Progressive synaptic degeneration and neuron loss are major structural correlates of cognitive impairment in Alzheimer's disease (AD). The mechanisms by which synaptic degeneration in AD occurs have not been established. The activation of proteins within the caspase family has been implicated in AD-associated neurodegeneration, and synaptically localized caspase activity could play a role in the synaptic degeneration and loss found in AD. We used synaptosomal fractionation with Western blotting and immunohistochemistry to examine the anatomical, subcellular, and subsynaptic expression patterns of caspase-3 in both the anterior cingulate cortex and hippocampus of control and AD patients. In both control and AD cases, there was a selective enrichment of caspase-3 at synapses, particularly in the postsynaptic density (PSD) fractions. Compared with controls, AD patients exhibited significant increases in synaptic procaspase-3 and active caspase-3 expression levels that were most evident in the PSD fractions. These data demonstrate for the first time the preferential localization and increase of caspase-3 in the PSD fractions in AD and suggest an important role for caspase-3 in synapse degeneration during disease progression. (Am J Pathol 2008, 173:1488–1495; DOI: 10.2353/ajpath.2008.080434)
implicated caspase-3 in various mechanisms of synaptic plasticity.37 There is relatively little information on caspase-3 in the AD brain in general and at the synapse in particular. Caspase-3 has been implicated in the processing of APP into amyloidogenic fragments and the accumulation of caspase-cleaved APP may be an early neurodegenerative event in the progression of AD.18,20,38 Immunohistochemical analysis has demonstrated increased levels of active caspase-3 in hippocampal neuron somata and neurites of AD brains with a high degree of co-localization with neurofibrillary tangles and plaques.39 Caspase-3 also has been reported to be present in synapses in response to apoptotic insults,39–41 but its local effects at the synapse have not been described.

To better understand the synaptic degeneration associated with AD, we examined the anatomical, subcellular, and subsynaptic expression patterns of caspase-3 in the anterior cingulate cortex and hippocampus of control and AD patients. We describe a selective enrichment of caspase-3 at synapses, especially in post synaptic density fractions (PSDs), and a significant increase in synaptic procaspase-3 and active caspase-3 expression in AD.

Materials and Methods

Cases and Controls

Human brain tissues used in this study were obtained from the Center for Neurodegenerative Disease Research collection at the University of Pennsylvania (Philadelphia, PA) and the Rush Alzheimer Disease Center at Rush University (Chicago, IL). All tissues were obtained in accord with institutional review board-approved protocols at the respective institutions. Summary case information is presented in Table 1. For the biochemical fractionation and Western blotting experiments, fresh frozen anterior cingulate cortex gray matter from 22 cases were used (12 AD and 10 normal controls). Anterior cingulate cortex was chosen as a region of interest because of extensive metabolic and molecular neuroimaging data highlighting its abnormality in AD and its role in the clinically relevant psychiatric, social, and executive behavioral abnormalities in AD.42–45 For immunohistochemistry and image analysis, fixed paraffin-embedded tissues from anterior cingulate (15 AD, 12 controls) and mid-hippocampus (22 AD, 23 controls) were used. Cases and controls were well matched for age, sex, and postmortem interval and there were no significant differences between groups for these variables.

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Age (SD)</th>
<th>Sex (M/F)</th>
<th>PMI (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>10</td>
<td>83.6 (8.8)</td>
<td>5/5</td>
<td>8.6 (5.7)</td>
</tr>
<tr>
<td>AD</td>
<td>12</td>
<td>83.6 (6.4)</td>
<td>6/6</td>
<td>8.1 (7.1)</td>
</tr>
<tr>
<td>Immunohistochemistry—anterio cingulated</td>
<td>C</td>
<td>12</td>
<td>80.9 (9.3)</td>
<td>5/7</td>
</tr>
<tr>
<td>AD</td>
<td>15</td>
<td>80.6 (9.8)</td>
<td>10/5</td>
<td>9.1 (6.7)</td>
</tr>
<tr>
<td>Immunohistochemistry—hippocampus</td>
<td>C</td>
<td>22</td>
<td>74.0 (13.6)</td>
<td>13/10</td>
</tr>
<tr>
<td>AD</td>
<td>22</td>
<td>74.9 (11.8)</td>
<td>12/10</td>
<td>11.6 (5.9)</td>
</tr>
</tbody>
</table>

Groups: C, control; AD, Alzheimer’s disease.

