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Aberrant GAP-43 Gene Expression in Alzheimer's Disease

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GAP-43 is a growth-associated phosphoprotein expressed at high levels in neurons during development, axonal regeneration, and neuritic sprouting. GAP-43 gene expression in mature neurons is probably functionally important for the structural remodeling of synapses as required for learning and establishing new memory. The widespread aberrant neuritic growth accompanied by impaired synaptic plasticity in Alzheimer's disease (AD) suggests that abnormal GAP-43 gene expression may contribute to the cascade of neurodegeneration. In the present study, end-stage AD brains exhibited reduced neuronal expression but increased glial cell levels of GAP-43 mRNA and protein. Glial cell localization of GAP-43 gene expression was confirmed by* in situ *hybridization of cerebral tissue, Northern blot analysis of microdissected cerebral white matter, and independent analysis of astrocytoma cell lines and primary malignant astrocytomas. In addition, in AD, GAP-43 immunoreactivity was translocated from the cytosol to membranes of swollen neuritic (dendritic) and glial cell processes throughout cerebral cortex and white matter. Downregulated and aberrant neuronal GAP-43 gene expression appears to reflect an important molecular lesion that precedes and progresses with the widespread synaptic disconnection and dementia in AD. At the same time, the presence of similar neuronal abnormalities in Pick's disease, diffuse Lewy body disease, Parkinson's disease, and Down syndrome suggests common mechanisms in the respective cascades of neurodegeneration. Finally, the finding of aberrantly increased glial cell GAP-43 gene expression in AD exposes a previously unrecognized neurodegenerative change that may account for the axonal loss and white matter atrophy detected early in the course of disease. (Am J Pathol 1995, 147:934–946)

GAP-43 (B-50, F1, pp46) is a growth-associated calmodulin-binding phosphoprotein and substrate for protein kinase C.[1–6] The probable importance of GAP-43 in the growth and maintenance of neuronal processes and interconnections is suggested by its (1) abundant distribution in growth cones,[3,4,6] (2) high level expression during developmental arborization of neurons,[7–14] (3) increased expression after neuronal injury in both the peripheral and central nervous systems,[15–18] (4) increased expression during regeneration and neuritic sprouting,[12,13,17,19,20] (5) relatively high level expression in neuronal populations that undergo constant vigorous synaptic remodeling throughout life,[9,21] and (6) ability to effect cellular filopodia formation and elongation after *in vitro* transfection.[22]

The clinical features of Alzheimer's disease (AD) neurodegeneration imply fundamental impairment in neuronal plasticity manifested as decreased cognitive skills and inability to learn and acquire new memory. Widespread neuritic dystrophy is one of the principal neuropathological lesions correlated with AD dementia,[19,23,24] suggesting that cognitive impairment is associated with aberrant sprouting and failure to maintain sound synaptic connections.[25–28] The probable physiological function of GAP-43, together with the observation that this gene is most abundantly expressed in areas of the adult brain that are severely affected by AD lesions,[9,21] prompted us to explore whether aberrant expression of GAP-43 is an important feature of AD neurodegeneration. To this end, levels of GAP-43 mRNA and immunoreac-

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tivity were evaluated in cerebral tissue from patients with AD and from nondemented aged controls. In addition, cerebral tissues from individuals with Down's syndrome and AD lesions, Parkinson's disease (PD), diffuse Lewy body disease, and Pick's disease were studied to determine the specificity of the findings in AD.

Materials and Methods

Source of Tissue

Fresh frozen and formalin-fixed postmortem brain tissue was obtained from demented patients with histopathologically diagnosed AD who had been evaluated with neuropsychiatric testing at the Alzheimer's Disease Research Center located at the Massachusetts General Hospital. Corresponding tissue was obtained from nondemented aged control patients who had been regularly followed at the Massachusetts General Hospital. Paraffin-embedded sections of cerebral cortex from patients with PD, Down's syndrome plus AD, Pick's disease, and diffuse Lewy body disease were also included in the immunocytochemical studies. All specimens were harvested within 18 hours of death, and they were processed and preserved according to the Massachusetts General Hospital Alzheimer's Disease Research Center brain banking protocol.[29]

Histopathological diagnoses were established with paraffin-embedded sections stained with luxol fast blue, hematoxylin and eosin, Bielschowski's silver impregnation, and Congo red. The diagnosis of AD was rendered by threshold criteria established by the NIA-NINCDS group[30] plus the requirement of abundant neocortical neurofibrillary tangles and dystrophic neurites. The diagnosis of PD was based upon neuronal loss, gliosis, and intraneuronal Lewy body inclusions in the substantia nigra, locus coeruleus, and dorsal motor nucleus of the vagus nerve. The PD cases were not classified as diffuse Lewy body disease due to the absence of numerous ubiquitin-immunoreactive cortical Lewy bodies. Pick's disease was diagnosed in brains with gross lobar atrophy, cortical Pick bodies, and achromasic neurons.

Patient Profile

GAP-43 gene expression was examined by Northern blot analysis, *in situ* hybridization, and immunohistochemical staining in 18 AD and 10 aged control brains. In addition, GAP-43 immunoreactivity was examined in histological sections from brains with PD dementia (n = 3), diffuse Lewy body disease (n = 2), Pick's disease (n = 2), Down's syndrome plus AD (n = 3). The AD group, composed of 11 females and 8 males, had a mean age of 76.3 ± 8.8 years (SD), a mean brain weight of 1116.7 ± 100.9 grams (range, 1050 to 1285), and a mean postmortem interval of 7.3 ± 3.9 hours (range, 4 to 14). The aged control group, composed of 8 males and 2 females, had a mean age of 78.0 ± 6.2 years, a mean brain weight of 1273.5 ± 115.1 grams (range, 1100 to 1360), and a mean postmortem interval of 8.3 ± 3.6 hours (range, 4 to 15). The differences were not statistically significant, save for the higher percentage of females in the AD group ($P < 0.01$).

Northern Blot Analysis

Northern blot analysis was performed with total cellular RNA extracted from frozen blocks of Brodmann areas 8/9 and 11 in the frontal lobe and area 21 in the temporal lobe, including cerebral cortex with underlying white matter. In addition, blocks of central frontal lobe white matter devoid of cerebral cortex and extracts of proliferating neuronal (PNET1, PNET2, and SH-Sy5y cells) and glial (A172, C6, Hgl-16, and Hgl-17) cell lines were studied. The tissues and cells were homogenized in 5 mol/L guanidinium isothiocyanate, and RNA was pelleted through a 5.7 mol/L cesium chloride step gradient.[31] Samples containing approximately 15 μg of RNA were electrophoresed in 1.2% agarose/3% formaldehyde gels.[31] The RNA was transferred to a nylon membrane (Nytran, Du Pont, NEN, Boston, MA) and hybridized with a probe generated from the C1a fragment of the human GAP-43 cDNA.[16] The probe was labeled with [α^{32}P]dCTP to a specific activity of $\sim 10^8$ cpm/μg DNA using random hexamers[32] (Amersham multiprime DNA labeling kit, Amersham, Arlington Heights, IL). The blots were washed in stepwise dilutions of 5X standard saline citrate (SSC; 1X SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate) containing 0.5% sodium dodecyl sulfate (SDS). The final wash was in 0.1X SSC/0.5% SDS at 65°C. The blots were exposed to Kodak XAR film to generate autoradiograms. To evaluate RNA content in each lane, the blots were stripped of probe and rehybridized with a synthetic 30-mer corresponding to 18s ribosomal RNA.[33] Multiple exposures were obtained and nonsaturated autoradiograms were subjected to volume densitometric analysis with ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA) to determine the relative abundance of GAP-43 mRNA.

