

Calpastatin modulates APP processing in the brains of β -amyloid depositing but not wild-type mice

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Abstract

We report that neuronal overexpression of the endogenous inhibitor of calpains, calpastatin (CAST), in a mouse model of human Alzheimer's disease (AD) β -amyloidosis, the APP23 mouse, reduces β -amyloid (A β) pathology and A β levels when comparing aged, double transgenic (tg) APP23/CAST with APP23 mice. Concurrent with A β plaque deposition, aged APP23/CAST mice show a decrease in the steady-state brain levels of the amyloid precursor protein (APP) and APP C-terminal fragments (CTFs) when compared with APP23 mice. This CAST-dependent decrease in APP metabolite levels was not observed in single tg CAST mice expressing endogenous APP or in younger, A β plaque predepositing APP23/CAST mice. We also determined that the CAST-mediated inhibition of calpain activity in the brain is greater in the CAST mice with A β pathology than in non-APP tg mice, as demonstrated by a decrease in calpain-mediated cytoskeleton protein cleavage. Moreover, aged APP23/CAST mice have reduced extracellular signal-regulated kinase 1/2 (ERK1/2) activity and tau phosphorylation when compared with APP23 mice. In summary, *in vivo* calpain inhibition mediated by CAST transgene expression reduces A β pathology in APP23 mice, with our findings further suggesting that APP metabolism is modified by CAST overexpression as the mice develop A β pathology. Our results indicate that the calpain system in neurons is more responsive to CAST inhibition under conditions of A β pathology, suggesting that in the disease state neurons may be more sensitive to the therapeutic use of calpain inhibitors. © 2012 Elsevier Inc. All rights reserved.

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1. Introduction

Disruption of multiple proteolytic systems contributes to Alzheimer's disease (AD) pathobiology, including alterations in the enzymes responsible for β -amyloid (A β) gen-

eration and clearance and dysfunction of the lysosomal system (Mathews et al., 2002a; Nixon et al., 2000). Calpains have also shown evidence of hyperactivity in human AD tissue (Grynspan et al., 1997; Liu et al., 2005; Nixon, 2003; Saito et al., 1993). The calpain family is a group of Ca²⁺-activated, cytosolic, neutral pH, cysteine proteases (Huang and Wang, 2001) which modulate, probably indirectly, the localization and proteolytic processing of the amyloid precursor protein (APP) in cultured cells (Mathews et al., 2002b). The most abundantly expressed calpains are m-calpain and μ -calpain, which are distinguished by their different affinities for Ca²⁺, each of which forms a functional heterodimer with the shared regulatory calpain small subunit 1 (Capn4) (Croall and DeMartino, 1991;

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Sorimachi et al., 1997). In addition to their Ca^{2+} -dependence, calpains are regulated by cytosol-to-membrane translocation and an endogenous inhibitor, calpastatin (CAST). The CAST protein consists of 4 calpain-inhibitory domains that are subjected to calpain cleavage and terminal inactivation by caspase (Croall and DeMartino, 1991; Goll et al., 2003; Maki et al., 1991; Sorimachi et al., 1997; Wang et al., 1998).

Activation of calpains in AD is evidenced by increased levels of activated μ -calpain (Saito et al., 1993) and m-calpain (Grynspan et al., 1997) in neurons. The role of the calpain system in normal brain function and in pathological conditions has also been examined in various mouse models with modified calpain and CAST expression. While genetic deletion of either the m-calpain large subunit or the single calpain small subunit is lethal (Arthur et al., 2000; Takano et al., 2005), deletion of the μ -calpain large subunit does not result in an apparent gross phenotype (Azam et al., 2001; Grammer et al., 2005). Mice lacking CAST, while showing decreased locomotor activity and a decreased acoustic startle response, have no change in hippocampal-dependent memory function (Nakajima et al., 2008). CAST overexpressing mice similarly do not have gross memory deficits (Higuchi et al., 2005; Takano et al., 2005), arguing that CAST deletion or overexpression produces, in general, mild effects in the normal mouse brain (Rao et al., 2008).

The role of the calpain system in AD pathobiology has been recently explored in β -amyloid depositing mouse models. In Tg2576 (Vaisid et al., 2007) and in APP/PS1 mice (Liang et al., 2010), calpain appears to be activated and CAST diminished, consistent with reports in human AD tissue (Grynspan et al., 1997; Liu et al., 2005; Nixon, 2003; Saito et al., 1993). In a mouse overexpressing wild type APP that does not develop β -amyloid pathology, calpains also appear to be activated in neurons (Kuwako et al., 2002). In APP/PS1 mice, chronic calpain inhibition has been shown to reduce amyloid plaque burden and improve memory and synaptic transmission (Liang et al., 2010; Trinchese et al., 2008). This is in contrast to previous results from multiple laboratories showing that the acute pharmacological inhibition of calpains in cell culture systems dramatically increases $\text{A}\beta_{42}$ generation (Klaffki et al., 1996; Mathews et al., 2002b; Yamazaki et al., 1997; Zhang et al., 1999), which is thought to be a more pathological $\text{A}\beta$ species. Here, we examined brain APP metabolite levels in mice overexpressing CAST in neurons compared with wild type mice, as well as CAST overexpressing mice crossed to the $\text{A}\beta$ depositing APP23 line. While CAST-overexpression-mediated changes were not seen in otherwise wild type mice, the development of $\text{A}\beta$ pathology in the APP23 mice corresponded to both an increased sensitivity of the calpain system to CAST inhibition and a reduction in APP metabolite levels.