Tissue Preparation and Biochemical Fractionation of Synaptosomes

Nuclear and cytoplasmic extracts were prepared with the NXRTRACT CelLytic nuclear extraction kit according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). Synaptosomes were prepared using a one-step synaptosome preparation based on the method of Phillips and colleagues.46 For each sample, 0.5 g of tissue was homogenized in a sucrose solution (0.32 mol/L sucrose, 0.1 mmol/L CaCl₂, 1 mmol/L MgCl₂) with a protease inhibitor cocktail (P8340, Sigma). The homogenate was brought to a final sucrose concentration of 1.25 mol/L by the addition of 2 mol/L sucrose and 0.1 mmol/L CaCl₂. The homogenate was overlaid with 1.0 mol/L sucrose and 0.1 mmol/L CaCl₂ and centrifuged at 100,000 g for 3 hours at 4°C. The synaptosomal fraction, which forms a band at the 1.25/1.0 mol/L sucrose interface, was collected and stored at −80°C until use.

Synaptosomal extracts were further fractionated to separate pre- and postsynaptic material using the method of Phillips and colleagues.46 Briefly, to obtain synaptic vesicle, presynaptic, and PSD fractions, synaptosomes were washed two times in 0.1 mmol/L CaCl₂ and pelleted at 40,000 × g. Synaptosomes were then solubilized in 20 mmol/L Tris-HCl, pH 6.0, 1% Triton X-100, 0.1 mmol/L CaCl₂, incubated on ice for 30 minutes, and centrifuged at 40,000 × g with the resultant supernatant being the synaptic vesicle fraction. The pellet contains the synaptic junction, consisting of pre- and postsynaptic membranes. This material was solubilized in 20 mmol/L Tris-HCl, pH 8.0, 1% Triton X-100, 0.1 mmol/L CaCl₂, incubated on ice for 30 minutes, and centrifuged at 40,000 × g resulting in the supernatant containing the presynaptic fraction and the pellet containing the PSD fraction. Synaptic vesicle and presynaptic fractions were processed with acetone precipitation, and all fractions were dissolved in 5% SDS. Protein concentration was determined by the BCA method (Pierce, Rockford, IL).

Western Blot Analysis

For the Western blot analysis validating the specificity of the subsynaptic fractions obtained by biochemical fractionation, 10 μg protein of synaptic vesicle, presynaptic, and PSD fractions from normal human brain tissue were separated on 10% Tris-glycine gels (Novex Invitrogen, Carlsbad, CA) and transferred to nitrocellulose-supported membranes. In all other Western blot analyses, 20 μg of protein of each sample was separated on 12% Tris-glycine gels (Novex) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% milk and incubated with primary antibody overnight at 4°C. Primary antibodies were used at the following dilutions in 3% milk in Tris-buffered saline with 0.1% Tween-20: caspase-3, 1:250 (IMG-144A; Imgenex, San Diego, CA); synapto-
physin, 1:3000 (MSB5258; Chemicon, Millipore, Billerica, MA); syntaxin-1, 1:1000 (sc-12736; Santa Cruz Biotechnology, Santa Cruz, CA), and NMDAR-1, 1:1000 (sc-9058, Santa Cruz Biotechnology); PSD-95, 1:1000 (US Biological, Swampscott, MA), and β-actin (A3853; Sigma, St. Louis, MO). After primary antibody incubation, membranes were incubated with a horseradish peroxidase-coupled secondary antibody (HRP-conjugated mouse IgG, NA931; Amersham, Piscataway, NJ) for 1 hour at room temperature and processed with the ECL Plus chemiluminescence system (Amersham). Band density was quantified by densitometric analysis with the GS-800 calibrated densitometer (Bio-Rad) using Quantity One 1-D analysis software (Bio-Rad).

