A disturbance of calcium homeostasis is believed to play an important role in the neurodegeneration of the brains of Alzheimer disease (AD) patients, but the molecular pathways by which it contributes to the disease are not well understood. Here we studied the activation of two major Ca\(^{2+}\)-regulated brain proteins, calpain and calcineurin, in AD brain. We found that calpain I is activated, which in turn cleaves and activates calcineurin in AD brain. Mass spectrometric analysis indicated that the cleavage of calcineurin by calpain I is at lysine 501, a position C-terminal to the autoinhibitory domain, resulting in a Ca\(^{2+}\)-calmodulin-independent phosphatase activity, but the phosphatase activity is remarkably activated upon truncation. The cleavage and activation of calcineurin correlate to the number of neurofibrillary tangles in human brains. These findings suggest that the overactivation of calpain I and calcineurin may mediate the role of calcium homeostatic disturbance in the neurodegeneration of AD.

Alzheimer disease (AD) is a progressive and irreversible neurodegenerative disorder characterized by extracellular deposits of \(\beta\)-amyloid in neuritic plaques, intracellular neurofibrillary tangles (NFTs) consisting of abnormally hyperphosphorylated aggregates of the microtubule-associated protein tau, and selective neuronal loss. The molecular pathogenesis of AD is not well understood. Disregulation of calcium is one of the major hypotheses that may explain the pathogenic mechanism of the disease (for review see Refs. 1 and 2). Many putative etiologic factors of AD, including excitotoxicity, \(\beta\)-amyloid neurotoxicity, and free radical injury, have in common the potential for disrupting intracellular calcium homeostasis (3–5). However, direct evidence of altered calcium homeostasis in human brain is difficult to obtain because of the postmortem alteration of the intercellular calcium level.

Calcium signaling is mediated through an array of calcium-dependent enzymes. In the central nervous system, the most important calcium-signaling mediators include calpain and calcineurin. Calpain is a family of calcium-activated intracellular cysteine proteases that catalyze limited proteolytic cleavage of a variety of cellular proteins in all eukaryotes (for reviews see Refs. 2 and 6). To date, at least 14 members of the calpain family have been identified (6). Among them, calpain I and II are the most predominantly and widely expressed in mammalian tissue, which are composed of a large catalytic subunit (80 kDa) and a small regulatory subunit (30 kDa) (2). They differ mainly in their calcium requirements for activation and their tissue distributions. Both calpain I and II are present principally as inactive precursors in the cell, and they are activated by calcium-stimulated autoproteolytic cleavage of the N-terminal sequence in response to calcium influx. Calpain I, which is the major calpain isoform in the neuron, is fully activated by low micromolar concentrations of calcium (hence, it is also called \(\mu\)-calpain), whereas calpain II requires low millimolar levels of calcium for optimal activity (hence, also called m-calpain). Calpain is thought to play a critical role in the activation of neuronal cyclin-dependent protein kinase 5 (7–9) and the mitogen-activated protein kinase pathway (10), as well as phosphorylation and truncation of tau, which in turn cause neuronal death (11–14). Altered calcium homeostasis as well as truncation and activation of calpain I have been reported in AD brain (15).

Calcineurin (CaN), also known as protein phosphatase (PP) 2B, is a Ca\(^{2+}\)/calmodulin-dependent phosphoseryl/phosphothreonyl protein phosphatase that is highly expressed in the central nervous system (16). It is a heterodimer, consisting of an A-subunit (CaN A, a 60-kDa catalytic subunit) and a B-subunit (CaN B, an 18-kDa Ca\(^{2+}\)-binding regulatory subunit) (17). CaN A has an autoinhibitory domain and a calmodulin-binding domain in the C-terminal part. The phosphatase is activated when Ca\(^{2+}\)/calmodulin binds to it, because the binding triggers the release of the autoinhibitory domain from the catalytic active site (16, 18). CaN A can also be activated by proteolytic cleavage of the autoinhibitory domain, resulting in a Ca\(^{2+}\)/calmodulin-independent, active phosphatase (16, 19, 20). It has been reported that calpain I cleaves and activates CaN (18, 21). Hence, the phosphatase activity of CaN is regulated by both Ca\(^{2+}\)/calmodulin and calpain activation.

