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Caspase Cleavage of the Amyloid Precursor Protein is Prevented After Overexpression of Bcl-2 in a Triple Transgenic Mouse Model of Alzheimer's Disease

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Original Article

Caspase cleavage of the amyloid precursor protein is prevented after overexpression of bcl-2 in a triple transgenic mouse model of Alzheimer's disease

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Abstract: A recent study demonstrated the lack of beta-amyloid (A β) plaque formation and accumulation of the amyloid precursor protein (APP) in a triple transgenic mouse model of Alzheimer's disease (3xTg-AD) following overexpression of the anti-apoptotic protein, Bcl-2 (Rohn et al., *J. Neurosci.* 28: 3051-9, 2008). The supposition from that study was the accumulation of APP resulted from a decrease in caspase-mediated processing of APP. To determine a direct role for the caspase-cleavage of APP in 3xTg-AD mice, we designed a site-directed caspase-cleavage antibody to APP and demonstrated it is a specific marker for caspase-cleaved APP. Application of this antibody revealed neuronal staining in the hippocampus and subiculum of 3xTg-AD mice. These results were confirmed utilizing a similar site-directed antibody to caspase-cleaved APP (APPneo). The caspase cleavage of APP as well as the formation of extracellular A β plaques was prevented in 3xTg-AD animals overexpressing Bcl-2. These results provide further support that caspases play a proximal role in promoting the pathology associated with AD.

Key Words: Amyloid precursor protein, beta-amyloid, caspase; mouse model, neurofibrillary tangles, plaques; tau; bcl-2; apoptosis

Introduction

In Alzheimer's disease, A β deposition is accompanied by the gradual replacement of the neuronal cytoskeleton with insoluble NFTs. NFTs are intracellular fibrillary inclusions composed of aggregations of paired helical filaments (PHFs), which are made up of abnormally phosphorylated and truncated tau [1]. According to the A β hypothesis, A β precedes NFT formation, suggesting that A β may be the earliest event that triggers downstream events [2]. Several studies have supported the hypothesis that A β formation and NFT formation may be closely linked through a common pathway of caspase-mediated proteolysis [3-5]. Direct evidence supporting a role for caspases in promoting

the pathology associated with AD come from several transgenic mouse studies: Galvan et al. demonstrated that deleting the C-terminal caspase-cleavage consensus site within APP reversed the pathology and behavioral deficits associated with human APP transgenic mice [6]. In a recent study by Spires-Jones et al., the authors used *in vivo* imaging in rTg4510 mice, a reversible mouse model of tauopathy, to demonstrate the activation of caspases within tangle-bearing neurons [7]. Finally, we have developed a triple-transgenic mouse model (3xTg-AD) that overexpress the anti-apoptotic protein, Bcl-2, in all postmitotic neurons of the CNS. Overexpression of Bcl-2 prevented caspase activation, the caspase cleavage of tau and improved place recognition memory in 3xTg-AD mice [8]. An

additional finding of that study was the accumulation of full-length APP and tau following overexpression of Bcl-2 in 3xTg-AD mice [8]. The interpretation of this finding was the observed accumulation of APP and tau resulted from an inhibition in caspase-mediated proteolysis following overexpression of Bcl-2. However, direct demonstration that APP is cleaved by caspases and is prevented following overexpression of Bcl-2 was not investigated. The goal of the present study was to examine directly a role for caspase-mediated cleavage of APP in 3xTg-AD mice utilizing a novel site-directed caspase-cleavage antibody to APP. Following application of this antibody and a similar antibody developed by Galvan et al. [6], we now demonstrate caspase-cleavage of APP does occur in 3xTg-AD mice and is prevented along with the formation of extracellular deposits of A β following overexpression of Bcl-2. These results provide further support for a role of caspases in the disease progression associated with AD.

