Calpain Inhibitor A-705253 Mitigates Alzheimer’s Disease–Like Pathology and Cognitive Decline in Aged 3xTgAD Mice

Calpains are cysteine proteinases that selectively cleave proteins in response to calcium signals. Exacerbated activation of calpain has been implicated as a major component in the signaling cascade that leads to β-amyloid (Aβ) production and tau hyperphosphorylation in Alzheimer’s disease (AD). In this study, we analyzed the potential therapeutic efficacy of inhibiting the activation of calpain by a novel calpain inhibitor in aged 3xTgAD mice with well-established cognitive impairment, plaques, and tangles. The administration of a novel inhibitor of calpain, A-705253, attenuated cognitive impairment and synaptic dysfunction in a dose-dependent manner in 3xTgAD mice. Inhibition of calpain lowered Aβ40 and Aβ42 levels in both detergent-soluble and detergent-insoluble fractions and also reduced the total number and size of thioflavin S–positive fibrillar Aβ deposits. Mechanistically, these effects were, in part, explained by a down-regulation of β-secretase 1 (BACE1) and an up-regulation of ATP-binding cassette transporter A1 (ABCA1) expression, which, in turn, contributed to reduced production and increased clearance of Aβ, respectively. Moreover, A-705253 decreased the activation of cyclin-dependent kinase 5 (CDK5) and thereby diminished the hyperphosphorylation of tau. Finally, blockage of calpain activation reduced the astrocytic and microglial responses associated with AD-like pathological characteristics in aged 3xTgAD mice. Our data provide relevant functional and molecular insights into the beneficial therapeutic effects of inhibiting calpain activation for the management of AD. (Am J Pathol 2012, 181:616–625; http://dx.doi.org/10.1016/j.ajpath.2012.04.020)
spinal cord injury, traumatic brain injury, and wallerian degeneration.8–11 Regarding the AD brain, calpains have regulated the proteolytic processing of the amyloid precursor protein (APP) and phosphorylation and proteolysis of tau, leading to a hypothesis that selective calpain inhibitors are potential therapeutic strategies for AD.12–16

However, the potential use of known calpain inhibitors as therapeutic tools in AD is limited because of their low cellular penetration, poor selectivity, and kinetics. More recently, A-705253, a novel calpain inhibitor capable of inhibiting calpain in nanomolar concentrations and with enhanced oral bioavailability, water solubility, and metabolic stability, has been characterized and may be a promising candidate of a therapeutic drug for AD.17 In this study, we sought to preclinically assess the therapeutic effects of A-705253 on aged 3xTgAD mice and to determine the possible molecular mechanisms involved in its actions.

### Materials and Methods

#### Animal Treatments

3xTgAD mice harboring a presenilin1 mutation (PS1M146V), the Swedish double mutation in APP (APP KM670/671ML), and a frontotemporal dementia mutation in tau (tau P301L) were used for all experiments.18 Strain-matched nontransgenic mice (nTg), 129/C57BL/6, were used as controls. Calpain inhibitor A-705253 [N-(1-benzyl-2-carbamoyl-2-oxoethyl)-2-[E-2-(4-diethylaminomethylphenyl)ethen-1-yl]benzamide] was dissolved in 2% sucrose solution and administered to the mice in their drinking water at a dose of either 40 or 80 mg/kg per day. A-705253 was kindly donated by Abbott Laboratories (Ludwigshafen, Germany). Mice were treated for 3 months, from the age of 15 months to the age of 18 months. No changes in total water consumption and body weight were found between vehicle and A-705253–treated mice. All procedures used in the present study followed the Principles of Laboratory Animal Care from NIH publication 85-23.

#### Behavior Paradigms

Behavior paradigms to assess cognition were performed for all groups of mice between the ages of 17 and 18 months, as previously described.19 Novel object recognition, Morris water maze, and contextual fear conditioning are briefly described herein.

#### Novel Object Recognition

Each mouse was habituated to an empty arena for 3 consecutive days. On the first day of testing, mice were exposed to two identical objects placed at opposite ends of the arena for 5 minutes. Twenty-four hours later, the mouse was returned to the test box, this time with one familiar object and one novel object. Time spent exploring the objects was recorded for 5 minutes. The recognition index represents the percentage of the time that mice spend exploring the novel object. Objects used in this task were carefully selected to prevent preference or phobic behavior.