In Situ *Hybridization*

Formalin-fixed, paraffin-embedded sections (8 μm thick) of frontal (Brodmann areas 8/9 and 11) and temporal (area 21) lobe neocortices were used to examine the cellular distribution of GAP-43 gene expression by *in situ* hybridization and immunocytochemical staining. The sections were de-waxed in xylenes, hydrated through graded alcohol solutions, and equilibrated in phosphate-buffered saline. GAP-43 antisense and sense (negative control) cRNA probes used for *in situ* hybridization were prepared from linearized pGEM4-C1a, with digoxigenin-11-UTP and either T7 or SP6 RNA polymerase.[34] Before hybridization, the sections were treated with 0.2 N HCl for 20 minutes at room temperature, followed by 2X SSC for 15 minutes at 70°C, and then acetylated with 0.025% acetic anhydride in 0.1 mol/L triethanolamine, pH 8.0 (room temperature). RNA was denatured in 50% formamide/2X SSC for 15 minutes at 50°C. The sections were dehydrated through graded alcohols and then hybridized with 250 ng/ml digoxigenin-UTP-labeled cRNA probe for 12 hours at 50°C in a buffer containing 50% formamide, 4X SSPE, 10 mmol/L sodium pyrophosphate, 1X Denhardt's solution, 200 mmol/L dithiothreitol, 300 μg/ml sheared (200 to 100 bp) denatured salmon sperm DNA, 100 μg/ml tRNA, 10% PEG 6000, and 1% SDS. The slides were washed for 2 hours at 50°C in 2X SSC/0.5% Triton X-100. Unhybridized cRNA probe was digested with 50 μg/ml RNAse A in 0.5 mol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0. The sections were additionally washed for 4 hours at 50°C in 0.5X SSC/1% SDS. The hybridized probe was detected with alkaline phosphatase-conjugated sheep $F(ab')_2$ anti-digoxigenin, and antibody binding was revealed with X-phosphate/5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium chloride. The sections were counterstained with 1% methylene blue and preserved with aqueous mounting medium (Glycergel, Dako Products, Carpinteria, Ca).

Immunocytochemical Staining

Formalin-fixed, paraffin-embedded tissue sections (8 μm thick) adjacent to those used for *in situ* hybridization and cytospin preparations of proliferating cultured glioblastoma cells were immunostained with rabbit polyclonal antibody to the chimeric GAP-43-β-galactosidase fusion protein generated in λ gt11.[9,20] The sections were de-waxed in xylenes, hydrated through graded alcohol solutions, and immunostained for GAP-43 by the avidin-biotin horseradish peroxidase complex method (Vector ABC Elite kit, Vector Laboratories, Burlingame, CA) following the manufacturer's protocol, with 0.05% 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as the chromogen. The sections were counterstained with hematoxylin, dehydrated in graded alcohol solutions, cleared in xylenes, and preserved with Permount (Fisher Scientific Products, Fair Lawn, NJ).

The binding specificity of the GAP-43 antibody was previously demonstrated.[9] In addition, in the present study, adjacent sections were immunostained with monoclonal antibodies to glial fibrillary acidic protein and Dengue fever virus as positive and negative controls. The cases were immunostained simultaneously under identical conditions and the slides were interpreted under code. GAP-43 immunoreactivity was evaluated by systematic examination of 20 adjacent microscopic fields at a magnification of ×400, encompassing the full thickness of cortex. The approximate densities of labeled neurons were scored as follows: absent, less than 10%, between 10 and 50%, or greater than 50% in most microscopic fields. In addition, the distribution of immunoreactivity was characterized in terms of its laminar organization within the cortex, cell types labeled in both the cortex and white matter, and neuritic or dendritic localization. The data were analyzed statistically with Student's *t*-tests and χ^2 contingency tables with the Number Cruncher Statistical System, version 5.01 (Dr. Jerry L. Hintze, Kaysville, UT).

Immunoelectron Microscopy

Postmortem cerebral cortex and underlying white matter from two control patients were initially fixed in 10% formalin and then postfixed with 4% paraformaldehyde/0.1% glutaraldehyde prepared in 10 mmol/L phosphate buffer, pH 7.3. Vibratome sections (80 μm thick) were immunostained for GAP-43 by the avidin-biotin horseradish peroxidase method described above, except that the sections were free-floating rather than mounted onto glass slides. The sections were exposed for 1 hour to 1% osmium tetroxide, followed by 10 minutes in 1% uranyl acetate prepared in 70% ethanol. The sections were dehydrated in graded alcohol solutions and embedded in an Epon-araldite mixture (Ladd, Burlington, VT). Sections 60 nm thick were examined and photographed in a Phil-