Methods and Materials

Animals

The generation and characterization of 3xTg-AD mice that overexpress the anti-apoptotic protein, Bcl-2, have been described previously [8]. In brief, 3xTg-AD mice harbor three known mutations, human APP^{Swe}, human tau^{P301L} and PS1^{M146V} known to give rise to the heritable forms of AD [9]. These mice, termed 3xTg-AD mice, progressively develop plaques and tangles in a manner that closely recapitulates the disease process in the human AD brain [9]. To generate 3xTg-AD mice that overexpress the anti-apoptotic protein, Bcl-2, 3xTg-AD mice were crossed with Tg mice that overexpress the human Bcl-2 gene in all post-mitotic neurons [10]. The progeny resulting from such a cross have been termed 3xTg-AD/Bcl-2 overexpressors (3xTg-AD/Bcl-2 OE). Littermates that were negative for the human Bcl-2 gene were used for comparison.

Tissue acquisition

Mice were anesthetized with pentobarbital, perfused with saline, and the brains rapidly removed. Brains were divided into hemispheres and one hemisphere was sunk in 4% phosphate-buffered paraformaldehyde, while the other hemisphere was snap frozen at

-50°C in isopentane. Mouse brains were mounted coronally and sectioned serially at 50 μ m on a vibratome, and stored for immunohistochemistry.

Generation of a caspase-cleavage, site-directed antibody to the amyloid precursor protein

Previous studies have demonstrated the caspase-3 cleavage of the 120 kDa peptide of APP results in two small fragments (23 and 25 kDa); both of these sites are within the first 220 amino acids while a third cleavage site at aspartic residue 739, creates a larger polypeptide (~85 kDa) [11]. A polyclonal antibody, herein termed APP caspase-cleavage product (ccp) was synthesized based upon a putative caspase-3 cleavage site (VEVD⁷³⁹) within APP [11]. A 7-mer peptide (HGVVEVD) corresponding to the upstream neopeptide fragment, that would be generated following cleavage, was coupled to a cysteine group to facilitate the conjugation of the adjuvant, KLH. The resulting peptide ([KLH] - CHGVVEVD) was injected into rabbits and the resulting sera was used to affinity purify antibodies using a sulfolink column coupled with the peptide (CHGVVEVD). Synthesis of peptides, injections of immunogens, and collection of serum was completed by Bethyl laboratories (Montgomery, TX).

Antibody dilutions

Primary antibodies used in immunohistochemical analysis were diluted as follows: APPccp, 1:100-150; mAB 1560 anti-A β (clone 6e10, Chemicon, Temecula, CA), 1:400; APPneo, 1:1000; and Tau C3 (Chemicon, Temecula, CA), 1:100.

Cerebral ischemia produced by middle cerebral artery occlusion, a model of apoptosis

Brain sections were generously provided to us by Dr. Sheng T. Hou (Experimental Neurotherapeutics Laboratory, National Research Council Institute for Biological Sciences, National Research Council Canada, Ottawa, Ontario, Canada). C57BL/6 mice (20-23 g) were subjected to middle cerebral artery occlusion (MCAO) as described previously [12]. Briefly, under anesthesia, mice were subjected to MCAO using an intraluminal filament for 1 hr. After 1 hr of MCAO, the filament was removed and blood flow was restored for 24 hr