#### Morris Water Maze

Mice were trained to swim to a circular clear Plexiglas platform submerged 1.5 cm beneath the surface. Four trials were performed per day, for 60 seconds each, with 5 minutes between trials. Mice were trained for as many days as needed for the group to reach the training criterion of 25 seconds. The probe test was assessed 24 hours after the last trial, with the platform removed. Performance was monitored with the EthoVision XT video-tracking system (Noldus Information Technology, Leesburg, VA).

#### Contextual Fear Conditioning

During training, mice were placed in the fear conditioning chamber and allowed to explore for 2 minutes before receiving three electric foot shocks (duration: 1 second; intensity, 0.2 mA; intershock interval, 2 minutes). Animals were returned to the home cage 30 seconds after the last foot shock. Twenty-four hours later, behavior in the conditioning chamber was videorecorded during 5 minutes and subsequently analyzed for freezing behavior.

#### Tissue Preparation

Mice were deeply anesthetized with sodium pentobarbital and sacrificed by perfusion transcardially with 0.1 mol/L PBS (pH 7.4) solution. The right brain hemispheres were fixed for 48 hours in 4% paraformaldehyde and cryoprotected in 30% sucrose for immunohistochemical (IHC) analysis. Frozen brains were divided coronally into sections (40 μm thick) using an SM2010R freezing microtome (Leica Microsystems, Bannockburn, IL), serially collected in cold 0.02% sodium azide, and stored at 4°C. The left hemispheres were snap frozen on dry ice and subjected to protein extraction sequentially using T-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL) and stored at –80°C. Protein concentration in the supernatant was determined using the Bradford assay.

#### Immunoblotting

Equal protein amounts were separated on a 4% to 12% gradient SDS-PAGE, transferred to a nitrocellulose membrane, and incubated overnight at 4°C with primary antibody. The following primary antibodies were used in this study: phospho-glycogen synthetase kinase (GSK3β; Ser9), human APP-CT20, Disintegrin and metalloproteinase domain-containing protein (ADAM)10, ADAM17, BACE1, GSK3β, CDK5 (Calbiochem, San Diego, CA), human tau (HT7), phospho-tau AT8 (phospho-Ser202/Thr205), phospho-tau AT180 (phospho-Thr231), phospho-tau AT270 (phospho-Thr181) (Thermo Scientific), Aβ 1-16 (6E10) (Covance Research Products, Denver, CO).
PA), protein phosphatase 2A (PP2A), p35, synaptophysin (Sigma-Aldrich, St. Louis, MO), neprilysin, insulin-degrading enzyme (IDE), ABCA1, apolipoprotein E, low density lipoprotein receptor-related protein (LRP), liver X receptor (LXR), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the membranes were incubated with adjusted secondary antibodies coupled to horseradish peroxidase. The immunocomplexes were visualized using the SuperSignal West Pico Kit (Thermo Scientific). Band density measurements were made using ImageJ 1.36b imaging software (NIH, Bethesda, MD).

**ELISA Data**

For the determination of the Aβ levels, T-PER soluble fractions were loaded directly onto enzyme-linked immunosorbent assay (ELISA) plates, whereas the formic acid supernatants (insoluble fractions) were diluted 1:20 in a neutralization buffer (1 mol/L Tris base and 0.5 mol/L NaH₂PO₄) before loading. MaxiSorp immunoplates (Nunc, Rochester, NY) were coated with mAb20.1 antibody (a gift from Dr. William E. Van Nostrand, Stony Brook University, Stony Brook, NY) at a concentration of 25 μg/mL in coating buffer (0.1 mol/L Na₂CO₃, pH 9.6) and blocked with 3% bovine serum albumin. Standard solutions for both Aβ₄₀ and Aβ₄₂ were made in the antigen capture buffer (20 mmol/L NaH₂PO₄, 2 mmol/L EDTA, 0.4 mol/L NaCl, 0.05% [3-(3-cholamidopropyl)dimethylammonio]propanesulfonate, and 1% bovine serum albumin, pH 7.0) and loaded onto ELISA plates in duplicate. Samples were then loaded (also in duplicate) and incubated overnight at 4°C. Plates were then washed and probed with either horseradish peroxidase–conjugated anti-Aβ₄₀ (C49) or anti-Aβ₄₂ (D32) (a gift from Drs. Vitaly Vasilevko and David H. Cribbs, University of California, Irvine) overnight at 4°C. The chromogen was 3,3',5,5'-tetramethyldiaminobenzidine (Vector Laboratories, Burlingame, CA), according to the manufacturer’s instructions. Sections were then mounted on gelatin-coated slides, dehydrated in graded ethanol, cleared in xylene, and coverslipped with DPX mounting medium (BDH Laboratory Supplies, Poole, UK).

