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Gamma secretase activating protein is a substrate for caspase-3: implications for Alzheimer's disease

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SUMMARY

Background—A major hallmark feature of Alzheimer's disease (AD) is the accumulation of amyloid β ($A\beta$), whose formation is regulated by the γ -secretase complex and its activating protein (also known as GSAP). Because GSAP interacts with the γ -secretase without affecting the cleavage of Notch, it is an ideal target for a viable anti- $A\beta$ therapy. However, despite much interest in this protein, the mechanisms involved in its neurobiology are not known.

Methods—Post-mortem brain tissues from AD patients, transgenic mouse models of AD and neuronal cells were used to investigate the molecular mechanism involved in GSAP formation and subsequent amyloidogenesis.

Results—We identify a caspase-3 processing domain in the GSAP sequence and provide experimental evidence that this caspase is essential for GSAP activation and biogenesis of $A\beta$ peptides. Furthermore, we demonstrate that caspase-3-dependent GSAP formation occurs in brains of individuals with AD and two different mouse models of AD, and that the process is biologically relevant since its pharmacological blockade reduces $A\beta$ pathology in vivo.

Interpretation—Our data by identifying caspase-3 as the endogenous modulator of GSAP and $A\beta$ production establish it as a novel, attractive and viable $A\beta$ lowering therapeutic target for AD.

Keywords

Alzheimer's disease; gamma secretase activating protein; gamma secretase; amyloid beta; caspase-3; transgenic mice

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Competing financial interests

The authors declare no biomedical financial interests or potential conflicts of interest..

INTRODUCTION

Alzheimer's disease (AD) is the principal cause of dementia of the elderly. With an increasing longevity and the absence of a cure, AD has become not only a major health problem but also a heavy economic burden worldwide. Accumulation of neurotoxic A β peptides is a major characteristic of the AD brain and responsible for its clinical manifestation. Since formation of A β is under the strict control of the γ -secretase complex, its pharmacological blockade is an attractive therapeutic approach for lowering A β (1). However, full blockade of γ -secretase has deleterious effects because this enzyme is also involved in the proteolytic processing of other substrates beside A β precursor protein (APP), such as Notch-1 and cadherins (2). Recently, a study identified a γ -secretase activating protein (GSAP) that facilitates A β production by interacting directly with this secretase without affecting the cleavage of Notch (3). Therefore GSAP is potentially a relevant target for a viable therapeutic strategy aimed at interfering with pro-amyloidogenic effectors. However, while we know that this protein is derived from a C-terminal fragment of a larger precursor called pigeon homologue protein (PION), a protein of unknown biological function expressed in various tissues, including the brain, nothing else is known about its neurobiology (4).

In this paper, we provide experimental evidence that caspase-3 is essential for GSAP formation and the biogenesis of the amyloidogenic A β peptides. Furthermore, we demonstrate that caspase-3-dependent GSAP formation occurs in brains of individuals with AD and two different mouse models of AD. Collectively our data indicate a crucial new role for caspase-3 in AD pathogenesis, and support the hypothesis that it is a viable target for the pharmacological therapy of this devastating disease.

MATERIALS and METHODS

Cell culture

The N2A (neuro-2 A neuroblastoma) neuronal cells stably expressing human APP carrying the K670 N, M671 L Swedish mutation (APP swe) were grown, as previously described (5). For transfection, cells were grown to 70% confluence and transfected with 1 μ g of vector (pcDNA3.1), human caspase-3 cDNA, or caspase-7 cDNA by using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h transfection, supernatants were collected, and cells pellets harvested in lysis buffer for biochemical analyses.

Cell treatment

N2A-APP^{swe} cells were grown to 70% confluence and then treated with the caspase-3 inhibitor (z-DEVD-fmk) (10 μ M, 25 μ M, 50 μ M, 100 μ M, 500 μ M) for 48 hours, after which supernatants were collected, and cells pellets harvested in lysis buffer for biochemical analyses. siRNA Knockdown studies. Caspase-3 siRNA (sc-29927) and a negative control siRNA (Control siRNA-A, sc-37007) were obtained from Santa Cruz Biotech. N2A-APP cells were reverse transfected with 100 nM siRNA using Lipofectamine® 2000 Transfection Reagent (11668-019, Invitrogen) according to the manufacturer's instruction and as

previously described (6). Cell toxicity was always monitored by measuring the amount of the lactate dehydrogenase enzyme released in the supernatant at the end of the incubation time by a colorimetric assay (Cell Biolabs, San Diego, CA).