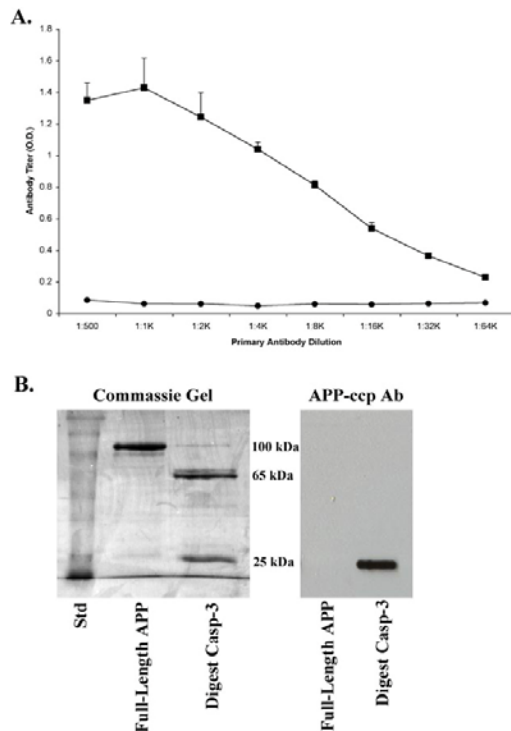


Figure 1. Characterization of the APPccp antibody *in vitro*. (A): ELISA results utilizing preimmune (circles) or immunized (squares) sera indicated a high titer of antibody following immunization of rabbits with the peptide, [KLH]-CHGVVEVD. Results are representative of three separate experiments ran in triplicate, \pm S.E.M. (B) Recombinant human APP was incubated in the presence or absence of caspase-3 for 2 hours at 37°C. Samples were separated by SDS-PAGE and gels were stained with coomassie blue (left panel) or transferred to nitrocellulose and probed with affinity-purified APPccp antibody (1:500). Incubation of full-length APP with caspase-3 led to efficient processing and formation of two prominent fragments (65 and 25 kDa, left panel). Western blot analysis indicated that the APPccp antibody recognizes only the caspase-cleaved fragment (~25 kDa) fragment and does not detect full length APP (~100 kDa).

at which time animals were sacrificed. Mouse brains were perfused with 10% formalin in PBS, then post-fixed in 10% formalin for 4 hr and cryo-protected overnight in phosphate buffer containing 30% sucrose at 4°C. Following fixation, brains were sectioned into 50 μ m free-floating sections to be processed by immunohistochemistry. Apoptotic areas were identified by Hoechst staining as described previously [12].

Fluoro Jade C labeling

To assess for neurodegeneration and apoptosis, the fluorescent dye, Fluoro Jade C (Millipore, catalog number AG325) was utilized as previously described by Bian *et al.* [13]. Briefly, fixed brain sections were mounted and pretreated for 5 min in a 80% alcohol solution containing 1% sodium hydroxide, followed by a 70% alcohol and a distilled water wash for 2 min. Sections were then incubated for 10 min in a 0.06% potassium permanganate solution followed by rinsing in distilled water for 2 min. Sections were then transferred into a 0.0001% solution of Fluoro Jade C containing 0.1% acetic acid for 10 min. Following 3 successive washes in distilled water for 1 min, slides were dried, dehydrated and cover-slipped with Depex.

Cell-free digestion of APP

To examine whether caspase-3 can cleave APP, 1 μ g of purified human recombinant APP (Calbiochem) was incubated with active human recombinant caspase-3 (Calbiochem) in 2x reaction buffer containing 10 mM DTT for two hours at 37°C. Reactions were terminated by the addition of 5x sample buffer and stored at -20°C until analyzed.

ELISA

Antibody titers were determined with an enzyme-linked immunoassay (ELISA). Samples were run in triplicate on ELISA plates coated with the peptide, CHGVVEVD, which was used as the immunogen. The peptide CHGVVEVD was bound in solid phase on high binding 96 well plates (Dynex Technologies). Serum was diluted 1:500 and then serially diluted to a final dilution of 1:64,000. Detection of the antibody was obtained using an HRP conjugated secondary antibody (1:5,000) and TMB micro well peroxidase substrate (KPL Protein Research Products).

Human Subjects

Autopsy brain tissue from the hippocampus and entorhinal cortex of five neuropathologically confirmed AD cases and five nondemented cases diagnosed as normal was studied. Human brain tissues used in this study were provided by the Institute for Brain Aging and Dementia Tissue Repositories at the University of California, Irvine.