The immunostaining was assessed at six brain coronal levels. Specifically, six alternate sections (40 μm thick) of the brain with an individual distance of approximately 160 μm were obtained between 1.34 and 2.54 mm posterior to the bregma. Images of stained hippocampal, entorhinal cortex, and amygdaloid area were acquired using an Axiocam digital camera and AxioVision 4.6 software connected to an Axioskop 50 microscope (Carl Zeiss MicroImaging, Thornwood, NY). A threshold optical density that best discriminated staining from the background was obtained using the ImageJ 1.36b imaging software (NIH). All histological assessments were made by an examiner blinded to sample identities (R.M., M.K., and D.B.V.).

**Immunofluorescence**

Sections were first blocked with 3% normal serum, 2% bovine serum albumin, and 0.1% Triton X-100 in TBS for 1 hour at room temperature. By using the same buffer solution, sections were incubated overnight at 4°C with the following primary antibodies: anti-Aβ₁₋₄₆ (6E10) (Covance Research Products), anti-postsynaptic density protein 95 (PSD-95), anti-gial fibrillary acidic protein (Milenipore, Billerica, MA), human tau (HT7) (Thermo Scientific) (1:1000), anti-ionized calcium binding adaptor molecule 1 (Iba-1) (Wako Chemicals, Richmond, VA) and/or phospho-tau PHF-1 (phospho-S396/S404) (a gift from Dr. Peter Davies, Albert Einstein College of Medicine, Manhasset, NY). Sections were then rinsed and incubated for 1 hour with secondary Alexa Fluor-conjugated antibodies (Invitrogen, Carlsbad, CA) at room temperature. Finally, sections were then mounted onto gelatin-coated slides in Fluoromount-G (Southern Biotech, Birmingham, AL) and examined under a Leica DM2500 confocal laser microscope using the Leica Application Suite Advanced Fluorescence software (Leica Microsystems). The immunofluorescence was assessed at the same brain coronal levels previously described. Confocal images were acquired by sequential scanning using a z-separation of 1 μm using the Leica Application Suite Advanced Fluorescence software (Leica Microsystems).

**Thioflavin S Staining**

Sections were incubated in 0.5% thioflavin S in 50% ethanol for 10 minutes, differentiated twice in 50% ethanol, and washed in PBS solution. Staining was visualized under a confocal microscope. Volumetric imaging and image measurements were made using Imaris software (Bitplane Inc., South Windsor, CT). The thioflavin S levels represent the average value obtained by the analysis of images of the hippocampus, entorhinal cortex, and amygdala.
Statistical Analysis

All data are expressed as mean ± SEM. The statistical evaluation of the results was performed using one- or two-way analysis of variance (ANOVA). After significant ANOVAs, multiple post hoc comparisons were performed using Bonferroni’s test. Some data were analyzed using the unpaired t-test. The accepted level of significance for the tests was $P < 0.05$. All tests were performed using the Statistica software package (StatSoft Inc., Tulsa, OK).

Results

Calpain Inhibitor A-705253 Improves Cognition in Aged 3xTgAD Mice

First, we tested the effect of calpain inhibition on the cognitive function in the 3xTgAD mice. As previously demonstrated, 18-month-old 3xTgAD mice exhibited marked impairments in cognition tasks dependent on brain areas mostly affected by AD pathological characteristics when compared with age-matched nTg mice (Figure 1). Treatment with A-705253 resulted in a dose-dependent improvement of spatial learning in the hippocampal-dependent Morris water maze (Figure 1A) and a significant increase in the time spent in the target quadrant during the probe session (Figure 1B). Similarly, 3xTgAD mice treated with calpain inhibitor explored the novel object significantly longer than the familiar one when compared with vehicle-treated 3xTgAD mice in the novel object recognition task, which was primarily dependent on the cortex (Figure 1C). A contextual fear-conditioning task, which was mainly amygdala and hippocampal dependent, also revealed a dose-dependent improvement of a fear-associated memory in the 3xTgAD mice when treated with A-705253 (Figure 1D). Notably, at a higher concentration of A-705253, 3xTgAD mice performed as well as age-matched nTg mice in all tasks we tested and were significantly different from vehicle-treated 3xTgAD mice.