To reveal the dysregulated calcium signaling in AD brain, we investigated the proteolytic cleavage and activation of calpain I and CaN, the two major calcium-signaling mediators, in autopsyed human brains with a postmortem delay of less than 3 h. We found that both calpain I and CaN were truncated and activated in AD brain and that CaN was truncated/activated by calpain I at lysine 501.

**EXPERIMENTAL PROCEDURES**

**Materials**—The longest isoform of human tau (tau\(_{441}\)) and cyclin-dependent kinase 5 (cdk5) and its activator p25 were cloned, expressed, and purified as described previously (22, 23). The catalytic subunit of
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| TABLE ONE |
|---|
| Human brain tissue used in this study |
| Case | Age at death | Gender | PMI* | Braak stageb | Tangle scoresc |
|---|---|---|---|---|---|
| AD 1 | 89 | F | 3 | V | 14.5 |
| AD 2 | 80 | F | 2.25 | VI | 14.5 |
| AD 3 | 78 | F | 1.83 | VI | 15.0 |
| AD 4 | 95 | F | 3.16 | VI | 10.0 |
| AD 5 | 86 | M | 2.25 | VI | 13.5 |
| AD 6 | 91 | F | 3 | V | 8.50 |
| Means ± S.D. | 86.5 ± 6.5 | 2.58 ± 0.54 | 12.67 ± 2.73 |

**Con**1 85 M 25 II 4.25
Con2 86 F 2.5 III 5.00
Con3 81 M 2.75 III 6.41
Con4 88 F 3 II 2.00
Con5 90 F 3 III 4.50
Con6 88 F 3.5 III 2.50
Con7 88 F 3 IV 4.50
Means ± S.D. 86.6 ± 2.9 2.89 ± 0.39 4.17 ± 1.50

* PMI means postmortem interval.

**a** Neurofibrillary pathology was staged according to Braak and Braak (27).

**b** Tangle score was a density estimate and was designated none, sparse, moderate, or frequent (0, 1, 2, or 3 for statistics), as defined according to CERAD AD criteria (28). Five areas (frontal, temporal, parietal, hippocampal, and entorhinal) were examined, and the scores were added up for a maximum of 15.

CAMP-dependent protein kinase and calpain I were purchased from Sigma. Protein G-agarose beads were purchased from Pierce. Monoclonal antibodies against CaN A, PP1, and calpeptatin were from Sigma and BD Biosciences. Polyclonal antibodies to CaN A, PP2A, and PP5 raised in rabbits were described previously (24, 25). Polyclonal tau antibody 92e was raised in our laboratory (26). Peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA). 125I-Labeled anti-mouse and anti-rabbit IgG and ECL kit were from Amersham Biosciences. \[^{32}P\]ATP was bought from ICN Biomedicals (Costa Mesa, CA). Bradford protein assay reagent was from Bio-Rad.

**Brain Tissue**—Medial temporal cortices of the 6 AD and 7 age-matched normal human brains used for this study (TABLE ONE) were obtained from the Sun Health Research Institute Donation Program (Sun City, AZ). All brain samples were pathologically confirmed and stored at −70 °C until used. The use of frozen human brain tissue was in accordance with the National Institutes of Health guidelines and approved by our institutional review committee.

**Immunoprecipitation of CaN**—Human brain tissue was homogenized in 9× volumes of buffer containing 50 mM Tris-HCl, pH 7.0, 8.5% sucrose, 10 mM β-mercaptoethanol, 2.0 mM EDTA, 2.0 mM benzamidine, and 2.0 µg/ml each of aprotinin, leupeptin, and pepstatin. The 16,000 × g extracts were prepared from the homogenates, and the protein concentrations were measured by the Bradford method (29). The extracts were then incubated with monoclonal antibody to CaN A that was pre-coupled to protein G-agarose beads for 4 h at 4 °C. The negative control was prepared with protein G-agarose beads without pre-coupling anti-CaN A. The immunoprecipitated complex was washed with Tris-buffered saline three times and with 50 mM Tris-HCl, pH 7.4, two times, and then used for Western blots and CaN phosphatase activity assays. The success of the immunoprecipitation was examined by Western blot analyses as described (30).