Immunohistochemistry and immunofluorescence

Free-floating 50 μm -thick serial sections were used for immunohistochemical and immunofluorescence studies conducted as previously described by Mouser et al., [14]. Antigen visualization was determined using ABC complex (ABC Elite immunoperoxidase kit, Vector labs), followed by DAB substrate (brown product) or with nickel chloride, which generates a black product or NovaRed, which generates an orange product (Vector Labs). MCAO brain sections were labeled with APPccp antibody (1:100) followed by streptavidin Alexa Fluor 555 (red, Ex/Em = 555/565). To visualize nuclei, sections were also incubated in Hoechst.

Western blot analysis

Full-length APP, or APP digested with caspase-3 were stored in 5x sample buffer at -20°C until analyzed. Samples were boiled for 5 minutes, placed on ice then loaded onto 12% SDS-PAGE gels. Gels were either stained with coomassie blue to detect total protein content, or slabs were transferred to nitrocellulose for Western blot analysis. Western blot analysis was performed utilizing the One-Step™ Advanced Western rabbit kit according to manufacturer's directions (GenScript Corporation, Piscataway, NJ). Samples were analyzed for protein content using the BCA assay (Pierce) to ensure equal protein loading.

Results

Characterization of a site-directed caspase-cleavage antibody to APP

To examine whether APP is a target for caspase cleavage in 3xTg-AD mice, we synthesized a site-directed antibody according to a putative caspase cleavage site (VEVD⁷³⁹) located at the C-terminal end of APP. The antibody was directed towards the upstream neo-fragment that would be generated following cleavage by caspase-3 at this site. Cleavage of APP by caspase-3 at this consensus site results in a predicted fragment of 25 kDa [11]. An 8-mer peptide was conjugated to KLH ([KLH]-CHGVVEVD) and injected into rabbits. As shown in **Figure 1A**, a strong antibody titer was obtained following immunization of rabbits with this peptide as compared to preimmune serum. Following

affinity purification, we tested the specificity of this antibody (herein termed the APP caspase-cleavage product (ccp) antibody) utilizing a cell-free system consisting of purified human recombinant APP incubated with or without caspase-3. Following digestion of APP, samples were separated by SDS-PAGE gel electrophoresis and either stained with coomassie blue (left panel, **Figure 1B**) or transferred to nitrocellulose and probed with the APPccp antibody (right panel, **Figure 1B**). While no immunoreactivity was evident in non-digested samples, a prominent band was visible at 25 kDa, corresponding to the 25 kDa fragment generated following digestion of APP with caspase-3 (**Figure 1B**, right panel). These results indicate the APPccp antibody recognizes only the caspase-cleaved fragment (~25 kDa) and does not detect full length APP (~100 kDa).

Experiments were performed to further characterize the APPccp antibody in an *in vivo* model system of ischemia, which has

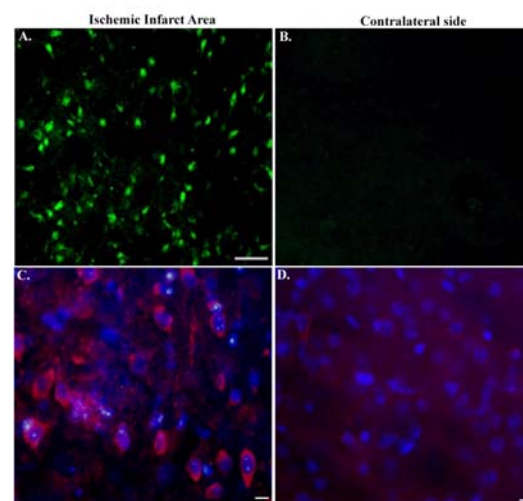


Figure 2. Detection of caspase-cleaved APP in an animal model of apoptosis. Mice were subjected to 1 hr MCAO, followed by reperfusion for 24 hr. (A and B): Brain sections incubated with Fluoro Jade C revealed labeling of shrunken, damaged neurons in the cortex of MCAO mice in ischemic infarct areas (A), while no staining with Fluoro Jade C was evident on the contralateral control side of the brain (B). (C and D): Identical to Panels A and B except sections were analyzed for caspase-cleaved APP using the APPccp antibody. In this case, APPccp staining (red) was evident in ischemic infarct areas (C) and was largely absent on the contralateral side of the brain (D). Nuclei were identified following staining with Hoechst (Blue). Scale bars represent 10 μm .