Co-immunoprecipitation studies

Cells were grown to 85-90% confluence and then lysed in 50 mM HEPES, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, 1% CHAPSO containing a protease inhibitor mixture. Prior to immunoprecipitation, cell lysates were diluted in lysis buffer lacking CHAPSO to give 0.25% final CHAPSO concentration. Cell lysates were incubated for 3 hr at room temperature with 5 µg of anti-Caspase-3 or anti-Caspase-7 antibodies. DynabeadsM-280 sheep anti-rabbit IgG (50 µl; Invitrogen) were added and samples were incubated overnight at 4°C. A control incubation of cell lysates with Dynabeads alone was also conducted. Dynabeads were collected and washed 5 times with lysis buffer containing 0.25% CHAPSO. Bound proteins were eluted with SDS sample buffer containing reducing agent and subject to Western blot analysis as described below.

Immunofluorescence microscopy

Immunofluorescence studies were performed as previously described (7). Briefly, cells were placed on glass coverslips and the following day fixed in 4% paraformaldehyde in PBS for 15 min at 22°C. For the brain tissues, sections were deparaffinized, hydrated and subsequently with 3% H₂O₂ in methanol, and then retrieved antigen with citrate (10mM). After rinsing several times with PBS, cells or sections were incubated in a blocking solution (5% normal serum/0.4% TX-100) for 1 h at 22°C and then with the primary antibody separately against GSAP, caspase-3 overnight at 4°C. After washing with PBS, samples were incubated for 1 h with a secondary Texas Red or Alexa Fluor 488-conjugated antibody (Invitrogen). Coverslips were mounted using VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA) and analyzed with Confocal Laser Scanning Microscope Carl Zeiss LSM 710 (Carl Zeiss, Germany). Control coverslips were processed as described above except that no primary antibody was added to the solution. Assay of caspase-3 activity. For determination of caspase-3 activity, cells were rinsed with PBS once and lysed in buffer A (50 mM Tris-HCl [pH 8.0], 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.02% sodium azide and freshly added protease inhibitors [100 µg/ml phenylmethanesulfonyl fluoride and 1 µg/ml Aprotinin]). Following incubation on ice for 0.5 h, the samples were centrifuged at 16,000g at 4 °C for 15 min and the supernatant was collected. The caspase-3 activity was measured by using a colorimetric substrate Ac-DEVDpNA and the production of pNA was monitored over 20 min by microplate reader at OD405. One unit activity was defined as the amount of the enzyme required to cleave 1 pmol of pNA/min/mg protein. The activity of caspase-3 was also measured indirectly by cleavage of PARP, which is a recognized substrate of caspase-3.

Mutagenesis

A putative consensus cleavage site specific for caspase3 was identified in the GSAP-FL amino acid sequence and located into the GSAP-16kDa C-terminal fragment (737DLD739). To obtain a caspase3 resistant GSAP mutant, the required aspartic acids (D) in position 737 and 739 were substituted with alanine (A) and glutamic acid (E), respectively, by direct

mutagenesis (Origene, Rockville, MD). In addition, both wild type and mutated GSAP were DDK tagged (FLAG tag) (Origene, Rockville, MD).

In vitro transcription/translation of wild type and mutated GSAP and cleavage by caspase-3 in a cell-free system

Wild-type and D737A/D739E-GSAP were transcribed and translated using the 1-Step Human Coupled IVT Kit (Thermo Scientific, Waltham, MA) as extensively described (7). In brief, 1µg of Wild-type and D737A/D739E-GSAP DNA were incubated with Hela lysate and reaction components for 3 hours at 30°C according to the manufacture's instruction (Thermo Scientific, Waltham, MA). Transcription/Translation reaction product proteins then were purified by immune-precipitation with Anti-DDK-4C5-magnetic beads (Origene, Rockville, MD) and incubating overnight at 4°C, and then competitively eluted by FLAG Peptide (Sigma Chem. Co., St. Louis, MO) from Anti-DDK-4C5-magnetic beads. To perform in vitro proteolytic analysis of caspase-3 on GSAP, purified Wild-type and D737A/D739E-GSAP were incubated with recombinant caspase-3 (Life science, St. Louis, MO) overnight at 37°C. Briefly, 8µl eluted wild-type and D737A/D739E-GSAP were incubated in 50µl of 25mM HEPES, PH7.5, 0.1% CHAPS, 5.0mM DDT with or without 50ng of recombinant caspase-3 overnight at 37°C. Protein samples were then separated by SDS-PAGE and the signals were detected by applying with HRP-conjugated anti-DDK antibody (Origene).