**Immunohistochemistry and Image Analysis**

Fixed, paraffin-embedded tissues were cut in the coronal plane at 6 μm on a rotary microtome that had been tested to assure invariant section thickness, and mounted on APES-coated slides. Dewaxed sections were immersed in 5% hydrogen peroxide dissolved in absolute methanol for 30 minutes to quench endogenous peroxidase activity. For antigen retrieval, the sections were boiled in 1 mmol/L ethylenediaminetetraacetic acid in 0.1 mol/L Tris buffer, pH 8.0, for 10 minutes. After cooling for 20 minutes and rinsing in water, followed by two changes of Tris-Triton (0.01% Triton X-100 in 0.1 mol/L Tris-HCL buffer, pH 7.6), sections were blocked for 45 minutes in 2% horse serum dissolved in Tris-Triton and incubated in the primary antibody, anti-cleaved caspase-3, 1:200 for hippocampal sections, 1:8000 for anterior cingulate sections (Asp-175; Cell Signaling Technology, Danvers, MA) overnight (14 to 18 hours) at 4°C. After Tris-Triton rinses, sections were incubated in a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for an hour at room temperature. Hippocampal sections were then treated for another hour at room temperature with an avidin-biotin-peroxidase complex made from a Vectastain Elite ABC kit (Vector Laboratories) and developed in a diaminobenzidine reaction, as described by the manufacturer (3,46). These sections were again treated for 30 minutes with a biotinyl tyramide solution. Anterior cingulate sections, which benefited from additional antigen retrieval, were treated for 30 minutes with a streptavidin-horseradish peroxidase complex from a TSA biotin system kit (Perkin Elmer, Waltham, MA) and incubated for 10 minutes with a biotinyl tyramide solution. These sections were again treated for 30 minutes with a streptavidin-horseradish peroxidase complex and then developed in a dianaminobenzidine reaction, as described for the hippocampal sections. After clearing in xylenes, all tissue sections were coverslipped under Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI). Specificity of the primary antibody for its antigen of interest was confirmed with a blocking experiment with recombinant activated caspase-3 (CASP3F, Sigma-Aldrich).

Immunolabeled slides were first qualitatively examined, and then immunoreactivity was semiquantitatively measured in extracellular neuropil regions of interest in anterior cingulate and the molecular layer of the dentate gyrus of the hippocampal formation by net optical density (OD), defined as the OD of the region of interest minus the OD of the background (obtained from blood vessel walls in the regions of interest). OD analysis was performed on high resolution, gray-scale photomontages captured on a Leica DMRBE microscope equipped with a motorized microscope stage with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). OD comparisons were performed on sections photographed at the same, verified light intensity. The operator was blind to any identifying information throughout data accrual and analysis.

**Statistics**

Statistical significance of differences between diagnostic groups was assessed with two-tailed Student’s t-test. Statistical significance was defined as P < 0.05.

**Results**

**Biochemical Localization of Caspase-3 in Nuclear, Cytosolic, and Synaptic Compartments**

Western blotting demonstrated that caspase-3, in its pro-caspase form, was present in cytosolic, nuclear, and synaptosomal extracts (Figure 1). In all cases, expression levels were highest in the synaptosomal extracts, followed by cytosolic and then nuclear extracts. Thus, a significant proportion of caspase-3 is localized at the synapse. Next, synaptosomal extracts were fractionated into subsynaptic compartments, and Western blotting was used to validate the efficiency of the method (Figure 2). The proteins used as markers for the different sub-synaptic fractions included synaptophysin for synaptic vesicles, syntaxin-1 for presynaptic plasma membrane and vesicle fusion protein, and NMDAR-1 and PSD-95 for postsynaptic density. The synaptic vesicle fractions showed a positive signal for both synaptophysin and syntaxin-1, although the signal for synaptophysin was much stronger. There was no evidence of NMDAR-1 or PSD-95 in the synaptic vesicle fraction. The presynaptic fraction showed a positive signal exclusively for syntaxin-1, and the PSD fraction displayed a positive signal for NMDAR-1 and PSD-95, as expected.

Caspase-3, in its pro-caspase form, was present in the synaptosomal extracts of all samples (Figure 3), and its
distribution in the various fractions of synaptosomal extracts was determined (Figure 4). There was a high level of expression of procaspase-3 in the PSD fractions and low levels of expression in the synaptic vesicle and presynaptic fractions. Active caspase-3 was observed only in PSD fractions and could be detected neither in synaptic vesicle and presynaptic membrane fractions nor in unfractionated synaptosomes in any case (Figure 4).

