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Neurobiology

β-Site Amyloid Precursor Protein Cleaving Enzyme 1 Increases Amyloid Deposition in Brain Parenchyma but Reduces Cerebrovascular Amyloid Angiopathy in Aging BACE × APP[V717I] Double-Transgenic Mice

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The generation of amyloid peptides (Aβ) from the amyloid precursor protein (APP) is initiated by β-secretase (BACE), whereas subsequent γ-secretase cleavage mediated by presenilin-1, produces Aβ peptides mainly of 40 or 42 amino acids long. In addition, alternative β′-cleavage of APP at position 11 of the amyloid sequence results in N-truncated Aβ(11-40/42) peptides, but the functional significance or pathological impact is unknown. Here we demonstrate that in the brain of BACE × APP[V717I] double-transgenic mice, amyloidogenic processing at both Asp1 and Glu11 is increased resulting in more and different Aβ species and APP C-terminal fragments. Pathologically, BACE significantly increased the number of diffuse and senile amyloid plaques in old double-transgenic mice. Unexpectedly, vascular amyloid deposition was dramatically lower in the same BACE × APP[V717I] double-transgenic mice, relative to sex- and age-matched APP[V717I] single-transgenic mice in the same genetic background. The tight inverse relation of vascular amyloid to the levels of the less soluble N-terminally truncated Aβ peptides is consistent with the hypothesis that vascular amyloid deposition depends on drainage of excess tissue Aβ. This provides biochemical evidence *in vivo* for the preferential contribution of N-truncated Aβ to parenchymal amyloid deposition in contrast to vascular amyloid pathology.
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Deposition of amyloid peptides (Aβ) in the brain of patients with Alzheimer's disease (AD) is the invariant pathological feature that has founded the hypothesis that Aβ is a critical and direct cause of this devastating neurodegenerative disease. Aβ peptides are generated from the amyloid precursor protein (APP) by sequential endoproteolytic cleavage by β- and γ-secretases.[1–8] The amyloid depositions in human brain contain Aβ peptides that are mainly 40 and 42 amino acids in length, but also various N-terminally truncated versions. The β-site APP cleaving enzyme (BACE) cleaves APP at Asp1 and Glu11, whereas subsequent cleavage by γ-secretase gives rise to Aβ1-40/42 and Aβ11-40/42 peptides. BACE was identified as a type I transmembrane aspartyl proteinase, with two active site motifs in its luminal domain that are the signature for aspartic proteinases of the type DT/SGT/S.[4–8] Besides APPs there are also the Aβ peptides, a potential substrate for this membrane-bound aspartyl proteinase, in addition to other proteins that appear limited in number.[9–15]

Mice deficient in BACE were devoid of Aβ production in brain, which conclusively identified BACE as the major β-secretase.[16–19] The inactivation of BACE in APP-Swe transgenic mice rescued their cognitive and electrophys-

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iological hippocampal deficits.[20] Moreover, the complete absence of an overt phenotype of BACE-deficient mice qualified this proteinase as the ideal drug target for therapy in AD[21] even more so because BACE activity appears up-regulated in the brain of sporadic AD patients.[22–24] The divergence in sorting and in cellular location of BACE and its substrate APP imposes, however physical constraints that cast doubt on the actual physiological relevance of the relation of APP to BACE.[25] That study, although conducted in polarized MDCK cells, indicated this major problem that should be analyzed in the most prominent cell type in this respect, ie, neurons, and in brain *in vivo*.

To this end, transgenic mice were generated with neuronal overexpression of human BACE that were crossed with APP[V717I] (London mutant) transgenic mice. Significantly, both the parent transgenic mice express the respective transgenes specifically in neurons only, both steered by the mouse *thy-1* gene promoter.[26] The parent APP[V717I] mice develop a robust AD-related phenotype with early cognitive and behavioral defects, followed by widespread amyloid plaques in brain parenchyma[26–28] and with cerebral amyloid angiopathy (CAA).[29] While this study was in progress, APP23 × BACE double-transgenic mice were reported to have increased amyloidogenic processing in brain.[30] Decreased levels of full-length APP were observed concomitant with increased levels of APPsβ, C99 and C89 C-terminal fragments (CTFs), and also of amyloid peptides, but no histological or immunohistological analysis of the amyloid pathology was presented. Most recently, BACE × APP-Sw double-transgenic mice were presented with increased intra- and extracellular amyloid depositions without differential biochemical characterization of the amyloid peptides.[31]

Here, we describe the in-depth biochemical and pathological analysis of aged BACE × APP[V717I] double-transgenic mice, demonstrating that increased BACE activity provoked increased neuronal amyloid processing of APP with overproduction of amyloid peptides that resulted in increased amyloid plaque formation. Significantly and unexpectedly, that was accompanied by a marked reduction in CAA in the brain of BACE × APP[V717I] double-transgenic mice. The comprehensive biochemical analysis of APP and its processing products demonstrated that BACE augmented the amyloidogenic processing at both Asp1 and Glu11 of the amyloid sequence. Completely in line with the resulting increased levels of Aβ, a dramatic increase in amyloid plaques was evident in the parenchyma by staining with thioflavin S and with pan-Aβ. Unexpectedly, however, this was accompanied by a pronounced and significant decrease in vascular amyloid. The biochemical composition of insoluble Aβ peptides was analyzed and correlated with the histological parameters to demonstrate a tight inverse relationship of vascular amyloid with levels of insoluble N-terminally truncated amyloid peptides. The combined biochemical and pathological data point to the differential contribution of amyloid peptides of different length to the development of vascular and parenchymal amyloid pathology. Significantly, the less soluble N-truncated amyloid peptides increased parenchymal amyloid deposition at the expense of vascular amyloid, as indicated by the current experimental findings. These should be taken into account when considering analysis or reanalysis of patients presenting with combined amyloid pathology in parenchyma and vasculature, patients with mixed types of AD, and patients with vascular dementia.