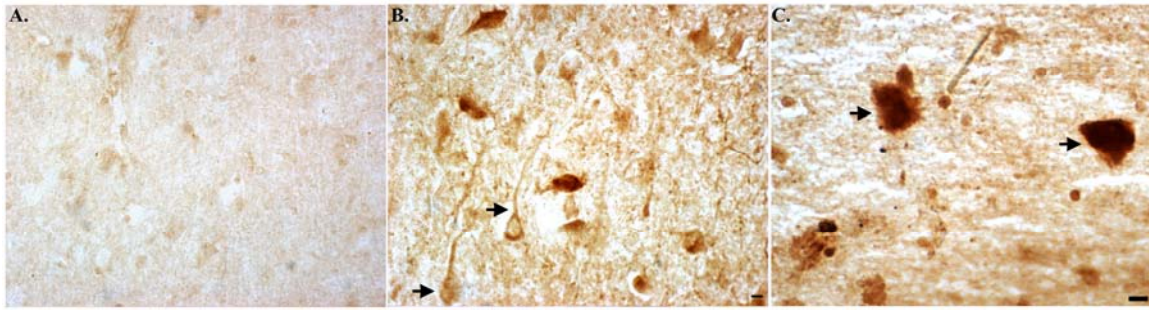


Figure 3. Characterization of APPcpc antibody in the human AD brain by immunohistochemistry. Data are representative staining from human postmortem hippocampal brain sections following application of the APPcpc antibody. (A): Representative age-matched control section following labeling with APPcpc indicating a general lack of staining. (B and C): Labeling of APPcpc in a representative AD case was evident within hippocampal neurons with apparent NFT morphology (arrow, B) and within plaque-rich regions (arrows, C).

previously been shown to activate apoptotic pathways [12]. The advantage of this model is that it creates an ischemic infarct confined to one side of the brain while leaving the other hemisphere undamaged, which thus can serve as an internal control. As an initial approach, we first determined whether we could detect neurodegeneration in ischemic infarct regions of MCAO mice. To accomplish this, we employed the use of a specific marker for neurodegeneration, Fluoro Jade C. Fluoro Jade C is a fluorescent dye that detects neuronal degeneration in animal models and displays a high degree of specificity and sensitivity [13]. Application of Fluoro Jade C to MCAO brain sections revealed widespread neuronal labeling in ischemic infarct areas (**Figure 2A**), while the contralateral side of the brain showed no evidence of labeling (**Figure 2B**). These data suggest that activation of apoptotic pathways and neurodegeneration does indeed occur in MCAO mice. Sections were further analyzed for caspase-cleaved APP using APPcpc and revealed the labeling of neurons in ischemic infarct areas. For these experiments, sections were also incubated with the nuclear label, Hoechst (shown in blue, **Figure 2C**) to indicate whether nuclei were condensed or fragmented, a characteristic morphological feature of apoptosis. In this regard, we found co-localization of APPcpc within neurons that were clearly displaying nuclear condensation and fragmentation (**Figure 2C**). In contrast there was no labeling of the APPcpc antibody on the contralateral side of the brain and the nuclei appeared normal (**Figure 2D**). These data provide further

support that the APPcpc antibody is a specific probe for caspase-cleaved APP.