**In Vitro Proteolysis of Calpain I and CaN**—Human brain extracts, prepared as described above except the protease inhibitors were omitted from the homogenizing buffer, were incubated in the presence of various concentrations of CaN A and/or protease inhibitors for 10 min at 30 °C. The reactions were terminated by addition of 4-fold concentrated SDS-PAGE sample buffer, followed by heating in boiling water for 5 min. The products of proteolysis were analyzed by Western blots (30) developed with antibodies to calpain I and CaN A.

**Mass Spectrometry (MS)**—The immunoprecipitated CaN A from AD brain extracts was resolved by 7.5% SDS-PAGE and stained with Coomassie Blue. The full-length and the truncated CaN A bands were excised and digested in gel with trypsin (20). Matrix-assisted laser desorption (MALDI)-MS was performed using an ABI 4700 (Applied Biosystems, Framingham, MA), as described previously (20). Briefly, spectra were obtained in the positive reflectron mode using external calibration. An LCQ (Thermo Electron Corp., San Jose, CA) nano-liquid chromatography (LC)/MS system equipped with a laboratory-made nano-spray ion source (spray voltage: 2.5 kV) and LC10 A gradient pumps (Shimazu, Kyoto, Japan) was used. The nano-LC column was a “stone-arch” column packed in-house with 100 × 0.1 mm C18 beads and was used at a flow rate of 200 nl/min after flow splitting. A linear gradient of B from 5 to 30% was run, using mobile phase A of 0.5% acetic acid and mobile phase B of 0.5% acetic acid/acetonitrile = 20/80. The MS scan range was 350–1500 m/z, and LC/MS spectra were collected in a data-dependent mode in which the highest intensity peaks in each MS scan were chosen for collision-induced dissociation, and the isolation window was 3 Da (precursor m/z 1.5 Da) to select a precursor ion. The dynamic exclusion option was selected with a repeat count of 1, a repeat duration of 0.5 min, and exclusion duration of 1 min.

**Calcineurin Phosphatase Activity Assays**—Unless specified otherwise, the phosphatase activity of CaN was assayed in a reaction mixture (20 µl) containing 50 mM Tris-HCl, pH 7.4, 1.0 mM MnCl₂, 10 mM β-mercaptoethanol, 1.5 mM CaCl₂, 1.5 µM calmodulin, 0.1 µg/ml \[^{32}P\]ATP, and the immunoprecipitated calcineurin complex. \[^{32}P\]-Tau was prepared by phosphorylation of recombinant human brain tau441 with CAMP-dependent protein kinase and cdk5/p25 and using \[^{32}P\]ATP, as described (30). When crude brain extracts were used for CaN activity assays, 100 nM okadaic acid was included in the reaction mixture to inhibit activities of other major brain phosphatases including PP1, PP2A, and PP5. After incubation at 30 °C for 20 min, the reaction was
terminated, and the released $^{32}$P, was determined by Cerenkov counting after separation from $^{32}$P-tau by ascending paper chromatography, as described previously (31).

**RESULTS**

**Both Calpain I and CaN A Are Truncated in AD Brain**—To investigate the proteolytic cleavages of calpain I and CaN A in AD brain, we employed Western blots to analyze the homogenates of temporal cortices from 6 AD and 7 age- and postmortem interval-matched control brains (TABLE ONE). In control brains, calpain I is displayed as two major bands (80 and 76 kDa) and a minor band (78 kDa) (Fig. 1A). This is consistent with previous observations that 80-kDa full-length calpain I can undergo calcium-stimulated autoproteolysis into 78- and 76-kDa truncated, activated forms (10, 15). The average ratio of the truncated over the full-length calpain I was 1.5 in control brains (Fig. 1B). In AD brains, almost all calpain I was proteolyzed into the 76-kDa truncated form, and the ratio was increased to ~12 (Fig. 1, A and B). CaN A in normal control brains was seen as a single 60-kDa band, whereas ~50% of CaN A was truncated into a 57-kDa form in AD brains, except the AD case 5 (Fig. 1, A and B). PP5, which is not regulated by calcium and hence was used as a sample loading control, showed no detectable cleavage in either AD or control brains (Fig. 1A). Because both calpain I and CaN A were found truncated in AD brain, we analyzed the levels of truncation of these two proteins and found a strong positive correlation ($r = 0.93$, $p < 0.01$) between calpain I truncation and CaN A truncation (Fig. 1C). This positive correlation was also seen when control cases were excluded from the analysis ($r = 0.81$, $p < 0.05$). These results suggest that CaN A truncation may be mediated by the proteolysis/activation of calpain I in AD brain.