Experimental Procedures

Transgenic Mice

The construct used to generate the BACE transgenic mice contained the human BACE cDNA inserted in the mouse *thy-1* gene cassette at the *Xho*I site of the pTSC plasmid.[26,32] Final constructs were verified by automated sequencing, linearized, and purified for microinjection of 0.5-day-old pronuclear DBA/C57BL6 embryos. Genotyping by polymerase chain reaction (PCR) using primers located in the mouse *thy-1* gene and the human BACE cDNA regions of the construct: 5′-ggctacaacattccacagaca and 5′-gttctgagatatttgaaggac. The five resulting BACE transgenic founders were expanded into the C57BL background and offspring screened for expression of BACE by Western blotting using standard protocols (see also below). The *thy-1* BACE line 16 with high expression of human BACE was selected and crossed with APP[V717I] transgenic mice, extensively characterized before.[26–29] Genotyping of double-transgenic mice was by PCR using the same primers for *thy-1* BACE as above in addition to the primer pair for the *thy-1* APP transgenic mice: 5′-ccgatgggtagtgaagcaatggtt and 5′-tgtgccagccaacacagaaaac.[26] In the experiments reported here offspring from the F1 generation were used that were heterozygous for both the human BACE and the human APP[V717I] transgene.

Biochemical Analysis of Brain Proteins by Western Blotting and Enzyme-Linked Immunosorbent Assay (ELISA) of Aβ Peptides

Biochemical analysis of APP and its processing intermediates was performed as described.[26–29] Briefly, mouse brains were homogenized in 6.5 vol of ice-cold buffer containing 20 mmol/L Tris-HCl (pH 8.5) containing a cocktail of proteinase inhibitors (Roche, Darmstadt, Germany). After centrifugation at 135,000 × *g* at 4°C for 1 hour, aliquots (20% of the volume) of the supernatant were saved for Western blotting for soluble APP derivatives. The remainder of the supernatant was applied on small reversed phase columns (C18 Sep-Pack cartridges; Waters, Milford, MA) and washed with increasing concentrations of acetonitrile containing 0.1% trifluoroacetic acid. The fraction containing amyloid peptides was dried under vacuum and used for measurements of amyloid peptides by Western blotting[33] and by specific sandwich ELISAs to measure full length (1-40/42) and total amyloid (×-40/42) peptides, the latter comprising both full length and N-terminally truncated Aβ peptides.[26–29] The membrane containing pellets from the first

extracts were resuspended in Tris-buffered saline with proteinase inhibitors and used for analysis of membrane-bound proteins and for extraction with guanidine-containing buffer. A quarter of the resuspended pellet was made 50 mmol/L Tris-HCl, 5 mol/L guanidinium-HCl, pH 8.0, and this extract was used to measure plaque-associated Aβ40 and Aβ42 levels by specified ELISA as detailed below.

The remaining resuspended pellets were biochemically analyzed for intact membrane-bound APP and for soluble APP derivatives generated by α- and β-secretase cleavage and of the respective CTF as described[26–29] with minor modifications. Briefly, proteins were denatured and reduced by heating (95°C, 5 minutes) in sodium dodecyl sulfate-sample buffer (final 2% sodium dodecyl sulfate, 1% 2-mercaptoethanol) and separated on 8% Tris-glycine gels and on 16% Tris-tricine gels. Intact APPm, APPs($\alpha+\beta'$), APPs, β-CTF, and total CTF were detected using specified antibodies: polyclonal antibody B10/4 and monoclonal antibodies (mAbs) WO2 and 22C11/1G5. Western blots were developed with the enhanced chemiluminescence detection system and photographically recorded. On each gel a dilution series of standard samples was applied to allow quantification by densitometric scanning as described[26–29] using a flatbed optical density scanner and dedicated software (Image Master; Amersham Biotech, Amersham, UK). Polyclonal antibodies to measure BACE by Western blotting were directed against the N-terminal amino acids 46 to 60 (EE-17) and C-terminal amino acids 482 to 501 (LK-16) (Sigma, St Louis, MO).

ELISA of Amyloid Peptides

Specific sandwich ELISAs were performed as described[27,28] for measurements of full length (denoted Aβ1-40 and Aβ1-42) and of total amyloid peptides, defined as the sum of full length and of N-terminally truncated peptides, the latter denoted as Aβ($\times$-40) and Aβ($\times$-42). Capture antibodies were Ab40/14 and 21F12[27,28] and detection mAbs were 3D6 (Innogenetics, Gent, Belgium) with an epitope located at residues 1 to 5 of Aβ and mAb 12B2, raised against residues 11 to 28 of the amyloid peptide (IBL Co., Fujioka, Japan). Synthetic peptides Aβ(1-40) (Bachem, Budendorf, Switzerland) and Aβ1-42) (Innogenetics) were used as standards.

Immunohistochemistry and Quantitation of Amyloid Load

Mouse brain was rapidly dissected and one hemisphere fixed in 4% paraformaldehyde in phosphate-buffered saline for 24 hours. Saggital sections (40 μm) were cut and stained by standard methods.[26–29] Immunohistochemical staining for amyloid peptides was performed with a variety of antibodies comprising Pan-Aβ (Oncogene, San Diego, CA), 3D6 (Innogenetics), and 6E10 (Chemicon, Temecula, CA). Dystrophic neurites were immunostained with antibodies against APP (22C11, Chemicon), synaptophysin (DAKO, Glostrup, Denmark), and phospho-tau (AT270) (Innogenetics).

Amyloid plaque load was determined on the one hand histochemically with thioflavin S for senile plaques and by immunohistochemistry for total amyloid load with the Pan-Aβ polyclonal antibody PC152 (Oncogene, La Jolla, CA, USA). Well-defined thioflavin S- and Pan-Aβ-stained sections (five per mouse) between bregma 2.04 and 1.08[34] were used for quantitation of the amyloid load in the subiculum and in the cortex. Images were acquired with a microscope (Leica DMR, Wetzlar, Germany) equipped with an analog 3 charge-couple device camera and analyzed with dedicated software (Leica Q-Win). Light intensity and condenser settings for the microscope were maintained throughout the image acquisition process and density slice threshold was applied uniformly throughout analysis. The brain surface of amyloid deposits staining with either thioflavin S or with pan-Aβ, was measured and expressed relative to the total surface. Quantitative analysis of vascular amyloid in the brain was performed on the thioflavin S-stained sections. The number of blood vessels with amyloid deposition in the cortex per section was counted manually. A total of five representative sections per mouse were included in the analysis.