Mice and treatments

All animal procedures were approved by the Animal Care and Usage Committee, in accordance with the U.S. National Institutes of Health guidelines. Tg2576, 3xTg and their matched wild type control mice used in this study were 15 and 13 month-old, respectively (n=6 mice per group; 3 males and 3 females). A separate group of 3xTg mice (age: 10 month, n=5 mice per group) were treated with artificial CSF or z-DEVD-fmk, the caspase-3 inhibitor (5mg/Kg body weight), as previously described (8). After one week treatment mice were euthanized and brain immediately harvested for biochemical analyses.

Human subjects

Human frontal cortices were obtained from patients with neuropathological confirmed AD (3 females and 4 males) (all Braak and Braak stage III-IV) and normal control subjects (3 females and 1 male). Averages of age in AD and control group (79±4 vs. 77±2 years) were not significantly different. Post-mortem diagnostic evaluation was performed in accordance with standard histopathological criteria and previously described procedures that have been used in earlier reports (9).

Western blot analysis

Proteins were extracted, sonicated, centrifuged at 13,000 rpm for 45 min at 4°C, and supernatants used for immunoblot analysis, as previously described (5,6). Actin was always used as an internal loading control. Primary antibodies used were as follows: anti GSAP full length (1:200) and GSAP-16kDa (1:150) (Thermo Scientific, Waltham, MA); anti-TMP21 (1:200), anti-CD-147 (1:200), anti-caspase-3, anti-caspase-7 (1:200) (Santa Cruz Biotech.,

Dallas, TX), anti-APP (1:200), anti CTF- α and CTF- β (1:200) (Millipore, Billerica, MA, USA); anti- β -actin (1:200). IRDye infrared secondary antibodies were from LI-COR Bioscience.

Quantitative analysis of A β peptides

Mouse brain homogenates were extracted in RIPA for the A β 1-40 and 1-42 soluble fraction, and then assayed by a sensitive sandwich ELISA kits (WAKO Chem., Richmond, VA), as previously described (5,6,9).

Real-Time Quantitative Reverse Transcription-PCR Amplification

RNA was extracted and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA), as previously described 5. Briefly, mouse GSAP gene was amplified by using the corresponding primers (SA Biosciences, Valencia, CA), and β -actin was always used as an internal control gene.

Data analysis

One-way ANOVA followed by the Bonferroni's Multiple Comparison tests were performed using GraphPad Prism 5.0. All data are presented as mean $\pm$ s.e.m. Significance was set at $p < 0.05$.

RESULTS

Caspase-3 is necessary for GSAP generation: in vitro evidence

Compared with empty vector, neuronal N2A-APP_{swe} cells transfected with caspase-3 cDNA produced significantly higher amount of A β 1-40 and A β 1-42, which was associated with an increment in the steady state levels of procaspase-3, active caspase-3, caspase-3 activity, and GSAP but not its precursor protein, GSAP-FL (Fig. 1a-d). By contrast, no significant changes were detected for two other γ -secretase modulatory proteins: TMP21 and CD147 (Fig. 1b) (4,10). No significant differences between the two groups were observed for steady state levels of APP, BACE-1, CTF-a, CTF-b, and the four components of the γ -secretase complex (supplemental figure S1A). In a similar fashion over-expression of caspase-3 did not influence Notch cleavage (supplemental figure S1B).