**Truncation of CaN A Activates Its Ca$^{2+}$/Calmodulin-dependent Phosphatase Activity in AD Brain**—We then investigated whether the truncation of CaN A in AD brain affects its phosphatase activity. In order to assay the specific CaN activity, we immunoprecipitated it from the equal amounts of brain extracts from 6 AD and 7 control cases and measured the phosphatase activity toward $^{32}$P-tau as a substrate. Western blot analysis indicated that the anti-CaN A immunoprecipitated CaN A from the brain extracts almost completely and did not co-immunoprecipitate any of the other major brain protein phosphatases (i.e., PP1, PP2A, and PP5) or tau (Fig. 2A). When the precipitated CaN A was assayed for phosphatase activity in the absence of Ca$^{2+}$/calmodulin, little activity in both control and AD brains was observed (Fig. 2B), which was consistent with the fact that the phosphatase activity of CaN A is Ca$^{2+}$/calmodulin-dependent (17). When Ca$^{2+}$/calmodulin was included in the assay mixture, 2-fold higher activity in control and 9-fold higher activity in AD cases were detected. In the presence of Ca$^{2+}$/calmodulin, the CaN phosphatase activity was 2-fold higher in AD than in control brains. The elevated CaN phosphatase activity in AD brains versus control brains was not because of any change of CaN level, because no difference in CaN levels was found between the two groups, as determined by dot blot assay (data not shown). These results suggest that the truncation of CaN A in AD brain activates its phosphatase activity. Hence, we analyzed the correlation between the CaN A truncation and the phosphatase activity in these human brain samples. We found that the CaN phosphatase activity was positively correlated to the level of CaN A truncation ($r = 0.95$), and the correlation was highly significant ($p < 0.01$) (Fig. 2C). This positive correlation was also seen when control cases were excluded from the analysis ($r = 0.89$, $p < 0.05$). These results indicate that the activation of CaN phosphatase activity in AD brain results from the truncation of CaN A.

**By using $^{32}$P-labeled phosphorylase kinase and p-nitrophenyl phosphate as substrates, it was reported previously that the phosphatase activity of CaN in AD brain extracts was unchanged (32) or slightly decreased (33, 34) as compared with controls. In order to understand the apparent discrepancy between the previous reports and our present findings, we measured the CaN phosphatase activities of the same brain extract samples without immunoprecipitation by using $^{32}$P-tau as a substrate and in the presence of 100 nm okadaic acid to inhibit other major brain protein phosphatase activities. Consistent with our previous finding with $[^{32}]$Pphosphorylase kinase as a substrate (32), we found no difference in the phosphatase activity between AD and control brain extracts (Fig. 2D). To reveal why the difference in phosphatase activity of CaN could not be observed when the brain extracts were used instead of immunoprecipitated CaN, we added the CaN-immunodepleted extracts back into the reaction mixture of the activity assay just before**
incubation for dephosphorylation reaction. We found that addition of the immunodepleted extracts back into the assay mixture activated the immunoprecipitated CaN phosphatase activity of the control group to the same level of the AD group, but it did not alter the activity of AD group (Fig. 2E).

Western blots of the assay mixtures of the control group indicated that the full-length CaN A was partially truncated into the 57-kDa form immediately after the extracts were added back into the reaction mixture where Ca2+/calmodulin was present (data not shown), whereas CaN A immunoprecipitated from AD brain extracts was already partially truncated (Fig. 1A).

These results suggest that in the presence of 1.5 mM Ca2+/calmodulin and 1.5 μM calmodulin in the assay mixture, calpain I in the brain extracts immediately cleaved CaN A and activated its phosphatase activity during assay. Hence, the previous failure of detecting the elevated calcineurin activity in AD brain was probably because CaN A in control samples was truncated and activated during assay incubation with the added Ca2+/calmodulin in the assay mixture.