More important, no significant changes in learning and memory functions were found between vehicle- and A-705253–treated nTg mice in the different cognitive tasks (Figure 1). Moreover, the improvement in the cognitive performance in the A-705253–treated 3xTgAD mice was not directly related to changes in motor function, because no significant alterations of the swimming speed and total distance traveled in the water maze were ob-

Figure 1. A-705253 improves cognition in aged 3xTgAD mice. A: The 18-month-old 3xTgAD and nTg control mice are treated orally with vehicle, 40 or 80 mg/kg per day A-705253, and escape latency times to the platform in Morris water maze are measured. B: Similar experiments are performed, and the amount of time in the target quadrant is measured during the water maze probe trial. C: In novel object recognition, a cortical-based task, mice are evaluated or time is spent exploring the novel object, showing memory retention. D: Contextual fear memory is also tested. A–D: Data represent mean ± SEM (n = 6 to 8 per group), one- or two-way ANOVA, followed by a post hoc Bonferroni’s test. $^*P < 0.05$, $^{**}P < 0.01$ between nTg and 3xTgAD mice; $^{**}P < 0.01$ between vehicle- and A-705253–treated 3xTgAD mice.

Figure 2. A-705253 increases levels of synaptic proteins in aged 3xTgAD mice. A and B: In brain samples from 3xTgAD mice, those receiving treatment with 80 mg/kg per day A-705253 express more presynaptic and postsynaptic proteins than those receiving treatment with vehicle, as shown by using Western blot analysis with synaptophysin (A) and IHC with PSD-95 (B). C: Quantification of Western blots in A (synaptophysin) is performed by densitometric analysis and is presented as a percentage of control, normalized to GAPDH. Quantification of the fluorescence intensity in B (PSD-95) is compared with vehicle. Data represent mean ± SEM (n = 6 to 8), unpaired Student’s t-test. $^{*}P < 0.05$, $^{**}P < 0.01$. 
served when compared with vehicle-treated 3xTgAD mice. Also, animals did not show differences in locomotor, exploratory, and anxiety-like behavior in an open field test (data not shown).

Calpain Inhibitor A-705253 Increases Levels of Synaptic Proteins in Aged 3xTgAD Mice

Given the marked recovery of cognition in treated 3xTgAD mice, we sought to determine the molecular correlate of this improvement. Synaptic dysfunction was an important phenotypic manifestation of AD neuropathological characteristics and was widely found in aged 3xTgAD mice.19 The systemic treatment with A-705253 (80 mg/kg per day, p.o.) significantly averted the decrease in presynaptic synaptophysin (Figure 2A) and postsynaptic PSD-95 (Figure 2B) levels in the 3xTgAD mouse brain when compared with vehicle-treated mice (quantified in Figure 2C). Remarkably, levels of PSD-95 in A-705253–treated 3xTgAD mice were similar to those in nTg mice, which was in agreement with a previous study that showed that calpain induced PSD-95 degradation20 (see Supplemental Figure S1, A and B, at http://ajp.amjpathol.org). These data provided evidence that calpain inhibitor A-705253 could halt the synaptic loss and, therefore, cognitive decline seen in 3xTgAD mice.

Calpain Inhibitor A-705253 Reduces Levels of \( \alpha\)/\( \beta\) in 3xTgAD Mice

The accumulation of \( \alpha\)/\( \beta\) and tau pathological characteristics coincided with the synaptic loss observed in 3xTgAD mice. 3xTgAD mice progressively accumulated \( \alpha\)/\( \beta\) as they aged, with fibrillar amyloid plaques starting to develop from the age of 12 months.21 Herein, we assessed the effect of long-term oral treatment with calpain inhibitor A-705253 on \( \alpha\)/\( \beta\) levels by biochemical and IHC analyses (Figure 3). The calpain inhibitor reduced the levels of \( \alpha\)/\( \beta\)-40 and \( \alpha\)/\( \beta\)-42 peptides in a dose-dependent manner in both detergent-soluble and detergent-insoluble fractions when compared with