Determination of Insoluble Amyloid Peptides in Cortex and in Meningeal Blood Vessels

Brain hemispheres were transferred to cold saline and the leptomeninges including the leptomeningeal blood vessels covering the superolateral cerebral surface were carefully separated from the cortex as described.[29] A small tissue sample of ~10 mg from the inferolateral temporo-occipital neocortex was dissected from the same hemisphere. In both instances care was taken to obtain leptomeninges free of cortex and cortex free of leptomeningeal blood vessels. The Aβ content was extracted in 5 mol/L of guanidinium-HCl (pH 8) for 1 hour at room temperature and subsequently analyzed by ELISA (see above).

Statistical Analysis

Poisson regression models were applied in the analysis of the relation between the amount of parenchymal and vascular amyloid with the relative levels of the different amyloid peptides. Correction was made for the total levels of Aβ, which are correlated per se with amyloid deposition, by including this as a covariate in the model. The reported P^* values in the Results section were moreover corrected for overdispersion, thereby representing the most stringent values. Spearman correlations were used to analyze the relation between the parenchymal plaque load determined by immunohistochemistry with the concentrations of full-length or of truncated Aβ40 and Aβ42 peptides.

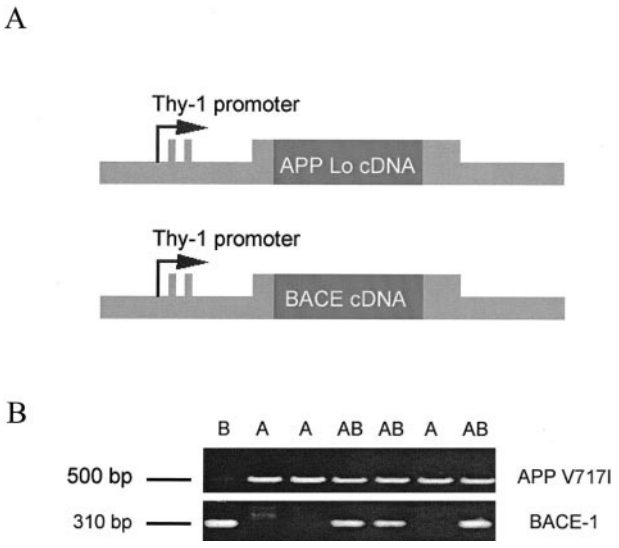

Figure 1. Constructs used to generate transgenic mice and their genotyping by PCR. **A:** Schematic representation of the mouse *thy-1* gene promoter construct that was used to steer expression of human APP [V717I] (APP London)[26] and human BACE-1 (this study) to neurons. **Arrow** denotes transcription initiation site. **B:** Genotyping by PCR of DNA isolated from tail biopsy to identify APP[V717I] or BACE single-transgenic mice, and BACE × APP[V717I] double-transgenic mice, denoted respectively by A, B, and AB.

Results

Generation of Transgenic Mice with Neuronal Overexpression of Human BACE

Transgenic mice were generated by standard methods, expressing human BACE cDNA under control of the mouse *thy-1* gene promoter using a typical construct (Figure 1A).[26] Offspring from two independent BACE transgenic founder lines were analyzed for expression in various areas of the brain and for processing of endogenous mouse APP (data not shown). This proved to be unaltered, as expected from the unfavorable amino acid sequence at the β-cleavage site of murine APP.[35] Therefore, human APP substrate was introduced by generating BACE × APP[V717I] double-transgenic mice and F1 offspring were genotyped by PCR and identified as either single- or double-transgenic mice (Figure 1B). Only offspring from the F1 generation was used in the current experiments to warrant that all double-transgenic mice were heterozygous for both transgenes and all in the identical FVB genetic background. In this study the brains of 16- and 22-month-old mice were analyzed biochemically for APPm and its known proteolytic fragments and derivatives, and correlated with histochemical and immunohistochemical observations of the amyloid pathology in the same mice.

Increased Amyloidogenic Processing of APP by Neuronal Overexpression of BACE in Brain

Membrane proteins extracted from three brain areas, ie, cortex, cerebellum, and midbrain, from APP[V717I] single and BACE × APP[V717I] double-transgenic mice were analyzed biochemically and quantified by Western blotting for APP and its processing products (Figure 2). The level of the human APP[V717I] transgene is ~2.5 times higher than the level of endogenous mouse APP[26–29] and different exposure times were needed to illustrate the different species (Figure 2). In brains of APP[V717I] transgenic mice, full-length APPm is detected as two species representing immature and mature APP (Figure 2B, insets).[26–29] Detailed analysis of total extracts of forebrain revealed a consistent set of consequences of BACE overexpression on APP processing.