Under this experimental condition, quantitative real time PCR did not show any significant difference in GSAP mRNA levels between the two groups (Figure 1g). Cells transfected with caspase-7 cDNA had no changes in GSAP-16kDa as well as its precursor protein levels (Fig. 1e, f). Immunofluorescence studies in these cells revealed a co-localization between GSAP and caspase-3, but not for GSAP and caspase-7 (Fig. 1 h, i). This observation was further substantiated by showing that caspase-3 and not caspase-7 co-immunoprecipitated with GSAP (Fig. 1j). Next, we incubated N2A-APP_{swe} cells with a specific cell permeable caspase-3 inhibitor, z-DEVD-fmk (11,12), and evaluated the effect on GSAP. As shown in figure 2, we observed that pharmacological inhibition of caspase-3 activation significantly reduced the level of GSAP but did not influence its precursor protein levels (Fig 2a, b). This treatment resulted in a decrease in the active form of caspase-3, but no changes in procaspase-3, TIMP-21 and CD147 levels were detected (Fig. 2a, b). In addition, we

observed a significant reduction of caspase-3 activity (fig. 2C), and A β 1-40 formation (Fig. 2d) and A β 1-42 (supplemental figure S2A), and consistent with a specific action on the γ -secretase pathway while we found no differences between the two groups in terms of A β precursor protein (APP) levels, an increase in the levels of C-terminal fragment α (CTF α) and CTF- β was detected (Fig. 2a,b). By contrast, pharmacological blockade of caspase-3 did not modify cleavage of Notch (supplemental figure S2C).

Next, we used caspase-3 siRNA to knock-down this protease and evaluated the effect on GSAP formation. N2A-APPswe cells receiving this siRNA showed a dramatic reduction in both pro-caspase-3 and active caspase-3 steady state levels and activity (Fig. 2 e,f). However, no differences in TIMP-21, CD147 and GSAP-FL were detected between control and siRNA-treated cells (Fig. 2 e, f). By contrast, cells treated with caspase-3 siRNA had a significant reduction in GSAP, a reduction in caspase-3 activity, and an accumulation of CTF- α and CTF- β , but no changes in total APP (Fig. 2 e-g). Under this condition, we observed a significant reduction in the levels of A β 1-40 (Fig. 2H) and A β 1-42 (supplemental figure S2B) in the supernatants. By contrast, caspase-3 siRNA did not modify cleavage of Notch (supplemental figure S2D).

To validate the necessary role of caspase-3 in the proteolytic cleavage of GSAP-FL to form its active fragment, we used a mutant form of the protein in which the identified caspase processing domain (cpd), ⁷³⁷DLD⁷³⁹, containing the required aspartic acids (D) were substituted with alanine (A) and glutamic acid (E), respectively (Fig. 2 i). This substitution allowed us to preserve the negative charge on the holoprotein and prevent any structure changes of it. Initially, we implemented a cell-free system and performed an in vitro transcription/translation experiment using the wild type and mutant GSAP. As shown in figure 2 j, we observed that Wt-GSAP was cleaved by recombinant caspase-3 and generated the 16kDa active form of it, however, the mutant D737A/D739E GSAP (-GSAP) fully resisted proteolysis by recombinant caspase-3. Similar results were obtained when Wt-GSAP and -GSAP were transfected in neuronal cells (Fig. 2 k).

Caspase-3 modulates generation of GSAP: in vivo evidence

Having shown that in vitro caspase-3 is directly involved in regulating GSAP expression levels and by doing so it modulates the formation of A β peptides, to further support our observation we embarked in a series of in vivo studies by using post-mortem AD brains as well as two different transgenic mouse models of AD, the Tg2576 and the 3xTg mice (13,14). Compared with age-matched controls, frontal cortex homogenates from AD brain tissues showed a significant increase in GSAP and active caspase-3 levels (Fig. 3a, b). By contrast, no significant differences were observed for GSAP precursor protein (GSAP-FL), TMP21, CD147, procaspase-7 and active caspase-7 (Fig. 3a, b). In a similar fashion, we observed that compared with wild type mice, brain homogenates from Tg2576 mice did not differ in the levels of GSAP-FL, procaspase-7, active caspase-7, as well as TMP21 and CD147 (Fig. 3 c,d). By contrast, they had a significant increase in the levels of GSAP-16kDa, active caspase-3 and caspase-3 ex vivo activity (Fig. 3 c-e), but not changes in GSAP mRNA levels (Fig. 3f). Immunofluorescence studies showed that caspase-3 and GSAP co-localize in the brains of these mice (Fig. 3g). By contrast, no co-localization was

detected between caspase-7 and GSAP (Fig. 3 h). Similar results were observed when 3xTg mice were investigated. Thus, while no difference were observed for GSAP-FL and caspase-7, compared with controls, brain extracts from 3xTg mice had a significant increase in the levels of GSAP-16kDa, active caspase-3 and caspase-3 ex vivo activity (Fig. 4 a-c), but not changes in GSAP mRNA levels (Fig. 4d). Immunofluorescence studies showed that caspase-3, but not caspase-7, and GSAP co-localize in the brains of 3xTg mice (Fig. 4 e,f).