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**Figure 3.** A-705253 reduces levels of \( \alpha\)/\( \beta\) in 3xTgAD mice. A: ELISA assesses levels of soluble and insoluble \( \alpha\)/\( \beta\)-40 and \( \alpha\)/\( \beta\)-42 in 3xTgAD mice receiving treatment with vehicle, 40 or 80 mg/kg per day A-705253. Data represent mean ± SEM (n = 6 to 8), one-way ANOVA, followed by post hoc Bonferroni’s test. **P < 0.01. B: Thioflavin S–positive plaques in 80 mg/kg per day A-705253–treated 3xTgAD mice are shown by staining and volumetric analysis. C: Quantification of the staining in B. Data represent mean ± SEM (n = 6 to 8), unpaired Student’s t-test. *P < 0.05, **P < 0.01. Levels of \( \alpha\)/\( \beta\) are assessed in 80 mg/kg A-705253–treated 3xTgAD mice by staining with 6E10 (D) and with an antibody specific for \( \alpha\)/\( \beta\)-42 (E) in both the cortex and hippocampus.
vehicle (Figure 3A). Of great relevance, 3xTgAD mice treated with A-705253 (80 mg/kg per day, p.o.) showed a significant reduction in the total number of thioflavin S–positive plaques. In addition, the size of the plaques was significantly different among vehicle- and A-705253–treated 3xTgAD mice; vehicle-treated 3xTgAD mice presented with more plaques with a volume $\geq 10,000 \mu m^3$ (Figure 3, B and C). Likewise, increased anti-6E10 (Figure 3D) and anti-Aβ42 (Figure 3E) immunoreactivity was found in the brains of 3xTgAD mice treated with vehicle when compared with A-705253–treated 3xTgAD mice. Levels of 6E10 and Aβ42 were reduced by 50% and 66%, respectively, in A-705253–treated 3xTgAD mice.

Calpain Inhibitor A-705253 Reduces BACE1 and Increases ABCA1 Levels in 3xTgAD Mice

To elucidate the mechanism by which calpain inhibition reduced Aβ levels, we determined whether APP processing pathways were modified (Figure 4, A and B). Steady-state levels of APP and α-APP cleaving enzymes ADAM10 and ADAM17 were unaffected by A-705253 (80 mg/kg per day, p.o.) treatment. Conversely, inhibition of calpain significantly diminished the expression of the putative β-secretase enzyme BACE1. Given the decline in BACE1 levels, we used the antibody 6E10 to detect full-length APP and its proteolytic fragments CTFβ [99–amino acid long C-terminal APP fragment produced by β-secretase cleavage (C99)] and CTFα [83–amino acid long C-terminal APP fragment produced by α-secretase cleavage (C83)]. As expected, the levels of C99, but not C83, were significantly diminished in the A-705253–treated 3xTgAD mice versus vehicle-treated 3xTgAD mice.

We also evaluated the effect of the calpain inhibitor in the major putative Aβ clearance pathways (Figure 4, C and D). A-705253 was not able to induce changes in the expression of the Aβ-degrading enzymes, neprilysin and IDE, nor in the levels of apolipoprotein E and LRP. ABCA1 levels, however, were significantly increased in the brains of A-705253–treated 3xTgAD mice versus vehicle-treated 3xTgAD mice. Surprisingly, treatment with A-705253 was also capable of increasing ABCA1 levels in nTg mice (see Supplemental Figure S1, A and B, at http://ajp.amjpathol.org). Furthermore, steady-state levels of LXR, a transcriptional factor indicating that activation was critical for up-regulation of ABCA1 expression, remained unaltered after treatment with A-705253. We concluded that the Aβ reduction seen in A-705253–treated 3xTgAD mice was the result of both a reduction in BACE1 and, therefore, a reduction in Aβ production, as well as increased clearance of Aβ through ABCA1. These data were consistent with previous findings that increasing ABCA1 expression can ameliorate amyloid burden.22,23
Calpain Inhibitor A-705253 Reduces Tau Phosphorylation through Blockage of CDK5 Activation in 3xTgAD Mice

We next investigated the effects of A-705253 (80 mg/kg per day, p.o.) on tau accumulation and phosphorylation in the 3xTgAD mice. No differences in the steady-state levels of tau were found among vehicle- and A-705253-treated 3xTgAD mice (Figure 5, A and B). However, Western blot analysis revealed a significant reduction in AT8, AT270, and AT180 immunoreactivity in the brains of 3xTgAD mice treated with calpain inhibitor, indicating a decrease in levels of tau phosphorylation. To further confirm these findings, we performed confocal studies using polyclonal tau and PHF-1 antibodies, which recognized normal and hyperphosphorylated tau, respectively. Reduced PHF-1 immunoreactivity was found in A-705253-treated 3xTgAD mice versus vehicle-treated 3xTgAD mice (Figure 5C).