2. Methods

2.1. Transgenic mice

All experiments involving mice received prior approval from the Nathan Kline Institute Animal Care and Use Committee. Neuron-specific overexpression of human CAST is driven by the Thy-1.2 promoter as previously described (Rao et al., 2008). APP23 mice, which overexpress Swedish-mutant human APP (K670N, M671L) under the Thy-1.2 promoter, develop $\text{A}\beta$ plaque pathology after the first year of life (Sturchler-Pierrat et al., 1997). The depositing APP23/CAST and APP23 mice used in this study were female 13-month-old mice. All mice were maintained on a C57Bl6 background.

2.2. Brain processing, Western blotting, and $\text{A}\beta$ ELISA measurements

Ten percent (weight-to-volume) homogenates were prepared from a hemibrain lacking the olfactory bulb and cerebellum, and used for biochemical analyses (Schmidt et al., 2005a). An aliquot of the homogenates was extracted in diethylamine (DEA) prior to sandwich enzyme-linked immunosorbent assay (ELISA) to detect soluble murine $\text{A}\beta$ in single transgenic (tg) CAST and wild type mice as well as soluble human $\text{A}\beta$ in the predepositing APP23/CAST and APP23 mice (Schmidt et al., 2005b). Diethylamine extracts of all genotypes were also used for secreted APP (sAPP) isolation prior to Western blot analyses as previously described (Morales-Corraliza et al., 2009). In aged APP23/CAST and APP23 mice, an aliquot of the homogenate was extracted in formic acid for $\text{A}\beta$ -associated $\text{A}\beta$ quantitation (Schmidt et al., 2005a, 2005b). For Western blotting, proteins were sized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane (Mathews et al., 2002b), and incubated with antibodies as previously described (Morales-Corraliza et al., 2009). For unbiased analysis of $\text{A}\beta$ species, $\text{A}\beta$ in brain homogenates was resolved by urea/SDS-PAGE as previously described (Klaffki et al., 1996) prior to Western blotting analysis.

2.3. Antibodies

Antibody C1/6.1 recognizes the carboxyl-terminal cytoplasmic domain of APP (Mathews et al., 2002b), and m3.2 recognizes residues 10–15 of murine $\text{A}\beta$, also detecting murine APP, sAPP α , and $\text{A}\beta$ (Morales-Corraliza et al., 2009). 22C11 was purchased from Millipore (Temecula, CA, USA) and detects APP N-terminal in both human and mouse epitopes. Monoclonal antibody 6E10 (Covance, Princeton, NJ, USA), which recognizes residues 1–16 of human $\text{A}\beta$, was used to detect human sAPP α and $\text{A}\beta$. The antibody 4G8 (Covance) recognizes residues 17–24 of $\text{A}\beta$ and was used to detect $\text{A}\beta$ and APP. Mouse monoclonal antibody 6A1 detects sAPP β containing the Swedish mutation but not the wild type sequence (Morales-Corraliza et

al., 2009). Neprilysin was detected with the monoclonal antibody 56C6 (CD10) (Novacastra, Newcastle, UK), and insulin degrading enzyme (IDE) with the rabbit polyclonal antibody IDE1 (Qiu et al., 1998; a gift of Dr. Dennis Selkoe). Cytoskeletal proteins and kinases were detected with antibodies against microtubule-associated protein 1 (MAP1) (Clone HM-1; Chemicon, Temecula, CA, USA), MAP2 (antibody 18–1; Rao et al., 2008), and α II-spectrin (MAB1622; Chemicon), cyclin-dependent kinase 5 (CDK5) (C-8: sc-173; Santa Cruz Biotechnology, Santa Cruz, CA, USA), glycogen synthase kinase 3 β (GSK-3 β) (27C10; Cell Signaling Technology, Danver, MA, USA), and ERK1/2 and phospho-ERK1/2 (9102 and 9101; Cell Signaling Technology). Phosphorylated tau was detected using PHF1 (Chemicon; phospho-epitope at Ser396/404) and CP13 (a gift of Dr. Dennis Selkoe; phospho-epitope at Ser202), while T57120 (BD Biosciences, San Jose, CA, USA), MN37 (a gift of Dr. Dennis Selkoe) and tau1 (Chemicon) were used to detect total tau independent of its phosphorylation state. Phosphorylated α -synuclein was detected using a phospho-Ser129-specific α -synuclein antibody (Abcam, Cambridge, MA, USA). Syn-1 antibody (BD Biosciences) was used for phospho-independent α -synuclein recognition. Activated calpain II immunolabeling was done using the rabbit polyclonal antibody C24 (Rao et al., 2008).

2.4. Thioflavin S staining

A β plaque burden was visualized by fluorescence microscopy of 35- μ m thick vibratome sections prepared from formalin-fixed hemibrains previously stained with Thioflavin S as described in Mi et al. (2007). Thioflavin labeling was quantitated by the program AxioVision 4.6 ($n = 5$ in APP23/CAST and $n = 5$ in APP23 mice; 5 sections per mouse).

2.5. Immunocytochemistry

Vibratome sections were blocked with 10% horse serum (Invitrogen, Carlsbad, CA, USA) at room temperature and incubated with the primary antibody C24 (Rao et al., 2008) overnight at 4 °C. Sections were immunostained using a biotinylated goat anti-rabbit secondary antibody and Vectastain ABC kit (both products, Vector Laboratories, Burlingame, CA, USA).