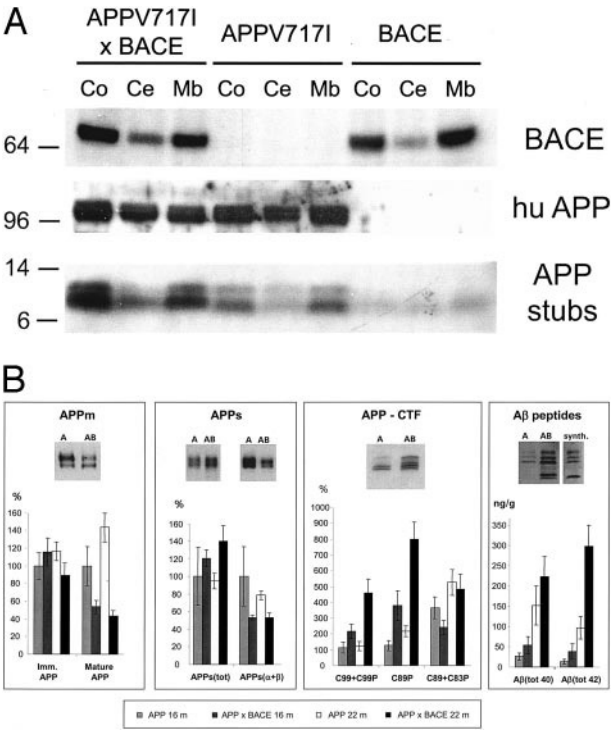

Figure 2. Analysis of APP processing in brain of aging single- and double-transgenic mice. **A:** Overview of Western blots of extracts of different brain regions from three individual transgenic mice with the three different genotypes generated and studied. Extracts of cortex (Co), cerebellum (Ce), and midbrain (Mb) were analyzed with antibodies specific for the proteins indicated on the **right**, ie, human BACE and APP[V717I] transgenes (huAPP) and human plus mouse APP-CTF (denoted as APP stubs). **B:** Quantification by Western blotting or ELISA (**right**) of APP metabolites and amyloid peptides as described in Experimental Procedures and as discussed in the text. **Insets** are representative Western blots, with A and AB denoting APP and APP × BACE transgenic mice, respectively. The histograms represent the mean ± SEM ($n = 4$) expressed either relative to the levels in the brain of the 16-month-old APP[V717I] single-transgenic mice or as absolute values for the soluble amyloid peptides (ng/gram brain tissue, **right**). Soluble amyloid peptides were measured by ELISA, detecting both full-length and N-terminally truncated amyloid peptides. The amyloid peptides were also analyzed by acid urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis[33] with mAb 4G8 Western blotting (**right**, **inset**) using as markers a mixture of synthetic amyloid peptides (Aβ38, Aβ40, Aβ42, Aβ11-42) (**right**, **inset**, lane denoted synth.). Note the presence of amyloid peptides with mobility similar to the synthetic N-terminally truncated Aβ in the brain extract of the BACE × APP[V717I] double-transgenic mice (**right**, **inset**, lane marked AB).

First, relative to APP[V717I] single-transgenic mice at age 16 months, the level of mature APPm was reduced considerably in the brain of BACE × APP[V717I] double-transgenic mice at age 16 and 22 months (Figure 2B, left) while the level of immature APPm was very similar at both ages in all four groups of mice (Figure 2B, left). This is consistent with similar expression of APP but increased turnover of mature APP by higher BACE activity.

Secondly, we measured the levels of the soluble, secreted ectodomain of APP resulting from cleavage by α-secretase and BACE, ie, total APPs(t) as the sum of APPs($\alpha + \beta + \beta'$) by Western blotting with mAb 22C11 and APPs($\alpha + \beta'$) with mAb WO2. The steady state levels of total secreted APP, denoted as APPs(tot) increased somewhat in brains of the oldest BACE × APP[V717I] double-transgenic mice relative to the APP[V717I] single-transgenic mice, whereas the levels of APPs($\alpha + \beta'$) decreased (Figure 2B, second panel from left).

Thirdly, important increased steady state levels were evident for the different APP-CTF, the cell-bound fragments remaining after secretion of the APP-ectodomain. Five distinct CTF species were identified in the membrane fraction with our polyclonal antibody directed against the C-terminal 20 amino acids of APP.[26,35] These represent α-, β-, and β'-CTF, additionally present as phosphorylated and nonphosphorylated isoforms, and denominated as C99P, C99, C89P, C89 + C83P, and C83 in order of decreasing apparent molecular mass and increasing mobility (Figure 2B, third panel from left).[36,37] The combined levels of C99 + C99P, determined with mAb WO2, and especially the levels of C89P or β'-CTF were increased more than fourfold in the oldest BACE × APP[V717I] double-transgenic mice relative to age-matched APP[V717I] single-transgenic mice (Figure 2B, third panel from left). On the other hand, the levels of C89 + C83P and of C83 were practically similar in the brain of BACE × APP[V717I] double-transgenic mice compared to age-matched APP[V717I] single-transgenic mice (Figure 2B, third panel from left).

To define the profile and concentration of the Aβ peptides resulting from cleavage by BACE at Asp1 or Glu11, we performed specific and sensitive sandwich ELISAs using detection with antibody 12B2 that binds to both full length and N-terminally truncated Aβ species. In combination with capturing antibodies that were specific for Aβ40 or Aβ42, the ELISAs measured, respectively, total Aβ40 and total Aβ42, ie, the sum of intact and N-truncated Aβ peptides. The analysis demonstrated very important increased levels of total Aβ40 and total Aβ42 in the brain of BACE × APP[V717I] double-transgenic mice, with the most significant threefold increase in total Aβ42 (Figure 2B, right). Cleavage of human APP by BACE at either position Asp1 or Glu11 of the amyloid sequence was thereby demonstrated to be selectively increased *in vivo* by the increased expression of BACE in the brain of old double-transgenic mice, resulting in increased levels of the amyloid peptides and corresponding APP-CTF.

Increased Amyloid Pathology in Brain Parenchyma of BACE × APP[V717I] Double-Transgenic Mice

Completely in line with the increased Aβ-levels, a dramatic increase in amyloid plaques was evident by thioflavin S staining and by pan-Aβ immunostaining of brain sections. Both senile plaques and diffuse plaques were dramatically enriched in the brain of BACE × APP[V717I] double-transgenic mice, particularly in the hippocampal formation and in the cortical regions, as described in detail further (Figure 3). The parental APP[V717I] transgenic mice develop brain amyloid pathology from the age of 10 to 12 months onwards that progressively comprises more and more diffuse and neuritic plaques in the parenchyma, followed by vascular amyloid angiopathy from 15 months onwards.[26,28,29] Similar to AD patients, the subiculum is the first brain region in APP[V717I] transgenic mice to become loaded with amyloid deposits[29] and is therefore the first and preferred region to be analyzed.

Fibrillar, thioflavin S-positive amyloid plaques in the subiculum were significantly increased in BACE × APP[V717I] double-transgenic mice, relative to sex- and age-matched single APP[V717I] transgenic mice (Figure 3; A to D). On the other hand, BACE overexpression augmented the immunoreactive amyloid load in the subiculum as defined by the pan-Aβ antibody, only minimally. This is because of a ceiling effect, ie, at the age of 15 months and older, the subiculum is practically totally covered with immunoreactive diffuse and senile plaques (Figure 3; E to H) primarily obscuring the additional effect of BACE overexpression, as well as of other genetic and epigenetic variables that we have tested (results not shown). In the cerebral cortex, however, the surface occupied by thioflavin S- and pan-Aβ-immunopositive amyloid deposits was increased threefold to fivefold depending on the particular region analyzed in the brain of old BACE × APP[V717I] double-transgenic mice, again relative to sex- and age-matched APP[V717I] single-transgenic mice (Figure 3, M and N).