To elucidate the mechanism underlying the reduction in tau pathological characteristics in A-705253-treated 3xTgAD mice, we assessed the activation of selected tau protein kinases and phosphatase (Figure 5, D and E). No changes in the activation of GSK3β were found in 3xTgAD mice treated with calpain inhibitor, as indicated by the comparable levels of phosphorylated GSK3β at Ser9 between vehicle- and A-705253-treated 3xTgAD mice. Similarly, steady-state levels of tau phosphatase PP2A remained unchanged in the 3xTgAD mice after A-705253 treatment. On the other hand, a significant decrease in the activation of CDK5 was found in the brains of 3xTgAD mice treated with calpain inhibitor versus vehicle, as determined by the changes in the levels of proteolytic fragment p25. These results showed that A-705253 effectively blocked the activity of calpains to cleave p35, thereby inhibiting the progression of tau pathological characteristics in 3xTgAD mice.

Calpain Inhibitor A-705253 Decreases Neuroinflammation in 3xTgAD Mice

Glial cell activation was one of the earliest pathological features of AD and might occur in response to the increasing number of degenerating neurons and synapses or the accumulation of Aβ. After treatment with A-705253, 3xTgAD mice had a significant reduction in the inflammatory response, as evidenced by the pronounced decrease in the glial fibrillary acidic protein–positive astrocytes (Figure 6, A and D), CD45-positive microglia (Figure 6, B and D), and Iba-1–positive microglia (Figure 6, C and D) immunoreactivities versus vehicle-treated 3xTgAD mice. Colocalization studies demonstrated that activated astrocytes and microglia were directly associated with Aβ deposits (data not shown). Moreover, A-705253 did not change basal levels of microglia and astrocytes in nTg mice (data not shown). These data provided strong evidence that specific calpain inhibition was beneficial to many relevant aspects of AD pathological characteristics, including neuroinflammation, which, in sum, restored cognition in 3xTgAD mice.

Discussion

Herein, we provide functional and molecular preclinical evidence that the novel calpain inhibitor A-705253 mili-
neuronal death and disrupts learning and memory. Evidence has also established that neurites and synaptic terminals are highly loaded with activated calpain, which may account for early synaptic loss and neuritic dystrophy in the AD brain.2,24,25 Indeed, calpain inhibitors impede the cleavage of p35 to p25 and the subsequent cell death induced by Aβ.26,27 Moreover, inhibition of calpain activity reestablishes normal synaptic plasticity in neuronal cultures from APP/PS1 mice through an enhancement in cAMP response element-binding (CREB) phosphorylation.28 Corroborating these findings, the calpain inhibitor A-705253 prevents N-methyl-D-aspartate-induced neuronal death and Aβ-induced synaptic dysfunction in hippocampal slice cultures.29 Similarly, the administration of this compound to rats diminishes the loss of cholinergic neurons in the nucleus basalis magnocellularis and prevents the cognitive dysfunction induced by the injection of Aβ1-42 oligomers.29 Notably, our study demonstrates that long-term oral treatment with A-705253 markedly reduces both Aβ and tau pathological characteristics in the brains of aged 3xTgAD mice. Of great relevance, inhibition of calpain also restored synaptic proteins and learning and memory to the level of nTg mice, which reinforce the idea that calpain may be important in the regulation of normal cognitive functions.7 These data suggest that A-705253 is neuroprotective and has disease-modifying effects in mice.

Further biochemical analyses reveal that A-705253 treatment resulted in a reduction in BACE1 levels, the putative β-secretase involved in APP metabolism and the rate-limiting factor in Aβ generation.30–33 Our findings are in agreement with previous evidence that has demonstrated that overexpression of m-calpain in APP695myc-expressing HEK293 cells augments the levels of BACE1 and APP CTFs. Likewise, Aβ-induced BACE1 up-regulation in cultured cortical neurons and Aβ deposition in APP/PS1 mice are attenuated by overexpression of endogenous calpain inhibitor calpastatin.16 More important, the calpain-mediated BACE1 induction and the consequent Aβ accumulation have been linked to the release of proteolytic fragment p25 from p35 and activation of CDK5.34,35 A second possible mechanism by which A-705253 reduces Aβ levels in the brain is mediated by the increase in ABCA1 levels. ABCA1 is a member of the ATP-binding cassette family of active transporters that gates the AD-like pathological characteristics in aged 3xTgAD mice. Inhibition of calpain by A-705253 restored the cognitive function and prevented the synaptic degeneration. Through a decrease in BACE1-mediated production and an increase in ABCA1-mediated clearance of Aβ, A-705253 promoted a decline in the number of fibrillar amyloid plaques in the brain parenchyma. In addition, the oral treatment with A-705253 reduced the hyperphosphorylation of tau through a CDK5-dependent mechanism.