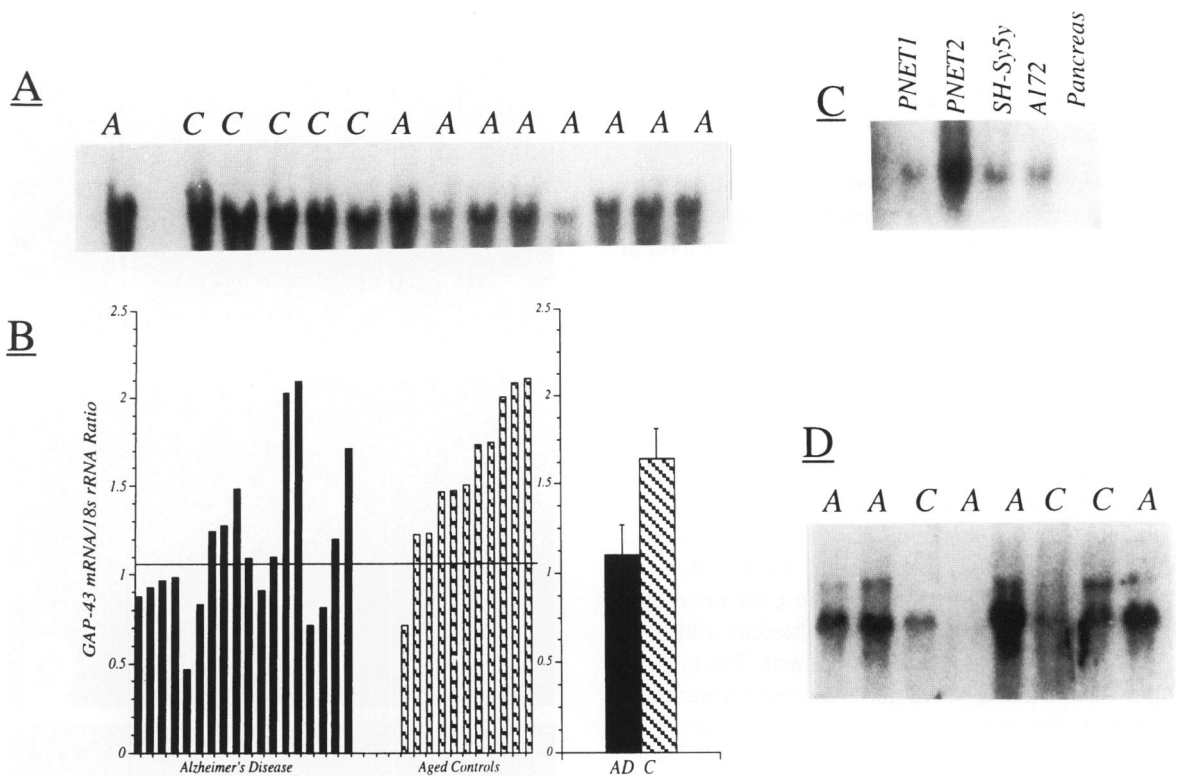

Figure 1. *Northern blot analysis demonstrating 1.6-kb mRNA transcripts corresponding to GAP-43 gene expression in the cerebral cortex with underlying white matter from Brodmann area 8/9 in the frontal lobe* (A), *neuroectodermal tumor cell lines of neuronal (PNET and SH-Sy5y) and glial (A172) origin* (C), *and microdissected central frontal lobe white matter* (D). *The faint upper band in* D *is often detected and may represent preprocessed GAP-43 mRNA. A, Alzheimer's disease; C, aged control. The pancreas in* C *served as a negative control.* B: *Densitometric scanning quantitation of GAP-43 mRNA levels in AD and aged control samples from Brodmann area 8/9, some of which are illustrated in* A. *The values reflect ratios of GAP-43 and corresponding 18s ribosomal RNA hybridization signals for individual cases as well as the group mean values ± standard errors. The horizontal line depicts the median GAP-43 level in the AD brains. The differences between the AD and aged control mean and median values were statistically significant* ($P < 0.05$).

lips 301 electron microscope without further contrast enhancement.

Results

GAP-43 mRNA Levels in Cerebral Tissue

Northern blot analysis demonstrated 1.6-kb GAP-43 mRNA transcripts in samples of cortex with underlying white matter (Figure 1A), in white matter alone (Figure 1D), and in both primitive neuroectodermal tumor and glioblastoma cell lines (Figure 1C), but not in hepatocellular carcinoma cells or human pancreas RNA (negative controls). Densitometric analysis of data obtained for Brodmann area 8/9 in the frontal lobe demonstrated reduced steady-state (net due to synthesis, processing, and degradation) levels of GAP-43 mRNA in 11 of the 18 AD compared with 10 of the 11 aged control brains (Figure 1B). To correct for differences in sample loading, the densitometry values depicted in Figure 1B represent ratios of corresponding GAP-43 and 18s ribosomal RNA hybridization signals obtained with the same blots. The differences between AD and aged control mean and median levels of GAP-43 mRNA were statistically significantly (both $P < 0.05$). Similar results were obtained for Brodmann areas 11 and 21 (data not shown).

At the same time, empirical observations suggested that in AD GAP-43 gene expression paradoxically might be upregulated in glial cells. To address this question specifically, central cerebral white matter dissected free of gray matter and snap frozen at the time of autopsy was used for Northern blot analysis. Specimens of uncontaminated anterior frontal lobe white matter demonstrated increased rather than decreased levels of GAP-43 mRNA in AD relative to aged controls (Figure 1D). Glial cell expression of GAP-43 mRNA was further confirmed by Northern blot analysis of RNA extracted from cultured A172 (Figure 1C), HGL16, HGL17, and C6 (rat) glioblastoma cell lines (data not shown). Previous studies by other investigators also demonstrated GAP-43 gene expression in glial fibrillary acidic protein-positive macroglial cells (astrocytes).[35,36]

In Situ *Hybridization Demonstrating the Cellular Distribution of GAP-43 mRNA in Aged Control Brains by Nonisotopic Detection Methods*

GAP-43 mRNA transcripts were difficult to detect by brightfield microscopy because of the generally low levels of gene expression in adult brains, although sparse intense labeling of individual neurons was observed in control brains. With darkfield microscopy, GAP-43 gene expression was readily detected in the normal adult cerebral cortex, although the density of labeled neurons and the intensity of hybridization signals were considerably lower than in postnatal developing brain, as described previously in experimental animals[9] (Figure 2, panels 1 and 2). Specific hybridization signals were not observed when sense cRNA probes were used (Figure 2, panel 3) or when the sections were pretreated with 100 μg/ml RNAse A before hybridization with antisense cRNA probes (data not shown). The highest regional levels of GAP-43 gene expression were observed in the temporal lobe neocortex (area 21), amygdala, hippocampal formation, and orbital frontal cortex (area 11). Lower levels were observed in primary sensory, primary motor, and association frontal and parietal cortex, whereas the lowest levels of GAP-43 gene expression were observed in the occipital cortex (area 17, primary visual) and cerebellum.

In Situ *Hybridization Demonstrating Aberrant GAP-43 mRNA Expression in Different Degrees of AD Neurodegeneration by Nonisotopic Detection Methods*

Corresponding with the results of Northern blot analysis, *in situ* hybridization studies demonstrated reduced neuronal GAP-43 mRNA expression in 10 of the 18 end-stage AD brains (Figure 3, panel 3), relative to all 11 normal aged control brains (Figure 3, panel 1). Reduced neuronal GAP-43 mRNA levels were manifested by lower densities of labeled neurons and lower intensities of *in situ* hybridization signals. In end-stage AD, the most striking reductions in GAP-43 gene expression were observed in temporal lobe structures, area 11 of the frontal lobe, and area 40 in the parietal lobe where the GAP-43 gene expression was readily detected in normal aged control brains. In regions where GAP-43 gene expression was normally low level, such as in the visual cortex and cerebellum, there were no clear differences between AD and control brains.