Because we observed that caspase3-GSAP pathway is up-regulated in human AD and two distinct transgenic mouse models of the disease, next we investigated whether this observation had a functional correlate. To this end, 3xTg mice were treated with the same caspase-3 inhibitor we used in the vitro studies (i.e., z-DEVD-fmk) to test whether this regimen would decrease GSAP levels in vivo and result in A β reduction. Since it is known that this compound does not penetrate the blood-brain-barrier very efficiently (12), we delivered it intrathecally over 7 days by means of implanted Alzet 1007D osmotic minipumps, which had an infusion rate of 0.5 μ l/hr (5 μ g/hr) and a 7-day pumping capacity. At the end of the treatment, the caspase-3 inhibitor significantly reduced the amount of soluble A β 1-40 in the brains of the treated mice (Fig. 5a). The drug also caused a significant reduction in GSAP, active caspase-3 protein levels and its ex vivo activity, but no changes were observed for GSAP-FL, procaspase-3, procaspase-7, active caspase-7, TMP21 and CD147 (Fig. 5 c-e). In addition, we observed a significant increase in the CTFs levels, but no changes in the steady state levels of APP (Fig. 5 c,d), BACE-1, and the four components of the γ -secretase complex (supplemental figure S5A). In a similar manner, the drug regimen did not influence Notch cleavage (supplemental figure S5B). Finally, we observed that mice receiving the active drug while did not have any changes in total soluble tau levels, had a significant reduction in the tau phosphorylation at Ser396/Ser404 as recognized by the antibody PHF-1 (supplemental figure S5C,D).

DISCUSSION

GSAP is a newly recognized protein derived from a C-terminal fragment spanning amino acid 733-854 of a larger precursor protein and a key molecule responsible for the rate-limiting step in A β production by interacting with key component for the γ -secretase complex, without altering Notch processing and subsequent signaling (2,3). For this reason, GSAP is potentially an ideal target molecule for the designing and development of therapeutic strategy aimed at interfering with pro-amyloidogenic effectors without the side-effects of the classical γ -secretase inhibition. Interestingly, in AD brains GSAP immunoreactive deposits are closely associated with A β -containing amyloid plaques (15). While conflicting reports on the influence that peripheral GSAP pharmacological blockade may have on A β levels (16,17), we recently showed that intra-cerebral injection of imatinib, a GSAP inhibitor, reduced A β levels and deposition in the 3xTg mice (18). However, despite its implication in AD pathogenesis the molecular mechanism controlling its expression is unknown. Because the amino-acids sequence of its precursor protein contains a putative caspase-3 processing site located into the GSAP C-terminal region of the protein, which originates the 16kDa fragment, we examined if this caspase was indeed involved in the formation of the active form of GSAP. In pursuing this hypothesis we discovered that caspase-3 directly controls GSAP levels and its biological effects in vitro and in vivo.

Thus, by using a genetic, pharmacologic and mutagenesis approach we accumulated consistent experimental evidence supporting the hypothesis that caspase-3 is essential for the formation of active GSAP, which then direct controls amyloidogenesis. To determine whether this finding was relevant to AD pathogenesis we investigated post-mortem human AD brain samples, AD transgenic mouse models and ultimately performed a pharmacologic intervention study. First, we observed that compared with matched controls caspase-3 and GSAP, but not its precursor protein or two other γ -secretase modulators, were significantly increased in the AD brains. These results were confirmed in two distinct AD transgenic mouse models where we also showed that caspase-3 and GSAP co-localize at the neuronal levels. Importantly, we next provided evidence that this association has a functional biological importance by showing that pharmacological blockade of caspase-3 activation in vivo resulted in a significant reduction in the GSAP levels and A β formation in the 3xTg mice. Previous evidence for a role of caspase-3 in AD included its activation in A β -treated neurons and immunodetection in AD brains, and in both cases its role was always considered as the terminal step in the biochemical cascade leading to apoptotic cell death (19, 20). While Gervais et al. originally showed that caspase-3 directly cleaves APP (21), under our experimental conditions we can rule out this mechanism since we never observed any changes in the steady state levels of APP. Interestingly, a recent paper provided evidence that caspase-3 is also involved in mechanisms important to synaptic function in a mouse model of AD, suggesting for the first time that this protease is more than just a mediator of programmed cell death (22).