Calpains are multifunctional enzymes unique in their capability to cleave many substrates and, thus, trigger a wide range of cellular responses. In the central nervous system, inappropriate activation of calpain stimulates g this compound to rats diminishes the loss of cholinergic neurons in the nucleus basalis magnocellularis and prevents the cognitive dysfunction induced by the injection of Aβ1-42 oligomers.29 Notably, our study demonstrates that long-term oral treatment with A-705253 markedly reduces both Aβ and tau pathological characteristics in the brains of aged 3xTgAD mice. Of great relevance, inhibition of calpain also restored synaptic proteins and learning and memory to the level of nTg mice, which reinforce the idea that calpain may be important in the regulation of normal cognitive functions.7 These data suggest that A-705253 is neuroprotective and has disease-modifying effects in mice.

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which inhibition of calpain activity, through the change in the intracellular distribution of APP toward the cell surface, increases both α- and β-cleavage and, consequently, Aβ levels. The reasons for such a discrepancy are unknown and may be the result of differences in the approaches applied. Additional studies are necessary to clarify the relationship between Aβ and calpain.

Dysregulation of kinase and/or phosphatase activity may lead to aberrant phosphorylation of tau in mature neurons, which, in turn, accumulates in the somatodendritic compartments and eventually develops into mature neurofibrillar tangles, resulting in neuronal degeneration and cognitive decline. In this process, calpain displays an essential role by generating p25 from the cleavage of p35. Unlike p35, p25 is not readily degraded, and binding of p25 to CDK5 constitutively activates CDK5, causing hyperphosphorylation of tau, collapse of the cytoskeleton, and neuronal dystrophy and death. Consistent with this idea, long-term oral treatment with A-705253 reduced the phosphorylation of tau at Ser202/Thr205 (AT8), Thr181 (AT270), Thr231/Ser235 (AT180), and Ser396/Ser404 (PHF-1) phosphorylated sites. Notably, such an effect was associated with a selective effect on p35 cleavage, because the brains of A-705253-treated 3xTgAD mice presented diminished levels of p25 fragment and unchanged levels of activated tau kinase GSK3β and tau phosphatase PP2A. More important, recent evidence has also reported that A-705253 prevents the cleavage of tau and the release of a cytotoxic 17-kDa fragment induced by Aβ in vitro. Taken together, these data clearly indicate that A-705253 is highly effective in preventing calpain-mediated tau pathological characteristics.

Several lines of evidence have shown that microglia and astrocytes are attracted to Aβ deposits in which their overactivation elicits detrimental responses and can potentiate the disease progression in the AD brain. Herein, we demonstrate that A-705253 reduces the inflammatory response associated with Aβ accumulation. This reduction in gliosis may be either indirectly through the inhibition of AD-like pathological characteristics or directly by the modulation of glial cells. In fact, recent studies have shown that activation of calpain in microglia and astrocytes contributes to the pathological processes after experimental allergic encephalomyelitis and spinal cord injury in rats. Moreover, the calpain inhibitor I exerts potent anti-inflammatory effects preventing the recruitment of neutrophils, expression of inducible nitric oxide synthase and cyclooxygenase-2 protein and activity, and, ultimately, the degree of peroxynitrite formation and tissue injury in vivo. The relevance of calpain on acute and chronic inflammation has been associated with its capability to modulate the degradation of 1kDa in the proteasome, which is a fundamental step in the activation of transcription factor NF-κB. Additional studies, however, are necessary to establish the role of calpain on NF-κB in the AD brain.

Overall, our study shows that the novel calpain inhibitor A-705253 reduces AD-like pathological characteristics in 3xTgAD mice. Because calpain overactivation contributes greatly to the enhancement of the neuropathological processes in the AD brain, the use of A-705253 could represent an attractive and clinically applicable therapeutic approach for AD.

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References


