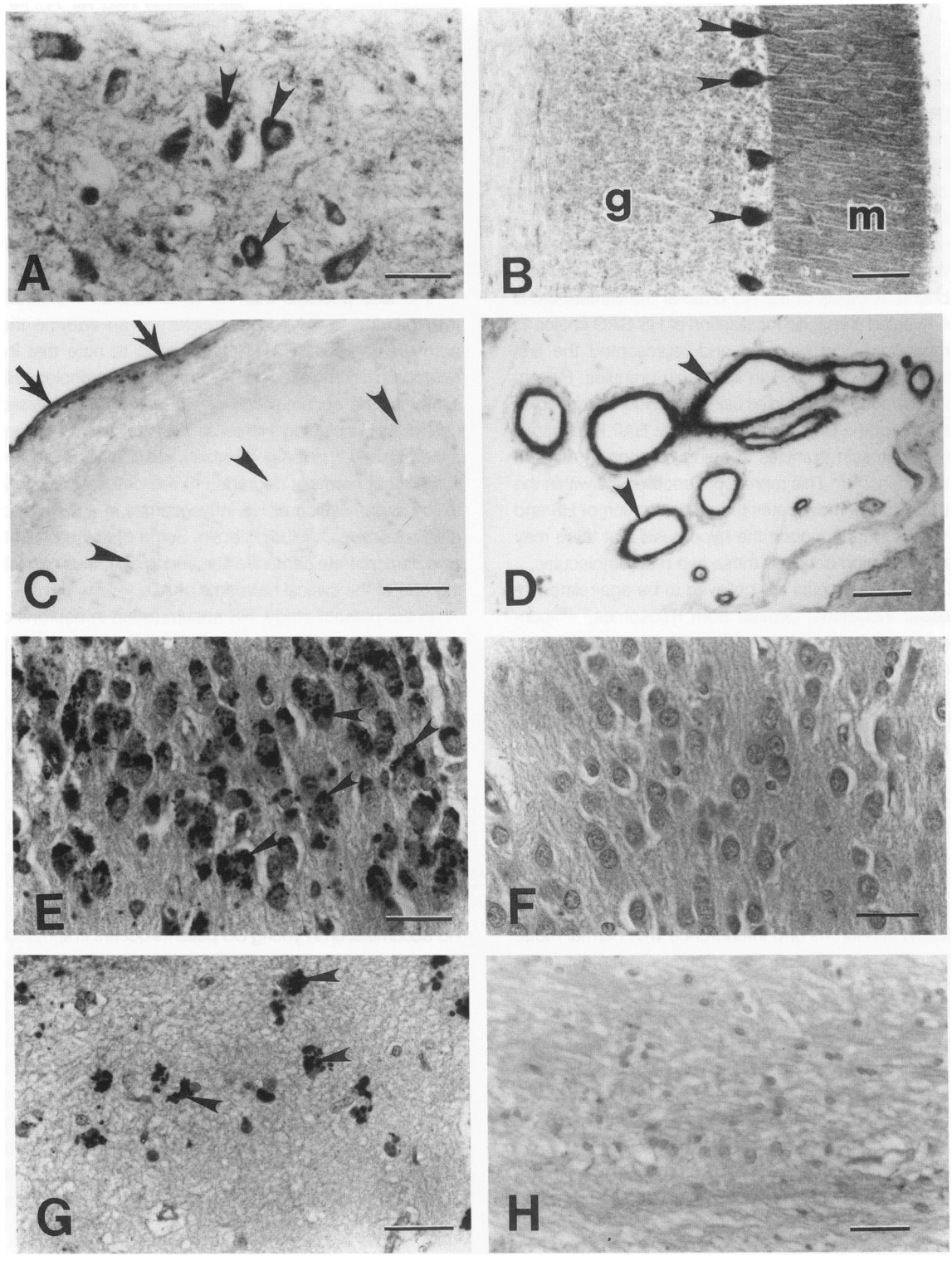

Figure 7. *Immunolocalization of heparan sulfate in young (<30 years) Down's syndrome and control brains.* **A:** *Positive HS immunostaining (HK-249, 1:5 dilution) in pryamidal neurons in the hippocampus (arrowheads) in a 1-day-old Down's syndrome brain. No counterstain. Bar, 33 μm.* **B:** *Positive HS immunostaining (HK-249, undiluted) in Purkinje cells in the cerebellum (arrows) in a 4-month-old Down's syndrome patient. The granular (g) and molecular (m) layers of the cerebellum are marked. No counterstain. Bar, 32 μm.* **C:** *Negative HS immunostaining (HK-249, undiluted) in pyramidal neurons in the hippocampus (arrowheads) in a 18-year-old with myocardiopathy. Note positive HS immunostaining of adjacent ependyma (arrows). No counterstain. Bar, 156 μm.* **D:** *In the same tissue section from* **C**, *although no HS immunostaining (HK-249, undiluted) is observed in hippocampal pyramidal neurons, strong positive HS immunoreactivity is present in the walls (arrowheads) of meningeal vessels. No counterstain. Bar, 104 μm.* **E:** *Positive HS immunostaining (HK-249, 1:10 dilution), within the cytoplasm of neurons (arrowheads) in the granular cell layer of the dentate gyrus in a 16-year-old girl with Down's syndrome. Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.* **F:** *Adjacent serial section of* **E** *immunostained with HK-249 (1:10 dilution) preabsorbed with excess HSPG antigen. No positive immunostaining of granule cell layer neurons is observed indicating the specificity of the HS antibody. Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.* **G:** *Heparan sulfate (HK-249, 1:10 dilution) immunostaining within the cytoplasm of astrocytes (arrowheads) in the white matter of the hippocampus of a 16-year-old girl with Down's syndrome. Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.* **H:** *Adjacent serial section of* **G** *immunostained with HK-249 (1:10 dilution) preabsorbed with excess HSPG antigen. No positive immunostaining of astrocytes is observed. Formic acid pretreatment; hematoxylin counterstain. Bar, 40 μm.*