Our study adds a new facet to this interesting protein by revealing a new cell death-independent role for it as an endogenous modulator of GSAP levels. Based on this new function, caspase-3 is an attractive and viable A β lowering therapeutic target because its inhibition would not affect other important functions of the γ -secretase complex. Consideration of these new aspects of GSAP neurobiology should encourage further studies on the rationale development of novel brain permeable and selective caspase-3 inhibitors for preventing or slowing AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Gandy S. Lifelong management of amyloid beta metabolism to prevent Alzheimer's disease. *N Engl J Med*. 2012; 367(9):864–866. [PubMed: 22931321]
2. He G, Luo W, Remmers C, Netzer WJ, Hendrick J, Bettayeb K, Flajolet M, Gorelick F, WEnnogle LP, Greengard P. Gamma-secretase activating protein, a therapeutic target for Alzheimer's disease. *Nature*. 2010; 467:95–98. [PubMed: 20811458]
3. Deatherage CL, Hadziselimovic A, Sanders CR. Purification and characterization of human γ -secretase activating protein. *Biochemistry*. 2012; 51:5153–5159. [PubMed: 22681044]

4. Chen F, Hasegawa H, Schmitt-Ulms G, Kawarai T, Bohm C, Katayama T, Gu Y, Sanjo N, Glista M, Rogaeva E, Wakutani Y, Pardossi-Piquard R, Ruan X, Tandon A, Checler F, Marambaud P, Hansen K, Westaway D, St George-Hyslop P, Fraser P. TMP21 is a presenilin complex component that modulates gamma-secretase but not epsilon-secretase activity. *Nature*. 2006; 440:1208–1212. [PubMed: 16641999]
5. Chu J, Praticò D. 5-Lipoxygenase as an endogenous modulator of amyloid- β formation in vivo. *Ann. Neurol*. 2011; 69:34–46. [PubMed: 21280074]
6. Chu J, Li JG, Ceballos-Diaz C, Golde T, Praticò D. The influence of 5Lipoxygenase on Alzheimer's disease-related tau pathology: in vivo and in vitro evidence. *Biol. Psych*. 2013; 2013; 74(5):321–328.
7. Kobayashi T, et al. An improved cell-free system for picornavirus synthesis. *J Virol Methods*. 2007; 142(1-2):182–188. [PubMed: 17320977]
8. Shineman D, Zhang B, Leight SN, Pratico D, Lee VM. Thromboxane Receptor Activation Mediates Isoprostane-Induced Increases in Amyloid Pathology in Tg2576 Mice. *J. Neurosci*. 2008; 28:4785–4794. [PubMed: 18448655]
9. Firuzi O, Zhuo J, Chinnici C, Wisniewski T, Praticò D. 5-Lipoxygenase gene disruption reduces amyloid- β pathology in a mouse model of Alzheimer's disease. *FASEB J*. 2008; 22:1169–1178. [PubMed: 17998412]
10. Zhou S, Zhou H, Walian PJ, Jap BK. Regulation of gamma-secretase activity in Alzheimer's disease. *Biochemistry*. 2007; 46(10):2553–2563. [PubMed: 17298085]