2.6. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

Brain homogenates were immunoprecipitated with a combination of 6E10 and 4G8 antibodies (Covance) using Dynabead M-280 sheep anti-mouse immunoglobulin G (IgG) (Invitrogen) (Tomidokoro et al., 2010). Proteins were eluted with C4 ZipTip (Millipore) in 90% acetonitrile and 0.1% trifluoroacetic acid (TFA), mixed 50:50 (vol:vol) with 10 mg/mL α -cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid and 50% acetonitrile, and air dried. Samples were analyzed using a Bruker Autoflex MALDI-TOF mass

spectrometer (Bruker Daltonics, Billerica, MA, USA) in positive ion linear mode using standard operating conditions at the NYU Protein Mass Spectrometry Core for Neuroscience.

2.7. Statistical analysis

Western blots were quantitated using ImageJ v.1.38x (rsb.info.nih.gov). ELISA measurements were analyzed using a nonparametric Mann-Whitney U test. All data were plotted on GraphPad Prism v.5 (GraphPad Software, San Diego, CA, USA) for statistical analysis. Throughout, results are expressed as the mean \pm standard error of the mean (SEM).

3. Results

To explore the relationship between neuronal calpain function and AD pathology, we crossed human CAST overexpressing tg mice (showing approximately 7 times the levels of the endogenous CAST expression in the brain; Rao et al., 2008) with an APP overexpressing tg line that develops A β plaques (APP23 mice; Sturchler-Pierrat et al., 1997). Brain plaque pathology in littermate 13-month-old female APP23/CAST and APP23 mice were visualized by Thioflavin S staining, with the area occupied by plaque in the double tg mice decreased by approximately 60% (plaque area as % of total brain: $0.13 \pm 0.02\%$ in APP23/CAST mice versus $0.34 \pm 0.03\%$ in APP23 mice; $p < 0.001$) (Fig. 1A and B). ELISA measurements of formic acid-extractable A β in the double tg APP23/CAST versus the single tg APP23 mice showed a decrease of $70 \pm 11\%$ for deposited human A β 42 ($p < 0.01$) and a similar decrease for human A β 40 levels ($77 \pm 15\%$; $p < 0.01$) (Fig. 1C). Similar decrease in codeposited murine A β 40 and A β 42 was also observed by sandwich ELISA (decrease of $61 \pm 22\%$ in A β 42 [$p < 0.05$] and $63 \pm 22\%$ in A β 40 [$p < 0.05$]; Supplementary Fig. 1A). Thus, the A β burden is reduced in aged APP23 mice overexpressing CAST, with the ratio of A β 42 to A β 40 remaining similar (APP23/CAST: 4.7 ± 0.3 and APP23: 4.1 ± 0.5 ; $p > 0.05$).

Because inhibiting calpain activity has been shown to change the ratio of A β 40 and A β 42 generated by APP overexpressing cells in culture (Klafki et al., 1996; Mathews et al., 2002b; Yamazaki et al., 1997; Zhang et al., 1999), we examined A β in these mice using urea SDS-PAGE in order to detect all A β species (Fig. 1D). In agreement with our A β ELISA findings (Fig. 1C), we observed a decrease in A β band densities in APP23/CAST versus APP23 mice, with the relative ratios of the 3 predominant A β species (A β 38, A β 40, and A β 42) not showing changes. Additionally, we analyzed A β peptides by MALDI-TOF mass spectrometry. While this technique does not allow for the direct quantification of different A β species (Tomidokoro et al., 2010), the ratio between the peak intensity of the A β species reflects the relative abundance of the peptides. No differences in the