All plaques in the brain of APP[V717I] single and BACE × APP[V717I] double-transgenic mice immunostained also with other antibodies that recognize full-length Aβ, although the overall intensity was less in brain of BACE × APP[V717I] double-transgenic mice, eg, with mAb 3D6 (Figure 3; I to L). Differential staining on serial sections with antibodies specific for either intact or N-truncated Aβ, or double immunostaining, did not reveal major differences in staining patterns, indicating that the amyloid plaques contained similar types of amyloid peptides in both single- and double-transgenic mice.

Neuritic pathology associated with Aβ depositions was analyzed by immunohistochemistry using antibodies against APP, against synaptophysin and against phospho-tau epitopes. In all of the transgenic mice analyzed, the different markers revealed immunoreactive clusters of swollen and distorted neuritic profiles (results not shown) corresponding to the dystrophic neurites in brain of the parental APP[V717I] transgenic mice and in brain of AD patients.[29] Quantitation of dystrophic neurites by image analysis proved not reliable, whereas visual scoring revealed a very similar pattern of dystrophic neurites associated with plaques in both single and double-transgenic mice. The apparent increased number of dystrophic neurites was very similar to the increased amyloid plaque load, which led us to conclude that no additional effects of overexpression of BACE became evident in this respect. These data further substantiated the notion that dystrophic neurites are qualitatively related and quantitatively directly proportional to the amyloid plaque load.[29]

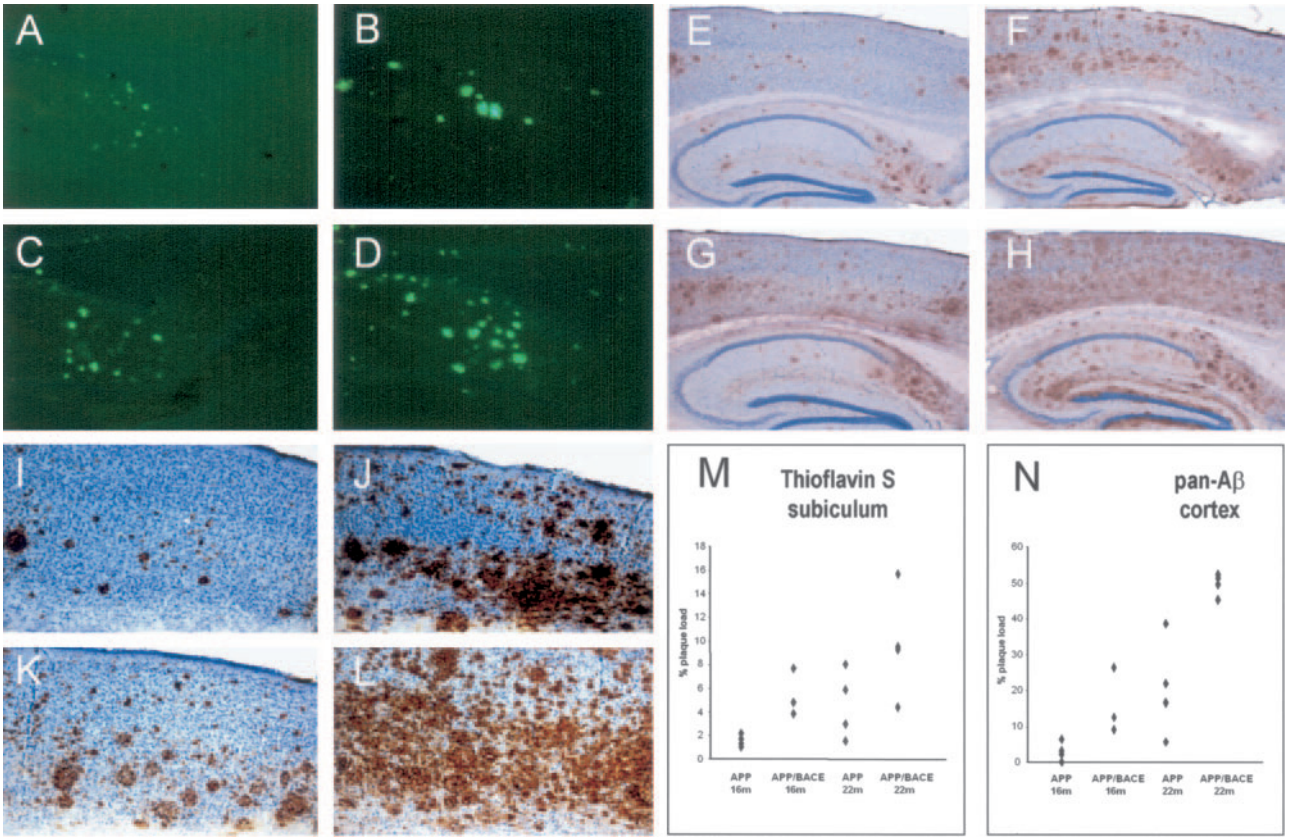

Figure 3. Amyloid pathology in brain of APP single-transgenic and BACE × APP double-transgenic mice. Amyloid deposits in subiculum, stained with thioflavin S (**A–D**) and in the cortex, immunostained with antibody pan-Aβ (**E–H**) and antibody 3D6 (**I–L**) of APP[V717I] single-transgenic mice (**A**, **C**, **E**, **G**, **I**, **K**) and BACE × APP[v717I] double-transgenic mice (**B**, **D**, **F**, **H**, **J**, **L**) at age 16 months (**A**, **B**, **E**, **F**, **I**, **J**) and 22 months (**C**, **D**, **G**, **H**, **K**, **L**). **M** and **N:** Quantitative analysis of amyloid load, expressed as relative surface area occupied by thioflavin S-positive plaques in subiculum and by pan-Aβ immunopositive plaques in the cortex of individual APP and BACE × APP transgenic mice of age 16 months and 22 months, as indicated.