CaN A Is Cleaved by Calpain I in Human Brain Extracts—To investigate further whether the 57-kDa truncation of CaN A is produced by activated calpain I in AD brain, we added various concentrations of calcium together with 2.0 mM EDTA to normal human brain extracts, and we incubated them at 30 °C for 10 min and then determined the truncation/activation of calpain I and the cleavage of CaN A by Western blots. We found that calpain I was proteolyzed and activated from 80 to 78 kDa and 76 kDa in a Ca2+ dose-dependent manner (Fig. 3A). In addition to the truncation/activation of calpain I, a 57-kDa truncated CaN A was also observed in the same Ca2+ dose-dependent manner. Because the incubation was carried out in the presence of 2.0 mM EDTA that chelated all endogenous Ca2+ when no additional Ca2+ was added and most of the added Ca2+ that allowed only free Ca2+ above 2.0 mM chelating capacity of EDTA, only micromolar levels of free Ca2+ were present in the reaction mixture during incubation of the brain extracts. Hence, these results suggest that the CaN A proteolysis in the human brain extracts resulted from activation of calpain I rather than calpain II, which requires millimolar concentrations of free Ca2+ for activation.

To confirm that CaN A is proteolyzed by calpain I in human brain, we studied inhibition of the Ca2+-stimulated proteolysis of calpain I and
CaN A with various selective protease inhibitors. When aprotinin, a serine protease inhibitor, and pepstatin, an aspartic protease inhibitor, were included in the normal human brain extracts during incubation, no significant inhibition of the proteolysis of either calpain I or CaN A was observed (Fig. 3B). These results excluded the involvement of serine proteases and aspartic proteases in the proteolysis of calpain I and CaN A. In contrast, when leupeptin, a selective inhibitor of cysteine and serine proteases, and N-acetyl-Leu-Leu-β-NA (ALLN), a calpain and cysteine protease inhibitor, were included in the incubation mixtures, a marked inhibition of calpain I proteolysis and an almost complete blockage of CaN A proteolysis were observed (Fig. 3B). A specific calpain inhibitor, calpepstatin peptide, also inhibited the autoproteolysis of calpain I and prevented CaN A from proteolysis. Taken together, these results indicate that in AD brain most probably the elevated Ca2+ induces autoproteolysis and activation of calpain I, which in turn cleaves CaN A into a 57-kDa truncated form.

To study the activation of CaN by calpain I, we also incubated CaN immunoprecipitated from human brains with various concentrations of purified calpain I, and we found a concentration-dependent CaN A cleavage (data not shown) and activation of CaN phosphatases activity (Fig. 3C). This activation was more remarkable for CaN from control brains than that from AD brains. At 2.14 μg/ml calpain I, the CaN phosphatase activity immunoprecipitated from both AD and controls reached the same level. This activation was completely blocked when calpain inhibitors ALLN or calpepstatin peptide were included in the reaction mixtures (Fig. 3D). These results further support our conclusion that CaN A is truncated and activated by calpain I in AD brain.

Fig. 3, A and B, also indicated that, in addition to the 57-kDa truncation, incubation of normal human brain extracts with Ca2+ also induced a 48-kDa truncated form of CaN A, which was not seen in the extracts of AD brains. To learn whether the 48-kDa truncation of CaN A is an in vitro phenomenon or represents a difference between normal control and AD brains, we incubated the control and AD brain extracts with Ca2+ in parallel. We found that the incubation of control brain extracts induced both a major 57-kDa and a minor 48-kDa truncated CaN A (Fig. 4A). However, the 48-kDa truncated CaN A was not seen in the incubated AD brain extracts. To learn why the 48-kDa truncation could not be seen in AD brain extracts, we studied the activation of a specific endogenous calpain inhibitor, calpseudatin, by quantitating the truncated active 50-kDa form of calpseudatin (35) in AD brain versus control brain. We found that calpseudatin was overactivated in AD brain (Fig. 4B). Thus, the lack of 48-kDa truncation of CaN A in AD brain might be
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FIGURE 4. Proteolysis of calpain I and CaN A and the activation of calpepstatin in human brain extracts. A, equal amounts of brain extracts from a control (control 7) and an AD patient (AD 3) were incubated at 30 °C for 10 min in the presence of 2.0 mM EDTA and various concentrations of CaCl2, followed by Western blots probed with anti-calpain I (upper panel) and anti-CaN A (lower two panels). Open arrowheads indicate the full-length calpain I or CaN A. Closed arrows and arrowheads indicate the truncated calpain I or CaN A. The bottom panel is the overexposed image of the blot shown in the middle panel in order to show the 48-kDa truncation of CaN A. B, the crude brain extracts of 7 control (Con) and 6 AD cases were analyzed by Western blots developed with anti-calpepstatin. The major activated form of calpepstatin (50 kDa, indicated by arrow) was then quantitated by densitometry. Data are presented as percentage of the activated calpepstatin in control brains. *, p < 0.01 as compared with controls.

because of the overactivation of calpepstatin that could prevent this specific truncation. We indeed observed that calpepstatin appeared to have a more potent inhibition to the 48-kDa truncation than to the 57-kDa truncation of CaN A (data not shown).