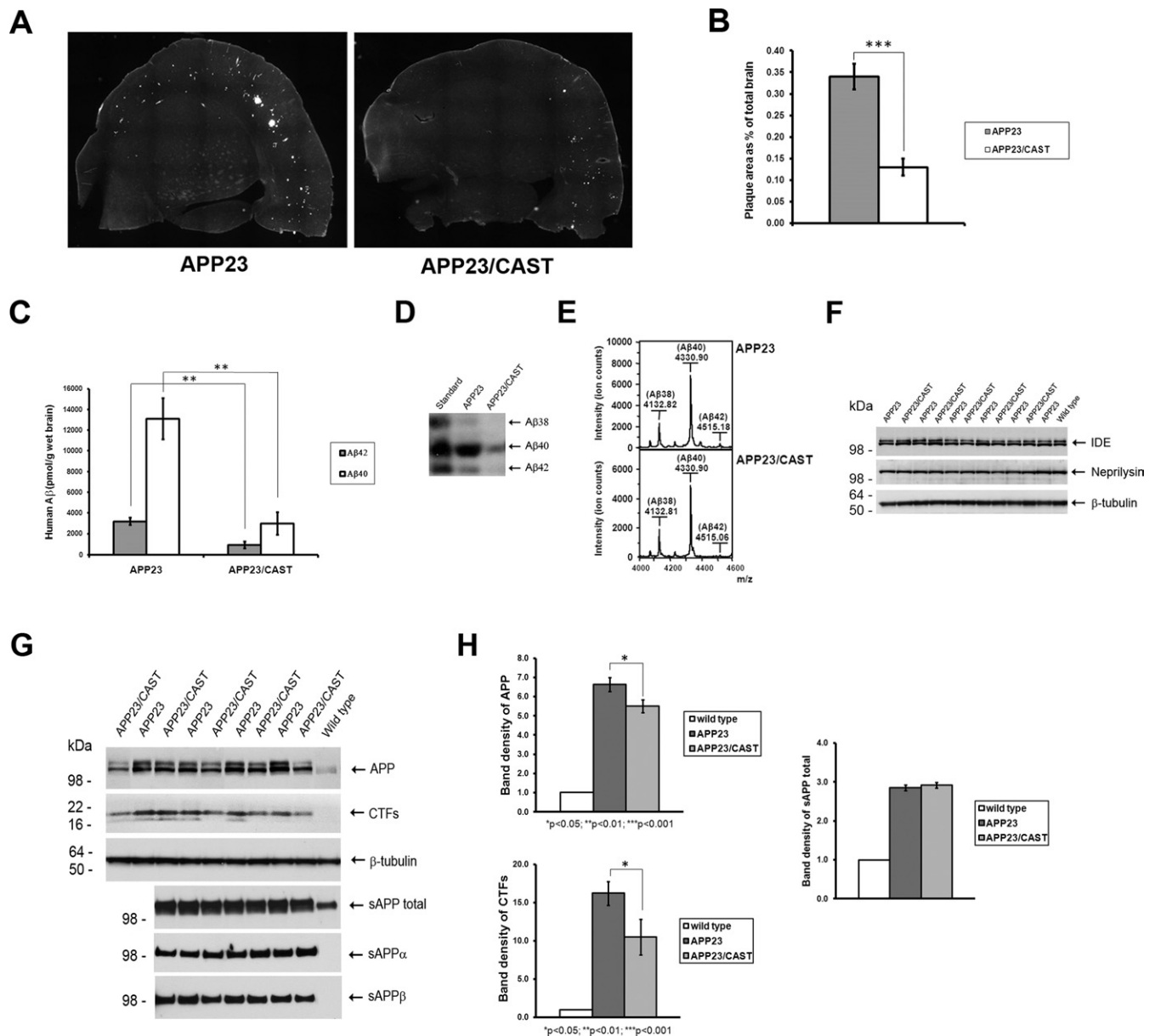


Fig. 1. Calpastatin (CAST) overexpression decreases amyloid precursor protein (APP) metabolite levels in depositing APP23/CAST mouse brain. (A and B) Brain sections of depositing 13-month-old APP23/CAST and APP23 mice were used to visualize and quantify β -amyloid ($A\beta$) plaque area by Thioflavin S staining. (C) Formic acid-extractable human $A\beta$ 40 and $A\beta$ 42 levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA). (D) Analysis of $A\beta$ 38, $A\beta$ 40, and $A\beta$ 42 using Western blots as previously described by Klafki et al. (1996) based upon migration of $A\beta$ standards. (E) Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of brain homogenates immunoprecipitated simultaneously with 6E10 and 4G8 antibodies. (F) Neprilysin and insulin degrading enzyme (IDE) levels are shown by Western blot analysis of brain homogenates. (G) By Western blot, total proteins probed for APP and C-terminal fragments (CTFs); soluble brain extracts lacking membrane-associated proteins probed for secreted APP (sAPP) total, sAPP α , and sAPP β , as indicated (Morales-Corraliza et al., 2009). (H) Graphic representation of the analysis of the band density of the blots of APP, CTFs, and sAPP total. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

relative abundance of $A\beta$ species were seen when comparing APP23/CAST with APP23 mice (Fig. 1E), consistent with the ELISA measurements (Fig. 1C) and the urea SDS-PAGE analysis (Fig. 1D).

Given this decrease in $A\beta$ in the APP23/CAST mice, we next determined the levels of two $A\beta$ -degrading enzymes within the brain: neprilysin and IDE. No changes in protein

levels in these proteases were observed when comparing APP23/CAST with APP23 mice (Fig. 1F). APP levels in the brain of 13-month-old $A\beta$ plaque-depositing APP23/CAST mice, however, showed a decrease of $25 \pm 7\%$ compared with APP23 mice ($p < 0.05$; Fig. 1G and H). Consistent with this reduction in APP, CTF levels were also decreased in APP23/CAST mice by $36 \pm 12\%$ ($p < 0.05$; Fig. 1G and