Decreased Angiopathy in BACE × APP[V717I] Transgenic Mice Correlates with N-Truncated Aβ Levels

Prominent CAA consisting of depositions of amyloid peptides on the abluminal surfaces of brain arterioles and leptomeningeal vessels develops later than parenchymal amyloid plaques in aging APP[V717I] transgenic mice as in other models.[29] The effect of BACE overexpression on vascular amyloid deposition was quantified by analysis of thioflavin S-stained brain sections as before.[29] From each individual mouse, five comparable brain sections were analyzed for the number of blood vessels that contained amyloid deposits staining with thioflavin S, either in a pattern of concentric rings or as focal accumulations (Figure 4). Unexpectedly, and in sharp contrast with the increased amyloid deposition in brain parenchyma, a dramatic decrease in vascular amyloid deposition was obvious in BACE × APP[V717I] double-transgenic mice, relative to sex- and age-matched APP[V717I] transgenic mice (Figure 4).

To find the biochemical correlate of that phenomenon, we analyzed the relative levels of intact and of N-terminally truncated Aβ40 and Aβ42, and defined the correlation and contribution to the amyloid pathology in brain of APP[V717I] single-transgenic and BACE × APP[V717I] double-transgenic mice. The guanidine-extractable amyloid peptides were isolated and quantified by ELISA using Aβ40- and Aβ42-specific capturing antibodies, in combination with two different detection antibodies reacting either with N-terminal or central epitopes on Aβ. Thereby we measured on the one hand the concentration of intact Aβ40 and Aβ42 peptides, ie, Aβ(1-40) and Aβ(1-42) and on the other hand the total of intact plus N-terminally truncated Aβ40 and Aβ42 peptides, ie, Aβ(tot 40) and Aβ (tot 42). By subtraction, the concentrations of N-terminally truncated amyloid peptides, ie, Aβ(×-40) and Aβ(×-42), were calculated.

The levels of guanidine-extracted intact amyloid peptides Aβ40 and Aβ42 were evidently increased by the co-expression of BACE with human APP[V717I] in all old transgenic mice, as expected (Figure 5). In addition, however, the prominent increase was noted in the levels of total Aβ40 and Aβ42, ie, these were approximately threefold higher in brain of BACE × APP[V717I] double-transgenic mice relative to APP[V717I] single-transgenic mice (histograms denoted Aβ(tot 40) and Aβ(tot 42) in Figure 5). The resulting calculated levels of N-terminally truncated Aβ were increased dramatically by the co-expression of BACE in double-transgenic mice in both age

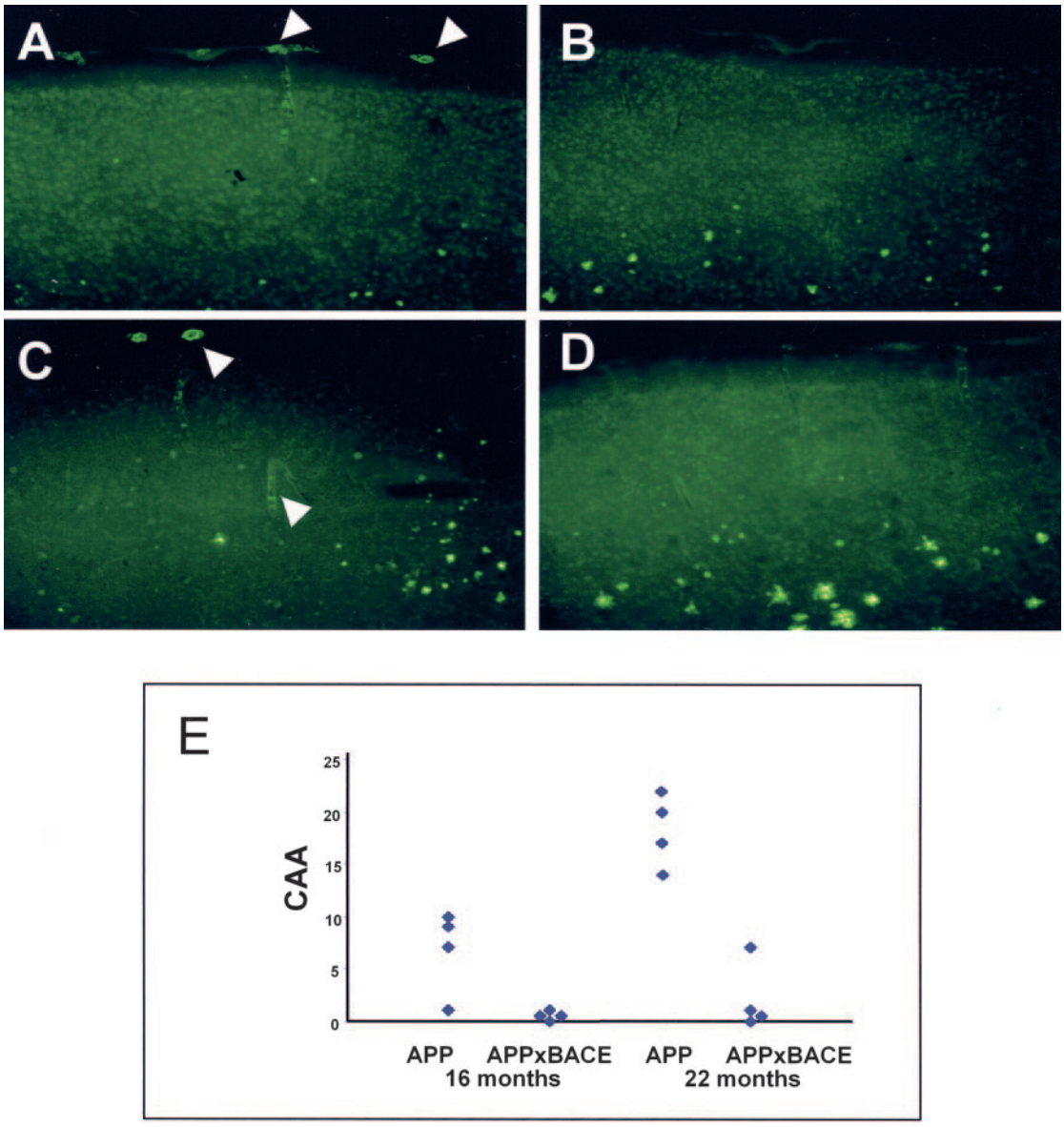

Figure 4. CAA in APP single-transgenic and in BACE × APP double-transgenic mice. **A–D:** Thioflavin S staining of vibratome sections revealing amyloid deposits in parenchyma and in cortical and leptomeningeal arterioles (**arrowheads**) in brain of APP[V717I] (**A**, **C**) and BACE × APP[V717I] (**B**, **D**) transgenic mice at 16 months (**A**, **B**) and 22 months (**C**, **D**) of age. **E:** Quantitation of CAA in brain of individual mice, represented as the number of blood vessels with amyloid deposits stained with thioflavin S, either in a pattern of concentric rings or as focal accumulations. The data represent the sum of vascular amyloid deposits in five brain sections from each mouse analyzed.

groups analyzed relative to age-matched APP[V717I] transgenic mice (histograms denoted Aβ(×-40) and Aβ(×-42) in Figure 5).