CaN A Is Truncated at Lysine 501, a Position C-terminal of the Autoinhibitory Domain, in AD Brain—To map the cleavage site of CaN A truncation by calpain I in AD brain, we immunoprecipitated both the full-length and the truncated CaN A from AD brain extracts. The immunoprecipitated CaN A was then separated by SDS-PAGE (Fig. 5, A and B). Both the full-length and the truncated CaN A bands were dissected from the gel and subjected to analyses by both LC-MS/MS and MALDI-MS, followed by fingerprint analysis of the MS data against the known human CaN A sequence (36). Of the full-length CaN A, the MS-detected fragments spread from leucine 11 to the C-terminal end glutamine 521 (Fig. 5C). In contrast, the C-terminal fragment starting from aspartic acid 488 was missing in the truncated CaN A, despite the other detected fragments being similar to those of the full-length CaN A. These results suggest that the cleavage site of the truncated CaN A in AD brain should be C-terminal to aspartic acid 488. The sequential determinants of calpain-mediated cleavages are reported to be C-terminal to lysine, tyrosine, or valine residues and, commonly, N-terminal to serine, threonine, or alanine residues (37). Because there was no tyrosine or valine residues and only one lysine residue at position 501 located at the missing C-terminal end (amino acids 489–521), we concluded that the truncated CaN A was cleaved C-terminal to lysine 501 (Fig. 5C, indicated by an arrow), which is C-terminal to the autoinhibitory domain (Fig. 5C, boxed sequence). Our results are consistent with a reduction of ~3 kDa apparent molecular mass by a loss of 20 amino acids from 60-kDa full-length to 57-kDa truncated CaN A and with the above findings that the AD-specific truncated CaN A still required Ca2+/calmodulin for its phosphatase activity (Fig. 2B). Our conclusion is also consistent with a recent observation that calpain cleaves CaN A at lysine 501 in vitro (20).

Truncation and Activation of CaN A Correlate to the Number of NFTs in Human Brains—To learn if the truncation and activation of CaN A correlate to the major AD brain pathologies (NFTs and β-amyloid), we performed linear correlation analysis. We found that both the truncation and activation correlate positively to the number of NFTs but not significantly to β-amyloid load in human brains (TABLE TWO). These results suggest that calpain-mediated CaN A truncation and activation might play a role in neurofibrillary degeneration of AD.

DISCUSSION

In this study, we found for the first time that CaN A is proteolyzed at lysine 501 into a 57-kDa truncated form by activated calpain I in AD brain, and this cleavage markedly activates the Ca2+/calmodulin-dependent phosphatase activity of CaN. Because calpain and calcineurin are the major mediators of calcium signaling in the brain, our findings are consistent with the notion that calcium homeostasis is dysregulated in AD brain (1, 2).

Elevated cleavage and activation of calpain has been reported previously in early stage AD (10, 15, 38). Because calpain cleaves a broad range of substrates in vitro, including the regulatory domain of protein kinase C, the activator of cdk5, calcium/calmodulin-dependent protein kinase II, and CaN (for review see Ref. 39), it is not known through which pathway the activation of calpain may play a role in the pathogenesis of AD. Our study demonstrates that calpain activation may play a role in neurodegeneration via cleavage and activation of CaN in AD brain.