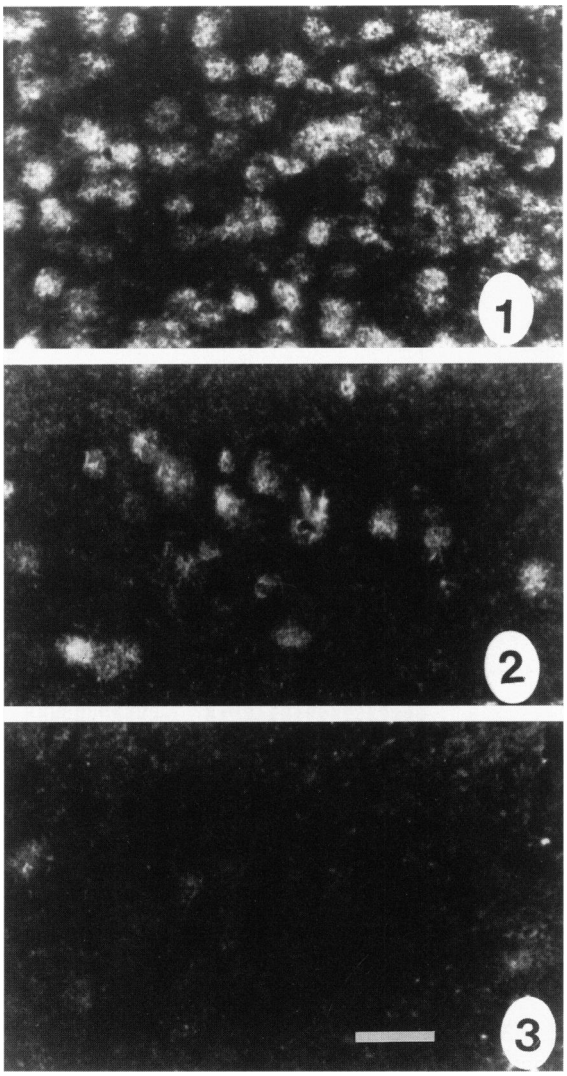

Figure 2. *GAP-43 gene expression demonstrated by* in situ *hybridization and darkfield photomicroscopy. The clusters of white granular signals over cells represent positive hybridization of digoxigenin-labeled probes detected by a nonisotopic method* (**panel 1**). *Abundant, widely distributed neuronal labeling in an infant cerebral cortex compared with normal adult temporal cortex* (**panel 2**). (**Panel 3**): *Section adjacent to that shown in* **panel 2** *but hybridized with a sense cRNA GAP-43 probe (negative control). Scale bar, 60 μm.*

Consistent with results obtained by Northern blot analysis of white matter, large numbers of cortical and white matter astrocytes exhibited GAP-43 gene expression in both end-stage AD and Down's syndrome plus AD brains (Figure 3, panel 4). Moreover, in two cases of early, asymptomatic AD, neuronal GAP-43 gene expression appeared normal, but white matter glial cell expression of GAP-43 was increased relative to control brains and similar to end-stage AD (data not shown). In aged control brains, GAP-43 mRNA transcripts were not detected in cerebral white matter or cortical glial cells (Figure 3, panel 2). With regard to other neurodegenerative

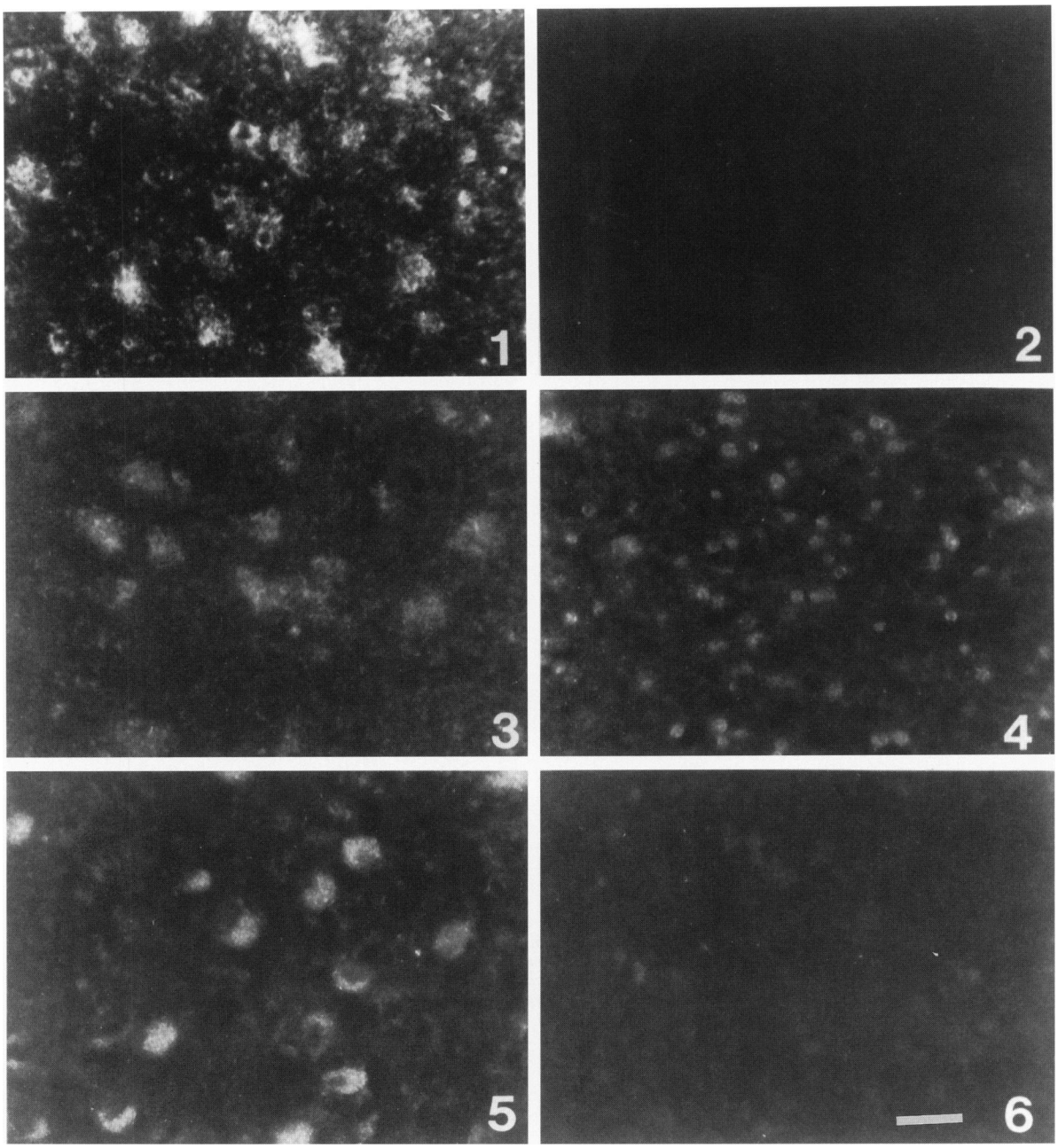

Figure 3. In situ *hybridization comparing GAP-43 gene expression in aged control* (**panels 1** *and* **2**), *end-stage AD* (**panels 3** *and* **4**), *and PD dementia* (**panels 5** *and* **6**) *in Brodmann area 11 of the frontal lobe.* **Panels 1, 3,** *and* **5** *represent cerebral cortex.* **Panels 2, 4,** *and* **6** *represent subcortical white matter. Increased GAP-43 mRNA in AD white matter correlates with the loss of white matter fibers and increased gliosis. The clusters of white granular signals over cells represent positive hybridization of digoxigenin-labeled cRNA probes detected by a nonisotopic method. Scale bar, 40 μm.*

diseases, PD with early clinical dementia (n = 2), Pick's disease (n = 2), and diffuse Lewy body disease (n = 2), all exhibited reduced neuronal GAP-43 gene expression similar to AD (Figure 3, panel 5). However, these cases also manifested low level white matter glial cell GAP-43 expression, similar to aged control brains (Figure 3, panel 6). The classification of GAP-43-expressing cells in white matter as astrocytes was based upon nuclear morphology and positive immunoreactivity for glial fibrillary acidic protein in adjacent sections.