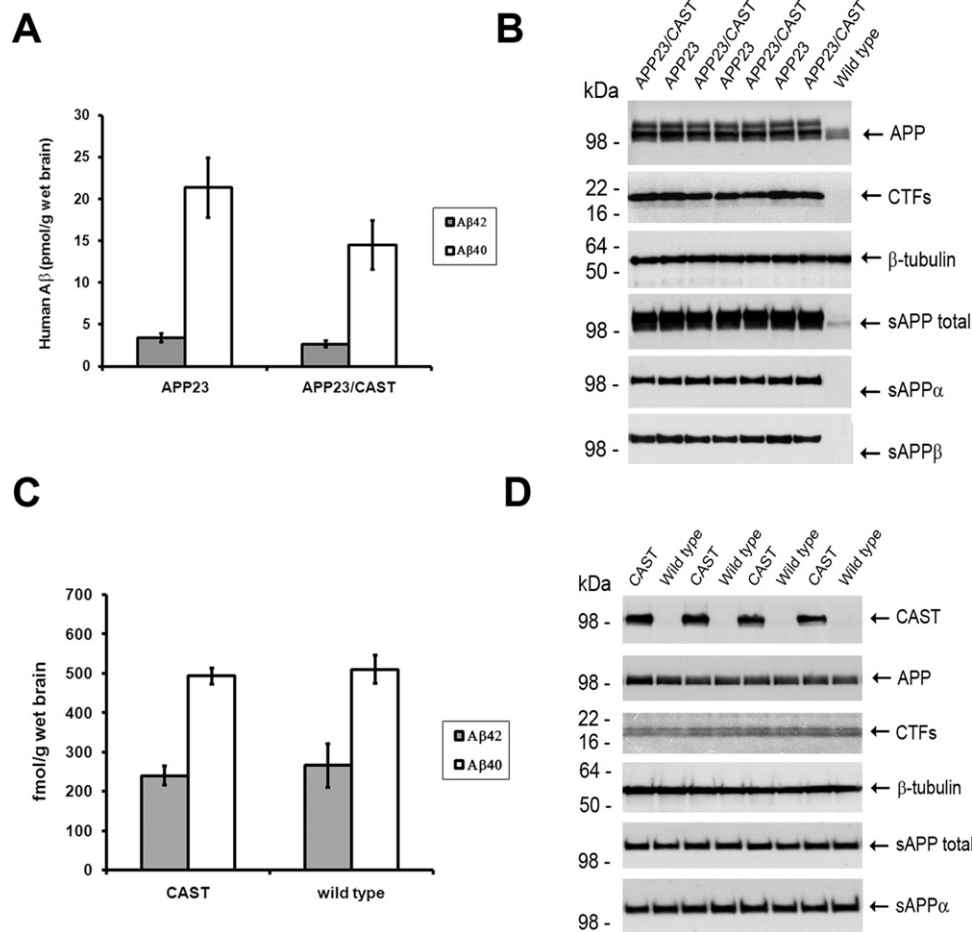


Fig. 2. CAST overexpression does not modify amyloid precursor protein (APP) metabolism in predepositing 4-month-old APP23 and wild type mouse brain. (A) Soluble human β -amyloid (A β)40 and A β 42 levels and (B) additional APP metabolites levels, including APP, C-terminal fragments (CTFs), sAPP total, sAPP α , and sAPP β in predepositing 4-month-old APP23/CAST and APP23 mouse brain. (C) Soluble endogenous A β 40 and A β 42 levels and (D) additional APP metabolite levels in 18- to 24-month-old CAST and wild type mice.

H). We determined that the highly stable sAPP metabolites (Morales-Corraliza et al., 2009) showed no changes between APP23/CAST and littermate APP23 mice, either when probing with an antibody that detect both sAPP α and sAPP β (sAPP total) or antibodies that only detect sAPP α or sAPP β (Fig. 1G). Relative levels of APP, CTFs, and sAPP are shown graphically in Fig. 1H.

We extended our study on CAST overexpression in vivo by examining APP metabolite levels in CAST mice without A β -plaque deposition by examining younger (4-month-old), predepositing APP23/CAST mice compared with APP23 mice and by comparing single tg CAST to wild type mice. Predepositing APP23/CAST mice did not show a significant change in human A β brain levels compared with littermate APP23 mice (Fig. 2A). At this age, no changes in the levels of APP, CTFs, sAPP total, sAPP α , and sAPP β were seen (Fig. 2B), which is in contrast to the reduction in APP and CTF levels in the 13-month-old mice (Fig. 1G and H). This suggests that calpastatin expression-mediated changes in neuronal APP processing are not prominent until

the mice are older and beginning to show brain A β pathology. To further examine this result, we analyzed at various ages APP metabolite levels in CAST mice expressing only the endogenous murine APP and therefore without A β accumulation or plaque pathology. At multiple ages from 4 to 24 months in single tg CAST mice (shown in Fig. 2C and D are findings from 18- to 24-month-old mice; other ages are shown in Supplementary Fig. 1B–D), no changes were seen in APP metabolite levels—including APP, CTFs, sAPP total, sAPP α , and A β —when compared with littermate non-tg mice.

By Western blot analysis, we also characterized in APP23/CAST and APP23 mice, calpain-mediated cleavage of cytoskeleton proteins, including MAP1 and MAP2, and α II-spectrin (Fifre et al., 2006; Pike et al., 2001; Warren et al., 2007). In Fig. 3A, Western blot analysis of the total protein and calpain-specific breakdown products of MAP1 (Fifre et al., 2006) showed lesser amounts of the breakdown products in APP23/CAST mice of either age when compared with APP23