Although calpain I is abnormally activated in AD brain, the truncation of CaN A we observed in AD brain was not an artifact due to postmortem delays or homogenization procedures. First, the CaN A truncation did not correlate to the postmortem delay in AD cases. Second, we used brain tissue with very short postmortem delays, and both the control and AD groups had very similar postmortem delays (average of <3 h). Third, there was no detectable truncation of calcineurin in control cases, even in the case with 3.5-h postmortem (Fig. 1A, case 6). Fourth, we included EDTA and leupeptin in the homogenizing buffer that prevented CaN A from cleavage by calpain, as demonstrated in Fig. 3, A and B, during the homogenization procedure.
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Previous studies have demonstrated that calpain can cleave and activate CaN A in vitro (18, 20, 21). When CaN A is incubated with calpain I, CaN A is truncated into a 55- to 57-kDa transient form and then further into a 43- to 49-kDa form (18, 21). The truncated forms of CaN A have increased phosphatase activity, but the 43- to 49-kDa form loses the ability to bind and to be activated by calmodulin. In the present study, we found that CaN A is truncated at lysine 501 to produce the 57-kDa form in AD brain but is not truncated further to the smaller 48-kDa form. This might be because of the overactivation of calpase-tin, a specific endogenous calpain inhibitor (35) in AD brain, which might preferentially inhibit the 48-kDa truncation.

The pathophysiological role of the cleavage and activation of CaN A by calpain in the pathogenesis of AD remains to be investigated. A recent study (20) has demonstrated that calpain-catalyzed cleavage and activation of CaN A mediates glutamate- and kainate-induced excitotoxicity in hippocampal neurons. A chronic persistent level of calpain activation develops at an early stage of AD (15). Therefore, cleavage and activation of CaN A and the resulting downstream cascade may underlie the role of calpain activation in the molecular mechanism of AD. Our findings that truncation and activation of CaN correlate to the numbers of NFTs, but not of β-amyloid plaques, suggest that calpain-mediated CaN A truncation/activation might play a role in neurofibrillary degeneration of AD.

Microtubule-associated protein tau is abnormally hyperphosphorylated in AD brain (see Ref. 40 for review). Although CaN A has been shown to dephosphorylate tau at several phosphorylation sites in vitro (41, 42), the present findings that CaN A phosphatase activity is up-regulated rather than down-regulated in AD brain suggest that CaN A is probably not involved in the up-regulation of tau phosphorylation in AD brain. Kinetic studies indicate that CaN A has a 5-fold lower affinity to tau (K_m = 53.5 μM) than PP2A, PP1, and PP5 (K_m = 7.6–11.6 μM) (43). When comparing the relative contributions of major brain protein phosphatases to dephosphorylation of tau, we found that CaN A contributes to only ~7% of total tau phosphatase activity (43). Hence, the cleavage and activation of CaN A probably play a role in the molecular mechanism of AD via other pathways than dephosphorylating tau.

By using p-nitrophenyl phosphate as a substrate, it was reported previously that the nickel- and manganese-stimulated protein phosphatase activity (that mainly represented CaN A activity) was decreased in some areas of AD brain as compared with controls (33, 34). When phosphorylated phosphorylase kinase was used as a substrate, we did not find any difference in CaN activity between AD and controls (32). In the present study, it was surprising that by employing tau as a substrate, we observed a 2-fold higher phosphatase activity of CaN immunoprecipitated from AD brain as compared with that from control brains. The discrepancy between the previous and the present studies was because of the fact that in the former, the CaN phosphatase activity was assayed in the brain extracts in the presence of added CaCl_2/calmodulin, which resulted in the cleavage and activation of CaN A in the control brain extracts, bringing the phosphatase activity to a level similar to that in AD brain extracts.

![FIGURE 5. Mapping of the truncation site of CaN A in AD brain by mass spectrometry.](image)

**TABLE TWO**

| Correlation (r values) between CaN A truncation/activation and AD pathologies | Tangle load^a (n = 13) | β-Amyloid load^b (n = 13) |
|---|---|---|
| CaN A truncation^c | 0.74^e | 0.52 |
| CaN A activity^d | 0.70^f | 0.51 |

^a Tangle load and β-amyloid load were numerical density estimates of NFTs and β-amyloid plaques, respectively, in the temporal lobes and were designated none, sparse, moderate, or frequent (0, 1, 2, or 3 for statistics), as defined according to CERAD AD criteria (28).

^b CaN A truncation was the ratios of 57/60-kDa CaN A.

^c, d p < 0.01.

^e CaN A activity was assayed after immunoprecipitation from the crude brain extracts.
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In conclusion, we have demonstrated that calpain I is truncated and activated in AD brain, which in turn truncates CaN A at lysine 501 and activates its phosphatase activity. Because overactivation of CaN has been shown to be cytotoxic, our findings suggest that the disturbance of calcium homeostasis may contribute to neurodegeneration in AD via overactivation of the calpain/CaN pathway.

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