Distribution of GAP-43 Immunoreactivity in Control Brains

The cellular and regional distributions of GAP-43 immunoreactivity corresponded with the patterns

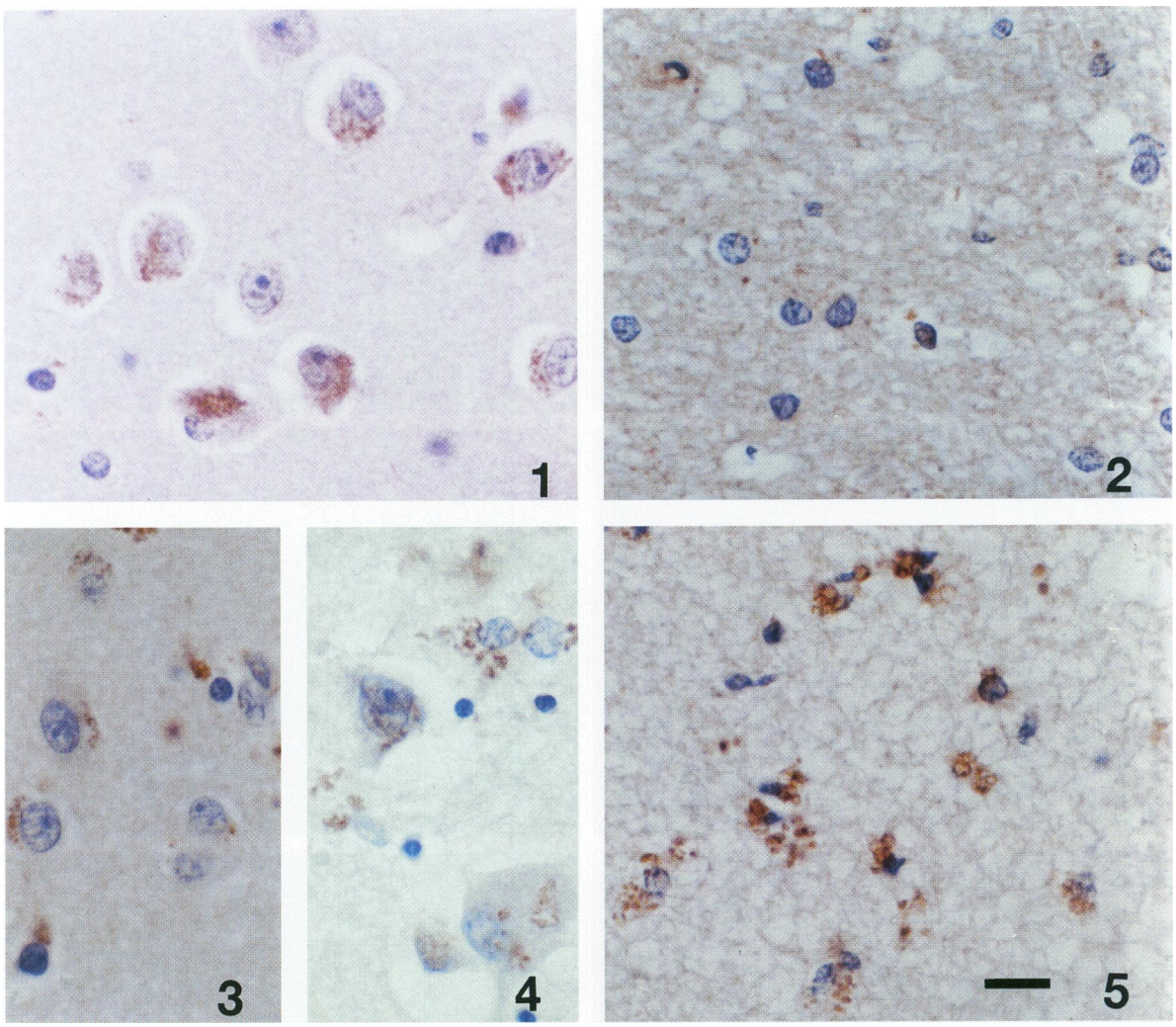

Figure 4. *Aberrant GAP-43 gene expression in AD demonstrated by immunocytochemical staining of sections from Brodmann area 21 of the temporal lobe neocortex.* Panels 1 *and* 2: *Aged control brain exhibiting GAP-43 immunoreactivity in neuronal perikarya* (panel 1) *and faint uniform labeling of white matter fibers* panel 2. Panels 3 *and* 4: *End-stage AD* (panel 3) *and Down's syndrome plus AD* (panel 4) *brains exhibiting reduced GAP-43 immunoreactivity in neuronal perikarya but increased vesicular labeling of cell processes. Enlarged neuron at lower right in* panel 4 *contains a neurofibrillary tangle.* Panel 5: *Central white matter in AD marred by numerous clusters of punctate to vesicular GAP-43 immunoreactive cell processes (probably glial). Scale bar, 20 μm.*

of gene expression detected by *in situ* hybridization. GAP-43 immunoreactivity was localized to neuronal perikarya (Figure 4, panel 1) and fibers throughout the neuropil and white matter. Neuronal perikaryal labeling was discrete and punctate but variable in intensity. Glial cell labeling was not detected in either the cortex or white matter of normal control brains (Figure 4, panel 2). The most abundant neuronal labeling was observed in the temporal lobe neocortex and in limbic structures, whereas less abundant labeling was detected in primary motor and sensory cortex, and the lowest levels of GAP-43 immunoreactivity were observed in the primary visual cortex and cerebellum. In 30% of the sections examined from Brodmann area 11 (frontal), area 21 (temporal), and area 40 (parietal), GAP-43 immunoreactivity was detectable in more than 50% of cortical neurons (Table 1), whereas in the remaining 70%, only 10 to 50% of neuronal cell bodies were labeled. In control cerebral cortex, the distribution of GAP-43 immunoreactivity was predominantly (28 of 30 sections, 93%) laminar as a result of prominent labeling of pyramidal neurons in cortical layers 3 and 5 and less abundant labeling of neurons in layers 2, 4, and 6. Throughout the cerebrum, GAP-43 immunoreactivity was finely and uniformly distributed in the neuropil and white matter fibers. Immunoelectron microscopy confirmed the localization of GAP-43 protein in myelinated axons. However,