Following confirmation that the APPcpc antibody is specific for the C-terminal caspase-cleavage fragment of APP, immunohistochemical analysis was performed using human postmortem hippocampal sections from AD and age-matched control brains. A previous study has shown that APP is cleaved by caspase-3 predominantly within the cytoplasmic tail of APP, a cleavage event, which also occurs in the human AD brain [11, 15]. In age-matched control sections, little labeling was observed following application of the APPcpc antibody (**Figure 3A**). In contrast, labeling of APPcpc was evident within hippocampal neurons, and within plaque-rich regions in cases exhibiting pathology consistent with AD (**Figure 3B and C**). Taken together (**Figs. 1-3**), these findings validate the APPcpc as a specific probe to detect caspase-cleaved APP.

Detection of caspase-cleaved APP in 3xTg-AD and its prevention following overexpression of Bcl-2

To test whether the caspase-cleavage of APP occurs in 3xTg-AD mice, brain sections of 18 and 24 month-old mice were analyzed by immunohistochemistry. Little labeling was observed in age-matched nonTg control mice (data not shown). Strong neuronal labeling was evident primarily within the subiculum in both 18 and 24 month-old 3xTg-AD mice (**Figure 4A and C**). Interesting, the APPcpc

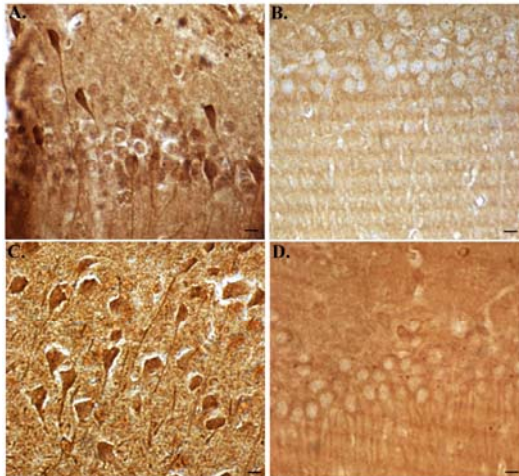


Figure 4. Evidence for the caspase-cleavage of APP in 3xTg-AD mice, which is prevented following overexpression of Bcl-2. Immuno-histochemical analysis following application of APPccp antibody in hippocampal sections from 18 month-old 3xTg-AD mice (A) or 24 month-old 3xTg-AD mice (C) revealed neuronal staining within the subiculum. In contrast, in age-matched 3xTg-AD mice overexpressing Bcl-2 there was no evidence for APPccp staining (B and D). Data are representative staining of a total of $n = 3$ mice from each age group. All scale bars represent 10 μm .

antibody did not immuno-detect A β plaques, suggesting that the C-terminal cleavage fragment of APP is not extracellularly deposited. In contrast to 3xTg-AD mice, there was no staining following application of the APPccp antibody in 3xTg-AD mice overexpressing the antiapoptotic protein, Bcl-2 (**Figure 4B** and **D**). To confirm these findings, we employed the use of a similar site-directed caspase-cleavage antibody to APP developed by Galvan et al. [6, 16]. This antibody, termed APPneo, selectively recognizes the neo-C-terminus fragment of APP generated by caspase-mediated cleavage of APP [16]. Application of APPneo in 3xTg-AD mice revealed labeling within neuronal cell bodies and dystrophic neurites in hippocampal and cortical regions (**Figure 5A** and **C**), which was absent in 3xTg-AD mice overexpressing Bcl-2 (**Figure 5B** and **D**).

In a previous study, overexpression of Bcl-2 in 3xTg-AD mice prevented the caspase cleavage of tau, the formation of extracellular plaques, and led to an accumulation of both APP and