play some role in predisposing these patients to the early age-related accumulation (ie, that observed as early as 35 years of age) of NPs, NFTs, and/or congophilic angiopathy.

The DS model was used also to determine whether the appearance of HS in conjunction with BAP, preceded or occurred after the initial appearance and accumulation of fibrillar amyloid. The 'amorphous' or 'diffuse' BAP-positive cortical deposits observed in the brains of DS cases of ages 18 and 24 represented the earliest detectable deposition of BAP, before the appearance of fibrillar amyloid (observed in DS patients 35 years and older).[27] These BAP-positive cortical deposits also contained HS suggesting that HS accumulation in conjunction with the BAP occurs before the appearance of fibrillar amyloid and is an early event in the pathogenesis of amyloid and/or NP development in Down's brain, and most likely in AD also.

The specific accumulation of HS in conjunction with the BAP and/or BAPP in amyloid deposits in blood vessels and plaques in AD and DS brains suggests that a particular interaction occurs between HS and portions of the BAPP. A recent study by Schubert et al[66] suggested that the core protein of a HSPG secreted from a pheochromocytoma cell line (PC 12) has an amino acid sequence and a size very similar to that of the BAPP and implied that these two molecules are antigenically related. Whether these two molecules are identical needs to be further evaluated because these authors also suggest that during purification and/or in gel analysis an interaction between the HSPG and the BAPP may occur. Recent *in situ* (ie, using tissue sections containing sites enriched in HSPGs) and affinity column chromatography studies suggest that there may be a characteristic binding affinity between HSPGs and the BAP (residues 1 to 17 and 1 to 38).[67] This specific interaction may help explain the colocalization of these macromolecules in a variety of BAP-containing deposits (described above).

The binding between HS and the BAP might be analogous to the binding of protease nexin to heparin and related molecules such as HS.[12,68] Recent studies suggested that the secreted version of the BAPP containing the Kunitz inhibitor domain is a serine protease inhibitor, known as protease nexin II.[10,11] In addition, protease nexin I has been localized to some NPs in AD brain, whereas almost all NPs and NFTs in AD brain were found to contain protease nexin I binding sites.[12] Because both protease nexin I and II contain heparin-binding sites,[68] one can envision binding interactions between some forms of BAPP (ie, protease nexin II) and heparin or related macromolecules (such as HS). In addition, heparan sulfate bound to BAPP molecules not containing the kunitz inhibitor domain may stabilize these deposits and protect them from proteolytic degradation at these sites, eventually leading to the formation and deposition of fibrillar amyloid. An analogous situation has been described in which HS derived from endothelial cells was found to protect basic fibroblast growth factor from proteolytic degradation by the protease plasmin.[69]

Heparan sulfate and related macromolecules colocalized to BAP/BAPP also may increase the affinity of a protease inhibitor for a target protease and therefore the rate of complex formation. For example, HS has been shown to accelerate the inhibition of thrombin by protease nexin I,[70] whereas heparin (related to HS) increases the rate of T-cell serine proteinase I inhibition by protease nexin I. In the brain, glia-derived nexin is a proteinase inhibitor that is homologous to, if not identical, protease nexin I.[71,72] Heparin has been shown to accelerate the inactivation of the protease thrombin by glia-derived nexin.[73] These studies not only demonstrate that HS and related macromolecules can participate in the inactivation of proteases but also raises the possibility that cells may actively control extracellular proteinases by modulating the amount and composition of cell-surface GAGs.

Besides the possibility of HS inhibiting the degradation of BAP and/or BAPP in the brain, it also may play a role in the ultimate formation of fibrillar amyloid. Highly sulfated PGs, such as HS, may influence the folding of the BAPP or derivatives thereof (ie, BAP) to form a beta-pleated sheet secondary structure characteristic of all amyloids. Recently HS (and not heparin or chondroitin-6 sulfate) was shown to increase significantly the beta-pleated sheet conformational structure of SAA_2, the specific AA amyloid precursor.[74] These *in vitro* studies imply that HS may play an important *in vivo* role in the ultimate folding of amyloidogenic precursors (such as the BAPP) into beta-pleated sheet structures characteristic of fibrillar amyloid.

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Acknowledgments

The authors thank Drs. Masato Kato and Sakaru Suzuki for establishing HK-249.