A

C

B

D

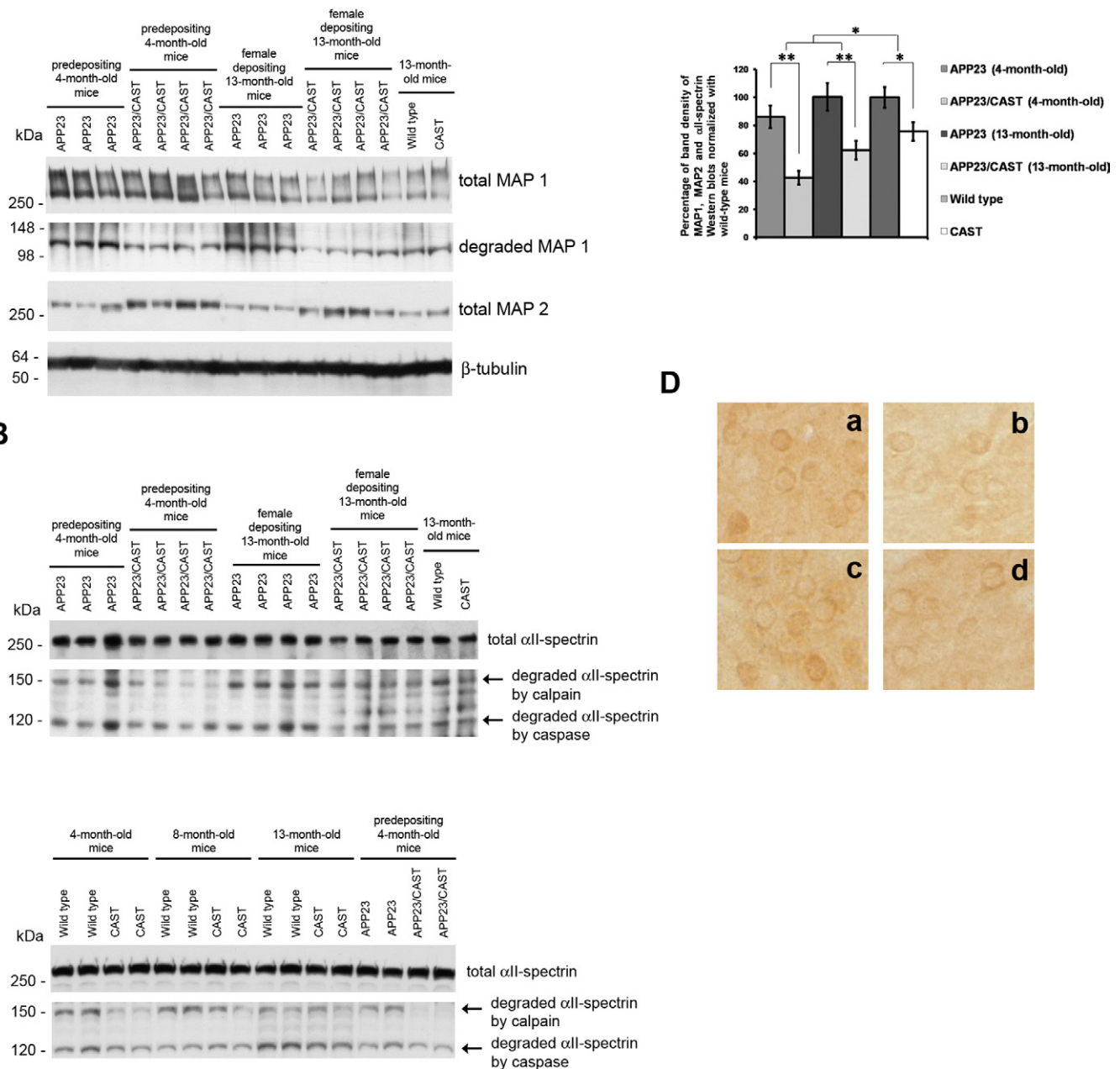


Fig. 3. Assessment of calpain activity in APP23/CAST and APP23 mouse brain. Characterization of calpain activity in APP23/CAST mice by Western blot analysis of calpain-mediated clearance/cleavage of cytoskeleton proteins (Fifre et al., 2006; Pike et al., 2001; Rao et al., 2008; Warren et al., 2007). (A) Blots show total protein remaining for microtubule-associated protein 1 (MAP1) and MAP2 and the major breakdown products of MAP1. (B) Total protein remaining and the 2 major breakdown products of α II-spectrin, including a 120 kDa band corresponding to α II-spectrin cleaved by caspase and 150 kDa band corresponding to α II-spectrin cleaved by calpain. (C) Graphic representation of the analysis of the band density of total proteins and breakdown products of MAP1, MAP2, and α II-spectrin in (A) and (B). The percentage of band density of single transgenic (tg) CAST and non-tg was calculated based on these 4 blots as well as additional blots. (D) Immunolabeling with activated calpain II-specific antibody (antibody C24) of serial brain coronal sections of APP23 (a), APP23/CAST (b), wild type (c), and CAST mice (d). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

littermates. Total MAP2 protein levels remaining in APP23/CAST were found to be greater than the levels seen in APP23 littermates, and these changes in MAP2 levels were not as great in single tg CAST compared with

non-tg mice. Fig. 3B shows that while α II-spectrin cleaved by caspase showed no significant changes between the 2 groups, calpain-mediated α II-spectrin breakdown product (Pike et al., 2001; Warren et al., 2007)

levels were reduced in the APP23/CAST mice compared with single tg APP23 mice. Fig. 3C shows a graphic representation of the analysis of the band density of total proteins and breakdown products of MAP1, MAP2, and α II-spectrin blots. Significant decreases in calpain activity were found in APP23/CAST and CAST mice compared with APP23 and non-tg mice, respectively. We also found greater CAST-mediated calpain inhibition when comparing APP23/CAST to APP23 mice than when single tg CAST were compared with non-tg mice (an apparent decrease in calpain activity of $41 \pm 10\%$ in APP23/CAST vs. APP23 mice and $24 \pm 7\%$ in CAST vs. non-tg mice; $p < 0.05$ between the groups). This is consistent with calpain inhibition itself being greater in A β -depositing mice expressing CAST (APP23/CAST vs. APP23 mice) compared with single tg CAST versus non-tg mice. In Fig. 3D, the immunolabeling pattern seen with an antibody that detects activated calpain II (C24; Rao et al., 2008) is in agreement with our findings above on calpain activity indicated by cytoskeletal protein Western blotting (Fig. 3A–C).