Samples from well-matched cases were divided into two groups (males and females), each with five control and six AD samples to measure differences in pro- and active caspase-3 expression levels between control and AD cases (Figure 3B and Figure 5). Extracts from the 11 cases in each group were run together on single blots. Caspase-3 band densities were normalized to β-actin. There were no differences in normalized density readings between males and females, so all cases were combined without adjustment for statistical analysis of diagnostic group differences. In synaptosomal fractions, procaspase-3 expression was significantly higher in AD by an average of 39% compared to controls (Figure 3). In PSD fractions, procaspase-3 was significantly higher in AD by an average of 84%, whereas active caspase-3 was significantly increased in AD by 147% (Figure 5). No significant differences in caspase-3 expression were found in the nuclear fraction between groups.

**Immunohistochemical Expression of Active Caspase-3 in Anterior Cingulate and Hippocampal Formation**

The cellular, laminar, and neuropil expression of active caspase-3 was examined in hippocampal formation and anterior cingulate cortex of control and AD cases with immunohistochemistry (Figure 6). Labeling was visually greater in AD than control cases in almost all instances. In both brain regions, variable numbers of neurons and glia exhibited immunostaining of varying intensities in cytoplasm, and infrequently in nuclei.

In anterior cingulate cortex, occasional neurons exhibited active caspase-3 immunoreactivity in cytoplasm that was clearly above background. This was chiefly observed in deeper layers. Background neuropil labeling was relatively homogeneous except for layer I and the superficial portion of layer II, where it was more intense. Numerous caspase-3-positive puncta and thread-like processes were present in the neuropil, suggestive of neuritic processes and spines. This was especially evident in the AD cases.

As in the anterior cingulate, occasional neurons in the hippocampal formation expressed caspase-3, both among granule cells in the dentate gyrus and pyramidal neurons in the ammonic subfields. In addition, most of these cells exhibited somatodendritic labeling without nuclear labeling. In the dentate gyrus of the hippocampal formation,
distinct neuropil labeling was present in both the inner and outer molecular layers, representing, respectively, the synaptic terminal fields of the hilar mossy cells and the perforant pathway of the entorhinal cortex, with especially high expression levels evident in the inner molecular layer. In the ammonic subfields, labeling was prominent in the neuropil of stratum pyramidale, stratum radiatum, and stratum lucidum (CA3). Neuropil labeling had a fine mesh appearance in the dentate gyrus molecular layer and was accompanied by puncta and thread-like processes in the hilus and ammonic subfields. Semi-quantitative analyses of optical density of active caspase-3 expression (minus background, measured in local blood vessel walls, which were unstained) in AD compared to control cases found significant increases in active caspase-3 expression in neuropil in AD compared to controls, with a mean 26% increase in the dentate gyrus molecular layer and a 37% increase in the extracellular neuropil of the anterior cingulate.

Discussion

The aims of this study were twofold: to compare the localization of caspase-3 in nuclear, cytosolic, synaptic, and subsynaptic compartments in human brain tissue; and to determine whether there are differences in the levels of synaptically localized caspase-3 between AD and control patients. Western blotting experiments demonstrated that a significant proportion of caspase-3 is localized at the synapse relative to the distribution levels in the nuclear and cytosolic extracts and that active caspase-3 is predominantly localized to the PSD fraction of synapses. In anterior cingulate cortex, Western blotting showed significantly higher levels of caspase-3 (in its pro-caspase form) in the synaptosomes of AD cases compared to controls and significantly higher levels of both pro-caspase-3 and active caspase-3 specifically in the PSD fractions in AD. Likewise, immunohistochemistry data indicated a greater degree of labeling for active caspase-3 in neuropil of synaptic fields in both the anterior cingulate and hippocampus of AD cases. To our knowledge, this study provides the first demonstration of subsynaptic localization of pro-caspase-3 or active caspase-3.