Statistical Correlation Analysis of the Biochemical and Pathological Data

To define the contribution of the specified species of amyloid peptides, ie, intact or N-truncated and ending at residues 40 or 42 in the amyloid sequence of APP, an extensive statistical correlation analysis was performed between the biochemical and pathological data. Particularly, we wanted to identify the Aβ species responsible for the decreased vascular amyloid deposition relative to the higher total amyloid load in brain parenchyma of the BACE × APP[V717I] double-transgenic mice.

The Poisson regression model was used to analyze the relation between the amount of vascular amyloid and the relative levels of the different amyloid peptides. Correction was made for the total levels of Aβ that are correlated per se with amyloid deposition, by including this as a covariate in the model. The reported P^* values (Figure 6) were corrected for overdispersion, thereby yielding the most stringent condition.

The statistical analysis revealed a significant positive correlation of vascular amyloid deposition with the relative levels of both the Aβ(1-40) peptides ($\beta = 0.05$, $P < 0.0001$, $P^* = 0.026$) and the Aβ(1-42) peptides ($\beta =$

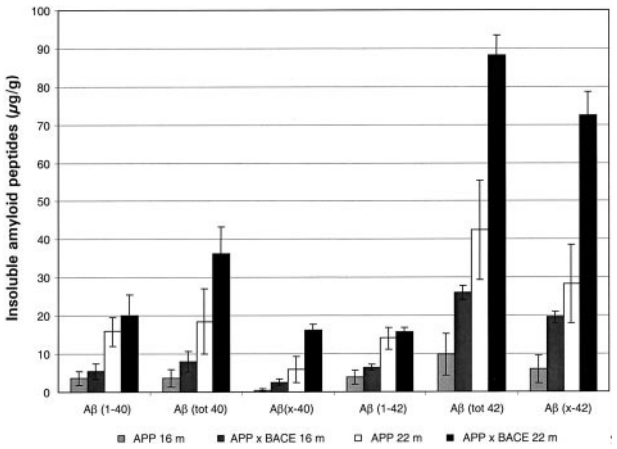

Figure 5. Insoluble amyloid peptides in brain of APP single-transgenic and BACE × APP double-transgenic mice. Amyloid extracted with guanidinium-containing buffer was quantified by ELISA detecting specifically Aβ40 or Aβ42 as either full length, denoted Aβ(1-40) or Aβ(1-42) or as total Aβ (full length plus N-truncated) denoted as Aβ(tot40) and Aβ(tot42). The levels of N-truncated Aβ, denoted Aβ(×-40) and Aβ(×-42), were calculated by subtraction. The data are expressed in μg per gram brain tissue and represent mean ± SEM ($n = 4$) in all age groups.

0.109, $P < 0.0001$, $P^* = 0.015$) (Figure 6, middle and right, respectively). A significant negative correlation was obtained with the relative levels of the truncated amyloid peptides Aβ(×-40) and Aβ(×-42) ($\beta = -0.092$, $P < 0.0001$, $P^* = 0.006$) (Figure 6, left).

Spearman correlation analysis demonstrated that the pan-Aβ immunoreactive parenchymal plaque load was highly and significantly correlated with both full length and truncated Aβ 40/42 peptides ($r = 0.78$, $P = 0.0006$; and $r = 0.93$, $P < 0.0001$, respectively) as expected and demonstrated before in our APP[V717I] amyloid mouse model.[26,29] Subsequently, we reanalyzed biochemically the composition of insoluble amyloid peptides in carefully dissected leptomeningeal blood vessels and in the neocortex of APP[V717I] as described.[29] The analysis revealed that the ratio of N-truncated to total amyloid peptides was ~0.3 in meningeal blood vessels in contrast to a ratio of ~0.8 in total tissue extracts of the neocortex.

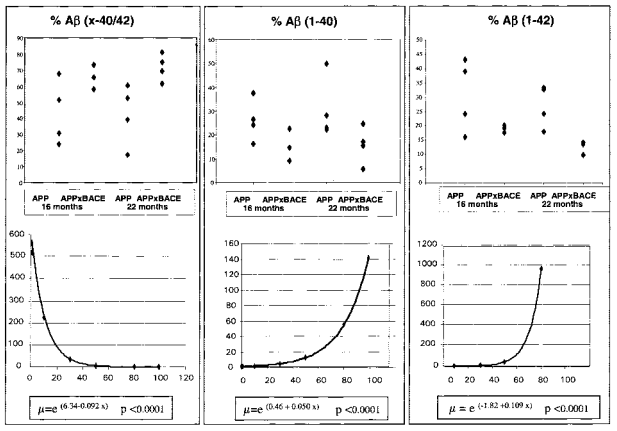

Figure 6. Statistical analysis of correlation of the levels of amyloid peptides with vascular pathology. Relative levels of truncated denoted as %Aβ(×-40/42) and of full-length amyloid peptides (**top**) and representation of the correlation analysis by Poisson regression (**bottom**). The equations of the regression curves and the associated statistical significance levels are indicated under each graph (see text for details and Discussion).

Taken together, the data underpin not only the role of full-length amyloid peptides in vascular amyloid deposition, but additionally point to a negative contribution of truncated amyloid peptides Aβ(×-40) and Aβ(×-42). This conclusion is in line with the higher propensity of truncated amyloid peptides to aggregate, forming more easily the nidi for amyloid plaque formation in the parenchyma close to their cellular origin, ie, neurons. This scheme also fits the hypothesis that vascular amyloid originates by drainage of amyloid peptides from interstitial fluid via the perivascular space, which will be lowered by increased deposition in the parenchyma.