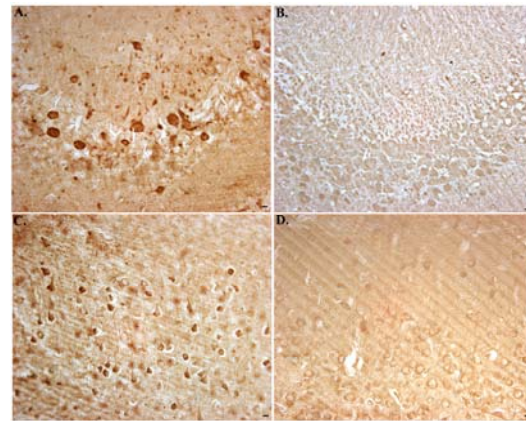


Figure 5. Confirmation of the caspase-cleavage of APP and its prevention following overexpression of Bcl-2 utilizing APPneo. Application of APPneo, an identical site-directed caspase-cleavage antibody to APPccp developed by Galvan et al., [6], indicated labeling in 3xTg-AD mice that was prevented following overexpression of Bcl-2. (A and B): Representative hippocampal staining in CA3 region of a 18 month-old 3xTg-AD mice with APPneo indicated strong labeling within neuronal cell bodies and dystrophic neurites (A) that was absent in 3xTg-AD/Bcl-2 OE mice (B). (C and D): Similar results were observed in the cortex of 24 month-old 3xTg-AD mice (C) and 3xTg-AD/Bcl-2 OE mice (D). All scale bars represent 10 μm .

tau [8]. To extend these findings, we performed double-label immunohistochemical experiments using the APPccp antibody together with anti-A β 1560 (clone 6E10), an antibody known to react with both the A β peptide as well as with full-length APP [17]. Strong staining of plaques with little neuronal staining was observed following application of the A β 1560 antibody in 3xTg-AD mice (brown labeling, **Figure 6A**). Evidence for caspase-cleaved APP was evident under the same conditions following application of APPccp (gray labeling, **Figure 6A**). In contrast, while there was strong intracellular labeling with A β 1560, there was little evidence for caspase-cleaved APP in 3xTg-AD mice overexpressing Bcl-2 (**Figure 6B**). These results suggest that overexpression of Bcl-2 prevents the caspase-cleavage of APP as well as the extracellular deposition of A β in 3xTg-AD mice.

Experiments were also performed to test whether APPccp co-localized with an antibody to caspase-cleaved tau (TauC3) developed by Gamblin et al [4]. Double-label immunohisto-

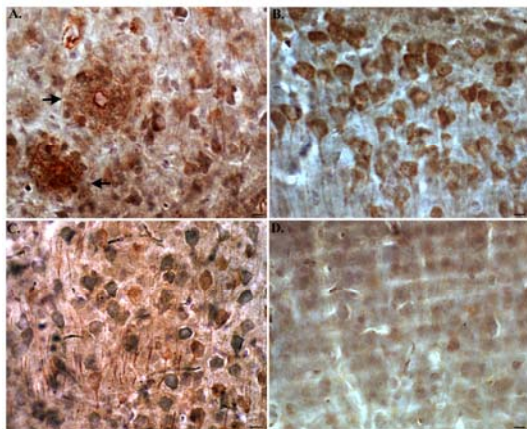


Figure 6. Prevention of extracellular plaque formation and the caspase cleavage of APP and tau in 3xTg-AD mice overexpressing Bcl-2. (A and B): Double-label immunohistochemical analysis in 24 month-old 3xTg-AD mice utilizing mAb anti-A β 1560 (red label) together with APPcpcp (blue) indicated a lack of extracellular plaque deposition and APPcpcp labeling in 3xTg-AD/Bcl-2 OE mice (B) compared to 3xTg-AD mice (A). (C and D): Double-labeling immunohistochemical analysis with APPcpcp (blue) and Tau C3 (red) shows co-localization of the two antibodies within the subiculum of 24 month-old 3xTg-AD mice (C). In 3x-Tg-AD/Bcl-2 OE mice, a reduction in the labeling of both antibodies was observed (D). Data are representative staining from $n = 3$ animals. All scale bars represent 10 μm .

chemical analysis with APPcpcp and TauC3 demonstrated co-localization of the two antibodies within the subiculum of 24 month-old 3xTg-AD mice (**Figure 6C**). In 3xTg-AD/Bcl-2 OE mice, a reduction in the labeling of both antibodies was observed indicating overexpression of Bcl-2 prevents caspase activation and subsequent cleavage of target proteins in 3xTg-AD mice (**Figure 6D**).