Table I. *Abnormal Levels and Distribution of GAP-43 Immunoreactivity in Alzheimer's Disease*

	Density of GAP-43$^+$ neurons					
	0 (0)	<10% (1)	10–50% (2)	>50% (3)	χ^2	Significance
Control	0%	0%	70%	30%	29.5	$P < 0.0001$
AD	13%	47%	27%	13%		

	Other parameters			
	Absent/ equivocal	Definite	χ^2	Significance
Laminar distribution of GAP-43$^+$ neurons				
Control	7%	93%	16.5	$P < 0.0001$
AD	51%	49%		
Intensity of GAP-43$^+$ neurons				
Control	20%	80%	25.7	$P < 0.0001$
AD	77%	23%		
Intensity of neuropil labeling				
Control	47%	53%	1.5	NS
AD	60%	40%		
Presence of GAP-43$^+$ neurites/cell processes				
Control	100%	0%	29.6	$P < 0.0001$
AD	42%	58%		

Control, n = 10 cases, 30 sections; AD, n = 18 cases, 53 sections. For each case, paraffin-embedded sections of Brodmann area 11 (frontal), area 40 (parietal), and area 21 (temporal) were stained for GAP-43 immunoreactivity. The degree of immunoreactivity was graded as absent/equivocal or definite. NS, not significant.

postmortem autolysis precluded unequivocal localization of GAP-43 gene expression in neuronal perikarya and dendrites.

Reduced Levels and Aberrant Distribution of GAP-43 Immunoreactivity in AD

In AD, the overall densities of GAP-43 immunoreactive neurons and the intensity of neuronal labeling were conspicuously reduced relative to aged control brains (Table 1). In 60% of the AD brain sections, less than 10% of cortical neurons expressed GAP-43 immunoreactivity ($P < 0.0001$ relative to control), and with respect to positive cells, the intensity of labeling was typically low level and difficult to discern (Figure 4, panel 3). Moreover, the laminar distribution of GAP-43 immunoreactivity observed in 93% of control brains was present in only 49% of the AD brains ($P < 0.0001$). Loss of the laminar distribution of GAP-43 immunoreactivity in AD was associated with reduced densities of GAP-43 immunoreactive neurons in layers 3 and 5, corresponding with the prominent distribution of neurofibrillary tangles as well as increased cortical glial cell labeling (see below).

The intensity of GAP-43 immunoreactivity in neuropil fibers was similar in AD and control brains (Table 1). However, instead of uniform neuropil labeling, AD brains exhibited striking vesicular and membranous labeling of cell processes throughout both gray and white matter. The GAP-43 immunoreactive cell processes measured between 0.4 and 2 μm in diameter, and they nearly always occurred in small aggregates between 5 and 20 in number (Figure 4, panels 3, 4, and 5). Reduced levels of GAP-43 immunoreactivity in neuronal perikarya *vis-a-vis* widely distributed GAP-43 immunoreactive vesicular cell processes was not unique to AD. Similar findings were observed in dementias resulting from Parkinson's disease (n = 3), diffuse Lewy body disease (n = 2), and Pick's disease (n = 1), and also in Down's syndrome associated with AD (n = 3) (Figure 4, panel 4).

GAP-43 Immunoreactive Gene Expression in Glial Cells

In histologically intact control brains, low level GAP-43 immunoreactivity was uniformly distributed throughout white matter fibers and distinct glial cell-associated labeling was not observed (Figure 4, panel 2). In AD, the cerebral cortex and white matter were marred by numerous clusters of vesiculomembranous GAP-43 immunoreactive cell processes (Figure 4, panels 3 to 5). Although in the cortex such structures were closely associated with both neurons and astrocytes, in deep white matter the cellular association was clearly glial and probably always astrocytic on the basis of nuclear morphology. Astrocytic gene expression of GAP-43 was further demonstrated by Northern blot analysis (Figure 1), immunocytochemistry (Figure 5), and Western blot analysis (data not shown) of the A172, HGL16, HGL17, and C6 astrocytoma cell lines (Figure 5, panels 1 and 2) as well as by positive immunocytochemical staining reactions in six of seven primary human cerebral malignant astrocytomas (Figure 5, panel 3).

GAP-43 Gene Expression Increases with Acute Neuronal Injury in AD

Although neuronal GAP-43 gene expression was generally reduced in AD, in two patients plus another with Down's syndrome and AD in which the brains contained multiple small ischemic lesions and recent infarcts, GAP-43 immunoreactivity was strikingly increased in neuronal perikarya and neuropil fibers in the immediate vicinity of the lesions (Figure 6, panel 2). In contrast, in remote uninvolved regions of the same brains, GAP-43 gene expression was low level as described above. Upregulated GAP-43 gene expression was also observed in the vicinity of acute

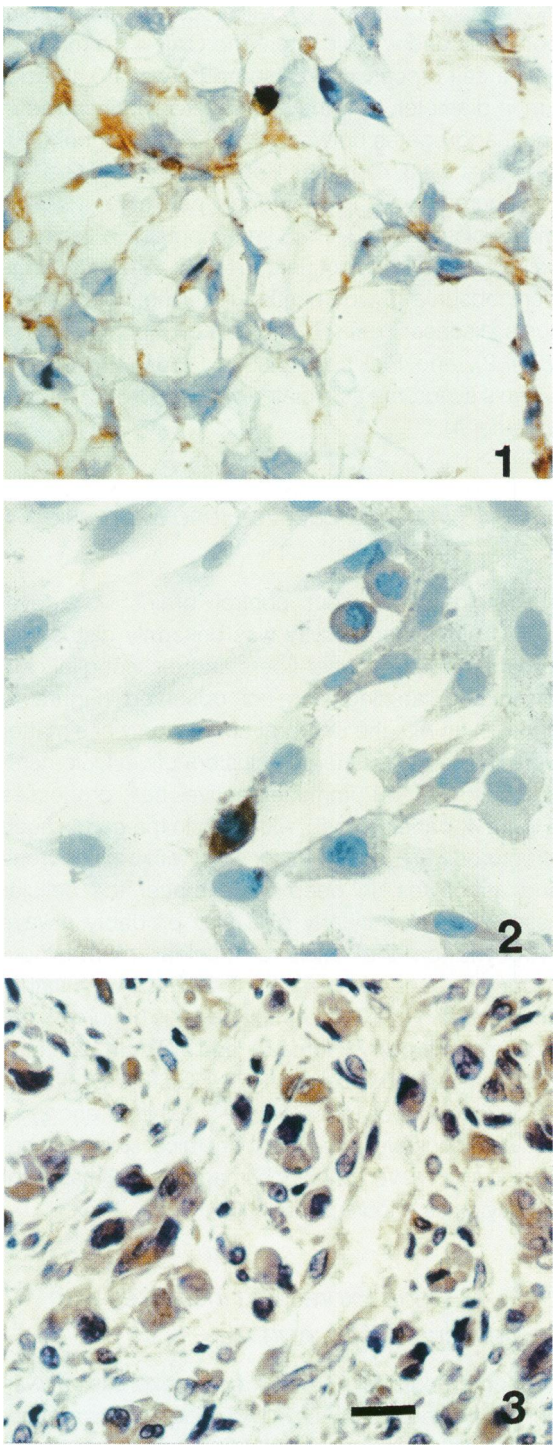

Figure 5. *GAP-43 immunoreactive gene expression in A172* (panel 1) *and Hgl-16* (panel 2) *glioblastoma cell lines and in a primary malignant astrocytoma* panel 3. *These findings were confirmed by Northern (Figure 1D) and Western blot analysis of these and other glial cell lines. Scale bar, 20 μm.*