The phosphorylation of tau can be driven by A β accumulation in the brain (Götz et al., 2001; Lewis et al., 2001), potentially by kinases whose activity can be modulated by calpains (Amadoro et al., 2006; Hye et al., 2005; Lee et al., 2000; Veeranna et al., 2004). Given the potential for aberrant activity of these kinases in AD, we determined the levels and activity/phosphorylation state of key tau kinases as well as tau using phospho-dependent and phospho-independent antibodies (Fig. 4). While the level of the catalytic domain of CDK5 and level of GSK-3 β did not change, a decrease in phosphorylated ERK1/2 levels (active form compared with nonphosphorylated ERK1/2) was seen in APP23/CAST compared with APP23 mice (Fig. 4). Additionally, the binding of 2 antibodies—PHF1 and CP13—that recognize tau phospho-epitopes of ERK1/2 was found to be significantly reduced in APP23/CAST animals, consistent with a decrease in phosphorylated ERK1/2 levels. The phospho-independent tau antibodies T57120, MN37, and tau1 showed no differences in total tau levels between APP23/CAST and APP23 mice. This is in contrast to the calpain-mediated turnover of other cytoskeletal proteins as shown in Fig. 3A–D, but consistent with the lack of tau-fragments seen in other mouse models of human β -amyloidosis (Liang et al., 2010; Roberson et al., 2007) and in agreement with the eventual accumulation of tau seen as paired-helical filament in human AD. To expand the potential disease relevance of these studies, we additionally examined the levels of phosphorylated α -synuclein (phospho-Ser129 α -synuclein) and total α -synuclein. No changes in α -synuclein signal were detected when comparing APP23/CAST with APP23 mice, consistent with the idea that the calpain system is particularly responsive to A β accumulation, and that this appears to have downstream effects on tau phosphorylation.

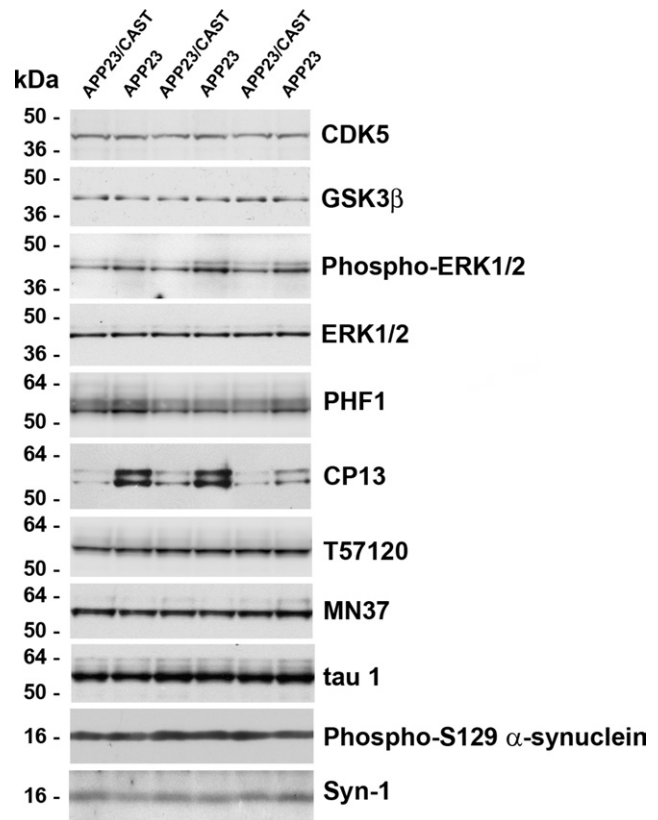


Fig. 4. Calpastatin (CAST) overexpression inhibits ERK1/2 dependent tau phosphorylation in APP23/CAST mouse brain. Brain proteins levels of tau kinases and tau in 13-month-old depositing APP23/CAST compared with APP23 mice were determined by Western blotting. Levels of the catalytic domain of cyclin-dependent kinase 5 (CDK5) (panel as indicated throughout) and glycogen synthase kinase 3 β (GSK-3 β) and levels of phospho-extracellular signal-regulated kinase 1/2 (phospho-ERK1/2) (phospho-dependent antibody) compared with total ERK1/2 (probed with a phospho-independent antibody; see 2. Methods). Levels of tau using 2 phosphorylation-dependent tau antibodies which recognize tau phospho-epitopes for ERK1/2: PHF1 (phospho-epitope at Ser396/404) and CP13 (phospho-epitope at Ser202) and 3 phospho-independent tau antibodies T57120, MN37, and tau1. Levels of α -synuclein using a phosphorylation-dependent α -synuclein antibody (which recognizes a phospho-epitope at Ser129) and a phospho-independent α -synuclein antibody (Syn-1).