11. Kishi T, Hirooka Y, Konno S, Ogawa K, Sunagawa K. Angiotensin II Type 1 receptor-activated caspase-3 through ras/mitogen-activated protein kinase/extracellular signal-regulated kinase in the rostral ventrolateral medulla is involved in sympatho-excitation in stroke-prone spontaneously hypertensive rats. *Hypertension*. 2010; 55:291–297. [PubMed: 20065158]
12. Stepanichev MY, Kudryashova IV, Yakovlev AA, Onufriev MV, Khaspekov LG, Lyzhin AA, Lazareva NA, Gulyaeva NV. Central administration of a caspase inhibitor impairs shuttle-box performance in rats. *Neuroscience*. 2005; 136:579–591. [PubMed: 16198488]
13. Hsiao K, et al. Correlative memory deficits, A β elevation, and amyloid plaques in transgenic mice. *Science*. 1996; 274:99–102. [PubMed: 8810256]
14. Oddo A, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kaye R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. Triple transgenic model of Alzheimer's disease with plaques and tangles: intracellular A β and synaptic dysfunction. *Neuron*. 2003; 39:409–421. [PubMed: 12895417]
15. Satoh J, Tabunoki H, Ishida T, Saito Y, Arima K. Immunohistochemical characterization of γ secretase activating protein expression in Alzheimer's disease brain. *Neuropathol. App. Neurobiol*. 2012; 38:132–141.
16. Sutcliffe JG, Hedlund PB, Thomas EA, Bloom FE, Hillbush BS. Peripheral reduction of b-amyloid is sufficient to reduce brain β -amyloid. Implications for Alzheimer's disease. *J. Neurosci. Res*. 2011; 89:808–814. [PubMed: 21374699]
17. Hussain I, Fabregue J, Anderes L, Ousson S, Borlat F, Eligert V, Berger S, Dimitrov M, Alattia J, Fraering PC, Beher D. The role of γ -secretase activating protein (GSAP) and imatinib in the regulation of γ -secretase activity and amyloid- β generation. *J. Biol. Chem*. 2013; 288:2521–2531. [PubMed: 23209290]
18. Chu J, Lauretti E, Craige C, Praticò D. Pharmacological modulation of GSAP reduces amyloid b levels and tau phosphorylation in a mouse model of Alzheimer's disease with plaques and tangles. *J. Alz. Dis.* Mar 24, 2014 2014 Epub ahead of print.
19. Marin N, Romero B, Bosch-Morell F, Lansola M, Felipe V, Romá J, Romero FJ. Beta-amyloid-induced activation of caspase-3 in primary cultures of rat neurons. *Mech. Ageing Dev*. 2000; 119:63–67. [PubMed: 11040402]
20. Stadelmann C, Deckwerth TL, Srinivasan A, Bancher C, Brück W, Jellinger K, Lassmann H. Activation of caspase-3 in single neurons and autophagic granules of granulovacuolar degeneration in Alzheimer's disease. Evidence for apoptotic cell death. *Am. J. Pathol*. 1999; 155:1459–1466. [PubMed: 10550301]
21. Gervais FG, Xu D, Robertson GS, Vaillancourt JP, Zhu Y, Huang J, LeBlanc A, Smith D, Rigby M, Shearman MS, Clarke EE, Zheng H, Van Der Ploeg L, Ruffolo SC, Thornberry NA,