Discussion

Neuronal overexpression of human BACE in double-transgenic BACE × APP[V717I] mice significantly increased all metabolites of APP generated by β-secretase cleavage, in the first instance APP-CTF C99 and C89 cleaved at Asp1 and Glu11 of the amyloid sequence, respectively. This was evidently accompanied by increased secretion of the APPsβ ectodomain and by increased levels of the Aβ peptides in the brain of old BACE × APP[V717I] double-transgenic mice. Thereby, the biochemical data demonstrated and confirmed explicitly the anticipated and wanted outcome, ie, overexpression of BACE increased the amyloidogenic processing of APP in brain *in vivo*, as reported by others.[30,31]

The significant reduction in the amount of mature APPm in the double-transgenic mice is in line with the concept that processing of APP by BACE occurs after glycosylation of APP, ie, in the post-Golgi compartments of constitutive exocytosis or in the endocytic pathway. The data indicate that the activity of BACE appears to be rate-limiting in the amyloidogenic processing of APP despite an already apparent excess of BACE activity in brain relative to the availability of its few known substrates.[9–15] On the other hand, we most recently observed that overexpression of ADAM10 as active α-secretase, increased the nonamyloidogenic processing of APP in the brain of ADAM10 × AP[V717I] double-transgenic mice.[38] Combined, the data demonstrate that besides the absolute levels of both these proteolytic activities, additional factors must decide on the actual type of processing of APP, ie, α- or β-secretase cleavage, an aspect that in the absence of relevant additional data, cannot be entertained further here.

The biochemical and pathological impact of increased BACE activity is not because of increased expression of APP, but must be because of increased cleavage of APP both at Asp1 and at Glu11, in the brain of old double-transgenic mice. Thereby, the relatively less soluble N-truncated amyloid peptides would increase the parenchymal amyloid deposition at the expense of the vascular amyloid load. This outcome of the current study is, to our knowledge, the first combined biochemical and pathological indication for a differential contribution of different Aβ amyloid peptides to parenchymal and vascular amy-

loid depositions, *in vivo*. Relevant data in patients recently defined a significant negative association between amyloid plaques and CAA in AD.[39] Unfortunately, no biochemical data from these patients were available, but the authors postulated that ". . . vascular-derived Aβ is composed mostly of Aβ($\times$-40) with only little Aβ($\times$-42) and with less N-terminal variability, ie, most species starting at Asp1 or Ala2."[39] Knowledge of the exact composition of vascular amyloid in the brain of AD patients is needed to clarify this issue, whereas the current model provides experimental support for that hypothesis.

We gained some additional insight into the preferential deposition in the parenchymal over vascular amyloid deposition from analyzing the composition of the insoluble, guanidinium-extracted amyloid peptides. The increased levels of total amyloid peptides, evident in all double-transgenic mice as compared to single-transgenic mice, were to a very large extent because of increased levels of N-terminally truncated Aβ species. Statistical correlation analysis of biochemical and pathological results revealed the significant inverse correlation between vascular amyloid deposition and the relative fraction of N-terminally truncated amyloid peptides. The data therefore demonstrate that the N-truncated amyloid peptides, and most likely the truncated Aβ($\times$-42) peptides, unexpectedly but effectively prevented the vascular amyloid deposition.

Whereas it cannot be excluded that some N-truncated amyloid peptides arise by proteolysis of full-length peptides subsequent to their excision from APP, the two major variants under discussion here, ie, Aβ(11-40) and Aβ(11-42) are considered to be generated directly from APP by BACE proteolysis, respectively, between Met^{-1} and Asp^{1} and between Tyr^{10} and Glu^{11}.[8,15,16] On overexpression, the preference for either β- or β'-cleavage appears to depend on the intracellular site of action, ie, within the endoplasmic reticulum β-proteolysis would predominate whereas in the *trans*-Golgi network β'-cleavage appears favored,[40] whereas endogenous cleavage occurs in the endosomes. In addition, besides APP also C99 and even Aβ peptides themselves constitute potential substrates for BACE,[9–15] which distorts or pre-empts a firm and even tentative conclusion toward these aspects under discussion. Nevertheless, it is important to note that in human brain, the insoluble amyloid pools comprise full-length Aβ peptides but also significant amounts of N-terminally truncated Aβ species, whereas the latter aggregate more rapidly than their full-length counterparts, at least *in vitro*.[41] The greater solubility of the Flemish synthetic variants of the amyloid peptide,[42] promoting angiopathy over parenchyma deposition of amyloid in the respective patients,[43] is completely in line with our findings and with the proposed explanation. It will be most interesting to see this correlation extended and confirmed *in vivo*, in familial AD cases originating from different mutations in APP and PS1, or from other genetic defects.

CAA is an important, but relatively neglected aspect of the AD pathology. The relative amounts of amyloid β-protein in cerebral blood vessels and parenchyma vary considerably among AD patients. Although several mechanisms have been proposed to explain the variability, the underlying genetic and environmental determinants are still unclear, as are the precise functional and pathological consequences.[39,44] Evidence from transgenic models indicates that CAA originates from amyloid peptides that are produced in neurons and drained from the tissue along the perivascular spaces into the cerebrospinal fluid.[29,45] Unknown factors, such as metal ions or ApoE or components of the extracellular matrix, could interfere with this drainage pathway by decreasing the solubility of the amyloid peptides, by chemical trapping, by complexation, or even by physical trapping. The subsequent deposition in the walls of cerebral arteries and fibrillization then distorts basal lamina and smooth muscle cells, causing vascular damage but only occasional aneurisms.[29,39,40]

In conclusion, we report the generation and characterization of BACE $\times$ APP[V717I] double-transgenic mice, wherein neuronal co-expression of BACE with a mutant human APP resulted in increased β-secretase cleavage of APP both at Asp1 and Glu11 *in vivo*. We demonstrate that the increased β-secretase cleavage resulted in increased amyloid deposition in the parenchyma at the expense of amyloid deposition in the vasculature. We believe that the BACE $\times$ APP[V717I] transgenic mice as presented, provide a very valuable model and tool to study the pathophysiological consequences and repercussions of parenchymal and vascular amyloid deposition that are not yet understood and not accessible in patients.[39,40]

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