ischemia in control brains (Figure 6, panel 1). In addition, the reactive hypertrophic astrocytes in white matter exhibited increased GAP-43 gene expression in injured control brains (Figure 6, panel 3). However, the immunostaining reaction was uniformly distributed in the cytoplasm, in contrast to the vesiculomembranous labeling observed in AD brains (Figure 6, panel 4). In both ischemic and uninjured AD brains, intense GAP-43 immunoreactivity was frequently distributed in irregularly swollen axons as well (Figure 6, panel 5).

Discussion

This study demonstrates reduced neuronal and increased astrocytic levels of GAP-43 gene expression in AD compared with aged control brains. The finding of abnormal GAP-43 mRNA levels is consistent with transcriptional regulation of the gene.[6,20] Reduced GAP-43 gene expression in AD cerebral cortex could not be attributed to neuronal loss *per se*, as *in situ* hybridization and immunocytochemical staining studies demonstrated downregulation of the gene in numerous histologically intact neurons. The importance of doing *in situ* hybridization and immunocytochemical staining in conjunction with Northern and Western blot analyses is underscored by the unexpected finding of glial cell expression of the GAP-43 gene. In fact, quantitative analysis of the Northern blot hybridization signals was confounded by the increased glial cell expression of GAP-43 present in AD brains. Although GAP-43 is generally regarded as a neuron-specific gene, evidence to the contrary is provided by the following: (1) GAP-43 gene expression was detected in glial cell lines and in primary astrocytomas; (2) the antisense hybridization signals in white matter and cortical astrocytes were abolished by RNAse A pretreatment, and sense GAP-43 cRNA probes failed to hybridize with glial cells in brain; and (3) Northern blot analysis of microdissected white matter samples yielded a single 1.6-kb mRNA transcript, the same size as that observed in neuronal cell lines and gray matter tissue. The findings indicate that the GAP-43 gene may be expressed in neoplastic as well as reactive or injured astrocytes. Increased GAP-43 gene expression in AD cerebral white matter correlated with the axonal loss and marked gliosis that occur in end-stage disease.[29] The prominent white matter glial cell expression of GAP-43 in early AD is also consistent with the finding of significant white matter degeneration in early and asymptomatic AD.[29]

The laminar distribution and regional variability in the levels of GAP-43 gene expression in control brains corresponded with the selectively vulnerable targets of AD neuronal degeneration, particularly

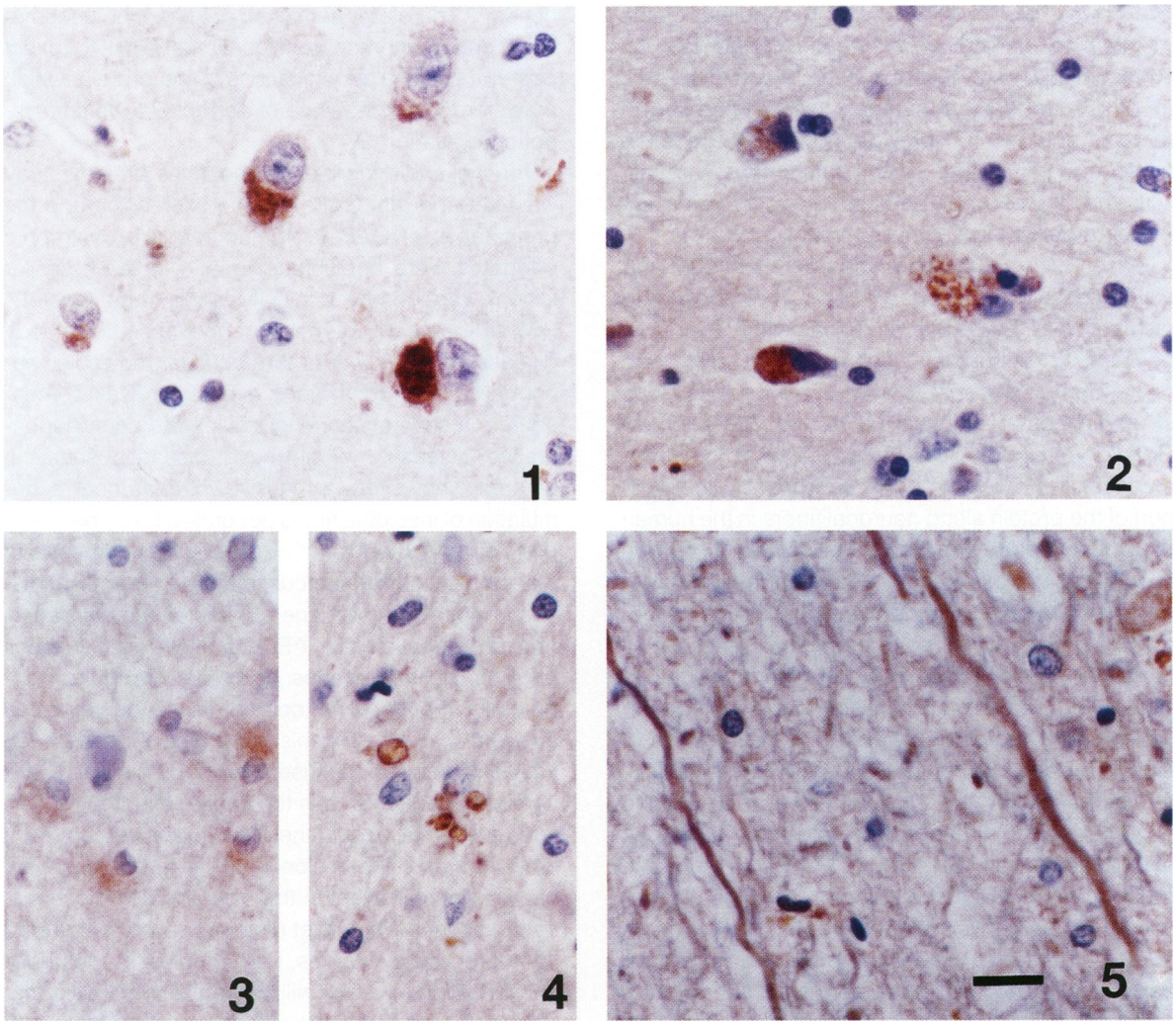

Figure 6. *Increased neuronal GAP-43 gene expression in the vicinity of acute ischemic injury in aged control* (panel 1) *and AD* (panel 2) *brains. Increased GAP-43 immunoreactivity in hypertrophic astrocytes in acutely ischemic white matter of an aged control brain* (panel 3), *contrasted with the membranous and vesicular labeling of cell processes prominent throughout AD white matter, independent of ischemic damage* (panel 4). *GAP-43 immunoreactive irregularly swollen axons were often detected in acutely injured AD brains* panel 5. *Scale bar, 20 μm.*