Our immunohistochemical data shows expression of active caspase-3 in synaptic fields and some neurons, whereas the biochemical data indicated presence of active caspase-3 only in the highly purified subsynaptic PSD fraction. That active caspase-3 is detectable only in the PSD fraction by Western blotting indicates the highly enriched concentration of active caspase-3 in this subsynaptic compartment relative to other subsynaptic compartments and the neuronal cell body. Our inability to detect active caspase-3 in these other fractions does not exclude its presence there; rather, it indicates that active caspase-3 was not expressed at a sufficiently high concentration to be detected by our Western blotting protocol. Our immunohistochemical and biochemical data complement one another, and particularly the biochemical studies emphasize the prominence of active caspase-3 in the postsynaptic densities of AD patients.

To understand the potential role of caspase-3 in AD-related synaptic degeneration, it is worthwhile to consider the relationships between caspase-3, APP, and Aβ. Caspase-3 has been shown to cleave APP giving rise to amyloidogenic fragments including Aβ, although not all have found this. In turn, exposure of cultured cortical neurons to Aβ or infection of rat hippocampal neurons with APP-expressing adenovirus (with associated increased Aβ accumulation) causes activation of caspase-3.

Figure 5. Caspase-3 expression in PSD subsynaptic fractions from control and AD brain tissue. The caspase-3 antibody recognizes both procaspase-3 (~32 kDa) and the large subunit of active/cleaved caspase-3 (~14 to 21 kDa). β-Actin was used as a loading control for normalization purposes. Only images of group 1 (N1 to N5 and AD1 to AD6) results are shown, but D includes data from both groups 1 and 2 (N6 to N10 and AD7 to AD13). A: Both pro- and active caspase-3 bands are displayed. B: Procaspase-3 bands are displayed more clearly with a shorter exposure time than in A. C: A longer exposure time than in A displays clearly the stack of bands (~20 kDa, 17 kDa, and 14 kDa) corresponding to active caspase-3. D: Densitometric analysis show increased expression levels in AD for procaspase-3 (t = 3.7, P < 0.002) and activated caspase-3 (t = 3.5, P < 0.004).
and neuron-specific apoptosis. In accordance with the prevailing amyloid hypothesis, the accumulation and toxicity of either fibrillar or oligomeric Aβ are thought to cause both neuronal and synaptic loss in AD. To a lesser degree, there has been some research examining Aβ as a trigger for synaptic degeneration and loss in AD.6,48 –51 Caspase-3 has also been implicated in mechanisms of tau-mediated neurodegeneration in AD. Tau is a substrate of caspase-3,31,33,52 and a mechanism linking Aβ deposition and neurofibrillary tangles in AD has been proposed: Aβ peptides promote neuronal pathological tau filament assembly by triggering caspase activation, leading to caspase cleavage of tau, which generates a proteolytic product that assembles more rapidly and extensively into pathological tau filaments.33,53 The fact that the rTg4510 tau transgenic mouse shows caspase-3 activation provides additional supporting evidence linking caspase-3 and tau-mediated neurodegeneration.54 Furthermore, it has been shown in the P301S tauopathy mouse model that synaptic loss occurs 3 months before fibrillary tau tangles appear.55 The occurrence of synaptic loss thus coincides with the conversion of normal tau to hyperphosphorylated insoluble tau, which destabilizes microtubules and impairs axonal and dendritic transport.56 Thus, it is possible that the abnormal accumulation of activated caspase-3 in synapses, particularly in the PSD fraction, reflects abnormal tau-mediated disruption of intraneuronal transport.

Previous immunohistochemical studies have shown immunoreactivity of active caspase-3 and caspase-3-cleaved substrates in neurons, senile plaques, and neurofibrillary tangles in AD,17,18,38,39,57–60 although not all investigators have reported these findings.61 Of note is that none of these previous studies have focused on caspase-3 in synaptic terminal fields or neuropil. The results of our study further highlight caspase-3 as a significant effector caspase involved in AD and contribute to a greater understanding of the role of synaptic degeneration.62–65 The fact that active caspase-3 is especially localized in the synapse, specifically postsynaptically, suggests that proapoptotic signaling is occurring at the synapse leading to synaptic degeneration. It also is consistent with the hypothesis that apoptotic signaling can be initiated at the synapse and be communicated to the cell body, which could contribute to neuronal cell death by apoptosis.41 Because caspase-3 activation has been proposed as an early neurodegenerative event in the progression of AD,38 it is important to elaborate the mechanisms underlying the synaptic loss and degeneration linked to active caspase-3.

Acknowledgments

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