- Xanthoudakis S, Zamboni RJ, Roy S, Nicholson DW. Involvement of caspases in proteolytic cleavage of Alzheimer's disease amyloid-beta precursor protein and amyloidogenic Aβ peptide formation. *Cell*. 1999; 97:395–406. [PubMed: 10319819]
22. Louneva N, Cohen JW, Han LY, Talbot K, Wilson RS, Bennett DA, Trojanowski JQ, Arnold SE. Caspase-3 is enriched in post-synaptic densities and increased in Alzheimer's disease. *Am. J. Pathol.* 2008; 173:1488–1495. [PubMed: 18818379]
23. D'Amelio M, Cavallucci V, Middei S, Marchetti C, Pacioni S, Ferri A, Diamantini A, De Zio D, Carrara P, Battistini L, Moreno S, Bacci A, Ammassari-Teule M, Marie H, Cecconi F. Caspase-3 triggers early synaptic dysfunction in a mouse model of Alzheimer's disease. *Nat. Neurosci.* 2011; 14:69–76. [PubMed: 21151119]

Figure 1. Over-expression of caspase-3 regulates GSAP levels and Aβ formation

N2A-APP_{sw} cells were transfected overnight with caspase-3 cDNA supernatant and cell lysates collected for analysis. **A.** Levels of Aβ 1-40 and 1-42 in conditioned media from N2A-APP cells transfected with empty vector (control) or caspase-3 cDNA (*p 0.03). **B.** Representative Western blot analysis of full length GSAP (GSAP-FL), GSAP-16kDa, procaspase-3, caspase-3, TIMP21 and CD147 in cell lysates. **C.** Densitometric analyses of the immunoreactivities to the antibodies shown in panel B (*p=0.03; **p=0.0003). **D.** Caspase-3 activity measured in lysates from N2A cells transfected with empty vector (control) or caspase-3 cDNA (*p=0.004). **E.** Representative western blot analysis of full length GSAP (GSAP-FL), GSAP-16kDa, procaspase-7, caspase-7, TIMP21 and CD147 in cell lysates transfected with empty vector (control) or caspase-7 cDNA. **F.** Densitometric analyses of the immunoreactivities to the antibodies shown in panel E (**p 0.001). Results are mean ± sem. **G.** GSAP mRNA levels in cells over-expressing caspase-3 or empty vector (control). **H.** Representative images of immunofluorescence analysis of the same cells incubated with primary antibody for caspase-3 (green), and GSAP (red). **I.** Representative images of immunofluorescence analysis of the same cells incubated with primary antibody for caspase-7 (green) and GSAP (red). **J.** Representative western blot analysis showing that GSAP co-immunoprecipitates with caspase-3 but not caspase-7.

Figure 3. Caspase-3 and GSAP are up-regulated in AD and Tg2576 mice brains. A Representative Western blot analysis of full length GSAP (GSAP-FL), GSAP-16kDa, TIMP21 and CD147, procaspase-3, caspase-3, procaspase-7 and caspase-7 in frontal cortex extracts from AD and matched control subjects. **B.** Densitometric analyses of the immunoreactivities to the antibodies shown in panel A (*p 0.04). **C.** Representative Western blot analysis of full length GSAP (GSAP-FL), GSAP-16kDa, TIMP21 and CD147, procaspase-3, caspase-3, procaspase-7 and caspase-7 in homogenates from cerebral cortices of Tg2576 mice and wild type (WT) controls (15 months of age, n= 6 animals per group). **D.** Densitometric analyses of the immunoreactivities to the antibodies shown in panel C (*p 0.04). **E.** Caspase-3 activity measured in brain homogenates obtained from Tg2576 mice and WT controls (**p=0.001). **F.** GSAP mRNA levels in brain tissues from Tg2576 and WT mice. **G.** Representative images of immunofluorescence analysis of brain sections of the same mice incubated with primary antibody for caspase-3 (green), and GSAP (red). **H.** Representative images of immunofluorescence analysis of brain sections the same mice incubated with primary antibody for caspase-7 (green) and GSAP (red).

Figure 4. Caspase-3 and GSAP are up-regulated and co-localize in the 3xTg mice brain
A. Representative Western blot analysis of full length GSAP (GSAP-FL), GSAP-16kDa, TIMP21 and CD147, procaspase-3, caspase-3, procaspase-7 and caspase-7 in homogenates from cerebral cortices of 3xTg mice and their wild type (WT) controls (13 months of age, n=6 animal per group). **B.** Densitometric analyses of the immunoreactivities to the antibodies shown in panel A (*p=0.003). **C.** Caspase-3 activity measured in brain homogenates obtained from 3xTg mice and WT controls (*p=0.001). **D.** GSAP mRNA levels in brain tissue from 3xTg and WT mice. **E.** Representative images of immunofluorescence analysis of brain sections of the same mice incubated with primary antibody for caspase-3 (green), and GSAP (red). Intensity histogram showing the colocalization of caspase 3 (green channel) and GSAP (red channel). **F.** Representative images of immunofluorescence analysis of brain sections the same mice incubated with primary antibody for caspase-7 (green) and GSAP (red). Intensity histogram showing the colocalization of caspase-7 (green channel) and GSAP (red channel).

Figure 5. In vivo pharmacological inhibition of caspase-3 modulates GSAP levels and Aβ formation

A-B. RIPA soluble Aβ 1-40 and Aβ 1-42 levels in brain cortices of 3xTg mice treated with the caspase-3 inhibitor z-DEVD-fmk (5mg/Kg body weight) or vehicle for one week (10 months of age, n=5 mice per group) (*p=0.002). **C.** Representative Western blot analysis of full length GSAP (GSAP-FL), GSAP-16kDa, TIMP21 and CD147, procaspase-3, caspase-3, procaspase-7 and caspase-7, APP and CTFs in brain cortex homogenates from 3xTg mice and their wild type (WT) controls. **D.** Densitometric analyses of the immuno-reactivities to the antibodies shown in panel C (**p 0.002). **E.** Caspase-3 activity measured in brain homogenates from 3xTg mice receiving the Caspase-3 inhibitor or vehicle for a week (*p=0.002).