4. Discussion

The pathological role of calpain hyperactivation following the unregulated increase in intracellular Ca²⁺ concentration that accompanies excitotoxicity has long been appreciated (Bartus et al., 1995; Wang, 2000). This activation of calpains results in the cleavage of a number of neuronal substrates that can negatively affect neuronal structure, function, and survival (Siman and Noszek, 1988). However, the involvement of calpains in mediating neuronal vulnerability in chronic, long-term disease is less well understood. Multiple studies suggest that in postmortem human AD brain the neuronal calpain system is upregulated (Grynspan et al., 1997; Liu et al., 2005; Nixon, 2003; Saito et al., 1993), albeit these findings do not necessarily argue for a

direct role for calpain activation in the disease. There are numerous studies, however, indicating that calpain activation may lead to pathological changes in tau phosphorylation both in vitro (Amadoro et al., 2006; Chung, 2009; Lee et al., 2000) and in vivo (Chung, 2009; Veeranna et al., 2004), as well as alterations in APP metabolism in vitro (Klafki et al., 1996; Mathews et al., 2002b; Yamazaki et al., 1997; Zhang et al., 1999). The growing evidence from transgenic mouse models, including our study, that calpain activation can result from A β accumulation and/or altered A β levels argues that the calpain system responds to AD-related pathological changes in the brain (Liang et al., 2010; Vaisid et al., 2007), thus positioning calpains as having a role in both driving and responding to the disease.

We initiated this study because of the evidence from multiple groups, including ours (Klafki et al., 1996; Mathews et al., 2002b; Yamazaki et al., 1997; Zhang et al., 1999), that the acute pharmacological inhibition of calpains in cell culture systems dramatically increases A β 42 generation, suggesting that inhibition of calpains in the brain might lead to greater A β pathology due to increased A β 42 levels. In vivo, however, we now report the opposite, with CAST-transgene overexpression-mediated calpain inhibition leading to a reduction in A β pathology while having no effect on the ratio of the various A β peptides detected in the brain. Indeed, we choose to use a model that develops A β pathology without artificial A β 42-drive, such as the mutant PS1 expression in the APP/PS1 Δ 9 x CAST crosses described by Liang et al. (2010), in order to be able to detect a CAST-overexpression effect on A β C-terminal cleavage. In addition to the evidence we present that there are no changes in the C-terminal cleavage-site of the A β derived from the human Swedish APP, we did not detect changes in the murine A β 40:A β 42 ratio from the endogenous, wild type APP in either single tg CAST mice or in the APP23/CAST and APP23 mice (see Figs. 1 and 2, and Supplementary Fig. 1).

We found that in A β -depositing APP23/CAST mice, CAST overexpression leads to a decrease in multiple APP metabolite levels, including APP, CTFs, and A β . This reduction in brain APP metabolite levels with CAST overexpression did not occur either in wild type mice throughout their life span or in predepositing APP23 mice. Indeed, our findings suggest that only with A β pathology in the aged APP23 mice, CAST overexpression produces changes in APP metabolism. Additionally, changes in the activity of the calpain system by CAST observed in vivo in this study are consistent with those seen in human AD tissue (Gryns-pan et al., 1997; Liu et al., 2005; Nixon, 2003; Saito et al., 1993) and in mouse models (Liang et al., 2010; Vaisid et al., 2007) subsequent to the formation of A β plaques, where calpains appear to be activated and CAST levels decreased. The influence of APP metabolism on calpain activity was also observed in a previous report in mice overexpressing wild type APP that showed that, although these mice do not

develop A β pathology, calpains are activated in neurons (Kuwako et al., 2002). In this study, APP-induced calpain activation, which was sensitive to calpain inhibitors in vivo, was not seen in mice expressing an APP mutant that is not processed to produce A β . This provides further evidence that calpain activation in these models is dependent upon altered brain A β levels. By examining the levels of calpain substrates and cleavage-products, our findings show that the inhibition of calpain activity by CAST is greater in the aged APP23 mice with A β pathology (and apparent in the predepositing APP23 mice), when compared with single tg CAST mice, which is consistent with the idea that the calpain system is more responsive to CAST overexpression when perturbed by excess brain A β . That CAST overexpression-mediated modulation of calpain occurs under conditions of A β -induced neuronal stress is also in agreement with the findings of Rao et al. (2008) showing that CAST overexpression inhibits pathologically elevated calpain activity in vivo following excitotoxicity, but less under baseline conditions. Our study supports the idea that, in vivo, there is crosstalk between developing A β pathology and the calpain/CAST system, which may show elevated specific activity to the disease that may render the calpain system more responsive to inhibition.

Our findings argue that restoring calpain homeostasis has multiple beneficial effects, including reducing A β accumulation and tau phosphorylation. While neither our study nor prior studies (Chung, 2009; Liang et al., 2010) differentiate between reductions in tau phosphorylation resulting from less A β accumulation or directly from modulation of calpain activity, either mechanism is potentially beneficial in the disease. Pathologically important disruption of calpain activity, including dysregulation of tissue-specific calpain family members, can occur in a number of aging-related diseases, including type 2 diabetes, cataracts, muscular dystrophy, Parkinson's disease, rheumatoid arthritis, ischemia, stroke and brain trauma, various platelet syndromes, hypertension, liver dysfunction, and some types of cancer (Carragher, 2006; Zatz and Starling, 2005). When associated with a specific calpain family member, disease development and progression appears to be directly linked to altered calpain expression and/or activity. Currently, the evidence from mouse models would suggest that changes in calpain activity in AD correlate with developing A β pathology. Such vulnerability of the calpain system in AD would appear to offer an opportunity for therapeutic modulation, with the CAST overexpression systems suggesting that inhibition and/or restoration of more normal calpain activity has benefits both by reducing β -amyloid accumulation and potentially by reducing tau phosphorylation.

Disclosure statement

The authors disclose no conflicts of interest.

All experiments involving mice received prior approval

from the Nathan Kline Institute Animal Care and use committee (AP2010-362).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.neurobiolaging.2011.11.023](https://doi.org/10.1016/j.neurobiolaging.2011.11.023).

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