with respect to neurofibrillary tangle formation.[37–39] Correspondingly, these regions and neuronal populations exhibited the greatest degrees of downregulated GAP-43 gene expression. These observations are consistent with the finding of downregulated gene expression with markedly diminished or nondetectable levels of GAP-43 mRNA in cortical neurons with neurofibrillary tangles in AD, as reported previously by Coleman and colleagues.[40,41] However, the present data indicate that molecular neurodegenerative changes in AD occur in a much broader population of neurons than those that develop neurofibrillary tangles. Juxtaposed to the similar abnormalities in Pick's Disease, PD, and diffuse Lewy body disease observed herein, the findings suggest that downregulated GAP-43 gene expression in neurons is not unique to AD and instead probably represents a common feature of filamentous neurodegenerative diseases. Another important observation in these studies was that downregulation of neuronal GAP-43 in AD was not fixed and immutable in all neurons, as increased levels of neuronal gene expression occurred in the vicinity of subacute resolving cerebral microinfarction in both AD and control brains. This result may indicate that reduced neuronal GAP-43 gene expression in AD represents an adaptive response to widespread synaptic disconnection.[26] Alternatively, diminished GAP-43 gene expression might reflect negative feedback from accumulated GAP-43 protein in dystrophic dendrites. Finally, aberrant, low level GAP-43 gene expression may result from reduced synthesis or half-life of other mRNAs,[42–44] downregulation of protein kinase C,[45,46] or decreased phosphorylation of

GAP-43 by protein kinase C.[47] Regardless of the mechanism, the findings with respect to nonspecific subacute injury suggest that, even in end-stage AD, at least some cortical neurons can be recruited to respond normally by upregulating GAP-43 gene expression during repair. This residual neuronal plasticity may therefore be harnessed to serve as a potential target of therapeutic intervention in AD.

GAP-43 is transported along neuronal fibers and distributed in growth cones.[3–6,10,17] The widespread distribution of GAP-43 immunoreactivity in neuronal perikarya (cell bodies), neuropil fibers, and white matter fibers is consistent with previous findings in experimental animals.[9,13,21] The detection of GAP-43 immunoreactivity in myelinated axons by immunoelectron microscopy is consistent with transport of the protein along neuronal fibers in the human brain. One feature that readily distinguished AD from control brains was the presence of abundant aggregates of GAP-43 immunoreactive dystrophic neurites/dendrites. Dystrophic dendrites constitute a fundamental and ubiquitous neuropathological lesion in AD,[23,24,48] and their abundant presence in superficial layers of the cerebral cortex is an important correlate of dementia.[48–50] Presumably, the aberrant neuritic sprouting and formation of dystrophic dendrites occur in response to widespread synaptic disconnection in AD.[25–28,48–50] GAP-43 gene expression is important for neuronal process elongation and the establishment of interneuronal connections, manifested by its distribution in growth cones,[3–6] high level expression in adult neurons that undergo active synaptic remodeling throughout life,[9,21,51] and upregulation during neuronal regeneration and repair.[12,13,15–20] Therefore, the finding of increased GAP-43 immunoreactive dendrites in AD cerebral cortex correlates with the lawless dendritic growth associated with AD neurodegeneration. It will be of interest to know whether abnormal expression of the GAP-43 gene is necessary or sufficient to precipitate the cascade of AD neurodegeneration.

In AD, GAP-43 immunoreactivity was distinctly localized in the membranes of dystrophic dendrites and did not correspond with lipofuscin granules or other intracytoplasmic (perikaryal) structures, eg, neurofibrillary tangles. This conclusion is supported by the frequent finding of GAP-43 immunoreactivity in processes distributed a few microns away from the neuronal perikarya rather than directly over the cell bodies and the previous demonstration of GAP-43 immunoreactivity in AD dystrophic dendrites by confocal laser microscopy.[52] Membranous localization of GAP-43 has been linked to fatty acylation (palmitolyation) of the protein in neuronal growth cones.[53] Therefore, persistent growth cone accumulation and membrane translocation of GAP-43 reflect post-translational modifications of the protein, which may stem from the failure of dendrites to reach synaptic targets in AD.

A novel and important finding in the present study was that aberrant GAP-43 gene expression and the proliferation of GAP-43 immunoreactive dystrophic dendrites were not unique to AD. Instead, similar lesions were observed in Down's syndrome plus AD, Pick's disease, PD dementia, and diffuse Lewy body disease. In other words, an identical molecular abnormality in neuronal gene expression was demonstrated in several seemingly disparate neurodegenerative diseases. What these diseases share in common, however, is massive intraneuronal accumulation of insoluble fibrils decorated by cytoskeletal proteins and ubiquitin.[54–57] Therefore, dementia in the context of filamentous neurodegenerative disease appears to be generally correlated with aberrant translocation of GAP-43 protein from the cell body to the membranes of swollen, irregular dendrites in the cerebral cortex. These observations suggest that AD, Down's syndrome plus AD, PD, Pick's disease, and diffuse Lewy body disease share common mechanisms in the cascade of central nervous system neurodegeneration. Moreover, aberrant GAP-43 gene expression is likely to represent an early molecular event in the cascade as it occurs in neurons that have not yet died or become entombed by neurofibrillary tangles, Lewy bodies, or Pick bodies. Our future studies will determine whether or not premature programmed cell death of neurons precedes the onset of aberrant GAP-43 gene expression and lawless dendritic growth in AD. In addition, it will be important to determine how changes in GAP-43 gene expression might alter the cellular localization and phosphorylation of neuronal cytoskeletal proteins such as τ, as paired helical filament-associated lesions, including neurofibrillary tangles, develop after GAP-43 gene expression becomes downregulated in AD.

A second important outcome of these studies was the clear demonstration of a significant glial cell abnormality in AD. Abnormal upregulation of cortical and white matter glial cell GAP-43 gene expression was detected early in the course of AD neurodegeneration before conspicuous down-regulation of neuronal GAP-43. These observations are consistent with previous reports of cerebral white matter lesions and atrophy in AD.[29,58,59] The failure to detect similar abnormalities in the context of obvious ischemic injury or infarction unassociated with AD negates the theory that white matter atrophy in AD is caused by

chronic ischemia or hypoperfusion. Upregulation of GAP-43 gene expression in glial cells is therefore a new molecular marker of glial cell pathology in AD. Impaired function of supportive glial cells may constitute an important pathogenic mechanism of progressive axonal loss and synaptic disconnection in AD.

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