Discussion

The amyloid precursor protein (APP) is a substrate for caspase-3 mediated cleavage, which may contribute to A β formation, synaptic loss, and the behavioral changes associated with AD [6, 11, 18, 19]. The cleavage of APP into A β represents an early stage in AD, and previous research in this regard has focused on secretase involvement (α , β and γ) [20]. Whether or not other proteases contribute to the processing of APP is unknown, however, studies have suggested that caspase-mediated cleavage of APP may contribute to

A β formation and lead to the generation of a cytotoxic C-terminal peptide termed C31 [21-23]. We recently demonstrated an accumulation of intracellular APP and a lack of extracellular plaque formation in 3xTg-AD mice following the overexpression of Bcl-2 [8]. Bcl-2 constitutes a critical control point in apoptosis residing immediately upstream of irreversible cellular damage, where it prevents the release of apoptogenic factors from the mitochondria [24]. One interpretation of these results showing an accumulation of APP in 3xTg-AD/Bcl-2 OE mice is that caspases play a role in the turnover of this protein. Blocking caspase activation by overexpressing Bcl-2 prevents cleavage and turnover of APP allowing it to accumulate intracellularly within neurons. However, direct evidence for the caspase-cleavage of APP and its prevention following overexpression of Bcl-2 was not demonstrated [8]. The goal of the present study was to examine this question directly by utilizing a site-directed antibody to caspase-cleaved APP based on a previous study by Gervais et al. [11].

Following the experimental procedures set forth in this study, we synthesized an antibody to the predicted upstream fragment that would be generated following cleavage of full length APP by caspase-3 at this site. To test the specificity of our antibody we utilized a cell-free system consisting of purified human recombinant APP incubated with and without recombinant caspase-3. Western blot analysis indicated that the APPcpcp antibody recognizes only the caspase-cleaved fragment (~25 kDa) but does not detect full length APP (~100 kDa), indicating that the antibody shows a high degree of specificity for the cleaved fragment. Following further verification of APPcpcp as a specific marker for caspase-cleaved APP *in situ*, we examined whether the antibody could detect cleaved APP in 3xTg-mice and whether this cleavage could be prevented following overexpression of the anti-apoptotic protein, Bcl-2. Utilizing this antibody as well as a similar antibody, APPneo, developed by Galvan et al. [16], we now provide evidence for the caspase-cleavage of APP in 3xTg-AD mice that is prevented following overexpression of Bcl-2. These results confirm and extend our previous findings and suggest that the caspase-cleavage of APP is a proximal event that may regulate the normal turnover of this protein and contribute to extracellular deposition of A β . The contribution to the formation of A β by

caspsases is predicted to occur by two different mechanisms. One, is a through a direct interaction of caspase-3 with the C-terminal region of APP, leading to proteolytic processing of APP [11]. Gervais et al., demonstrated that cleavage of APP by caspase-3 enhanced A β production five-fold [11]. Alternatively, caspsases may contribute to the formation of A β , indirectly through cleavage of the beta-site APP cleaving enzyme (BACE) trafficking protein, GGA3. In a study by Tesco and colleagues, caspase activation led to the cleavage and stabilization of the BACE trafficking molecule, GGA3. GGA3 plays a role in sorting BACE to lysosomes allowing for turnover of the enzyme. Consequently, after caspase-3 activation and cleavage of GGA3, there is increased availability of BACE leading to enhanced production of A β [25]. Taken together, our findings along with previous studies, suggest that caspase activation may be an early and important event associated with the pathological mechanisms underlying AD. Therefore, therapeutics aimed at inhibiting members of the caspase family may provide a means of treating AD.

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Caspase, APP, bcl-2 and AD

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