BACE1 is the major β-secretase for generation of Aβ peptides by neurons

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Two β-secretases, BACE1 and BACE2, are involved in generation of Alzheimer's disease Aβ peptides[1–3]. We report that secretion of Aβ peptides (Aβ1–40/42 and Aβ11–40/42) is abolished in cultures of BACE1-deficient embryonic cortical neurons, and that whereas both human and murine BACE1 can cleave either human or murine β-amyloid precursor protein (APP) at the +1 site of Aβ, cleavage at the +11 site is species specific. We establish that BACE1 is the principal neuronal protease required to cleave APP at +1 and +11 sites that generate N-termini of Aβ.

Alzheimer's disease, a progressive neurodegenerative disorder, is characterized by deposition of Aβ-amyloid and neurofibrillary tangles in many brain regions, particularly the hippocampus and cerebral cortex[4,5]. Endoproteolytic cleavage of APP by β- and γ-secretase generates toxic Aβ peptides. Both β-secretases are type I transmembrane aspartyl proteases[1–3,6]. BACE1 is synthesized as a pro-enzyme, and furin cleaves pro-BACE1 to generate the mature enzyme[7,8]. In brain, *BACE1* mRNA is high, whereas *BACE2* mRNA is relatively low[9]. BACE2 can cleave APP more efficiently at sites within the Aβ domain as compared to the +1 site of Aβ, suggesting that BACE2 could limit Aβ secretion[10].

We used homologous recombination in embryonic stem cells to generate BACE1-deficient mice. (For methods, see the *Nature Neuroscience* web site.) We derived primary cortical cultures from control, *BACE1*$^{+/-}$ and *BACE1*$^{-/-}$ embryos at day 16.5 *post coitum*. Immunoprecipitation-mass spectrometry (IP-MS) analysis of conditioned culture media from control or *BACE1*$^{+/-}$ neurons after five days in culture, using an antisera (4G8) specific to epitopes between residues 17–28 of Aβ, revealed two prominent Aβ species with mass values of 3171 and 4233, corresponding to murine Aβ11–40 and Aβ1–40 respectively, and several minor species, including Aβ11–42 and Aβ1–42 (Fig. 1b). Secretion of these Aβ species was abolished from *BACE1*$^{-/-}$ neurons, except for the Aβ17–40 (p3) fragment (Fig. 1b). Thus BACE1 is the major β-secretase required for cleavage of βAPP at the +1 and +11 sites of Aβ peptide in embryonic cortical neurons. Because BACE2 primarily cleaves at +19/+20 of Aβ[10] and we fail to detect Aβ20–40/42 or Aβ21–40/42, we infer that BACE2 is not significantly involved in APP cleavage in neurons.

We examined processing of APP in control and *BACE1*$^{-/-}$ cultures infected with a recombinant adenovirus expressing a humanized APP cDNA (murine APP cDNA with the human Aβ1–42 region) bearing the Swedish variant (APPswe). Quantitative sandwich ELISA analyses of conditioned media from *BACE1*$^{+/+}$ cultures expressing APPswe showed high levels of Aβ1–40 (798 ± 291 pg/ml; n = 3) and Aβ1–42 (71.4 ± 43.2 pg/ml; n = 3), whereas media of *BACE1*$^{-/-}$ cultures showed undetectable levels. Metabolic labeling of control and *BACE1*$^{-/-}$ cortical neurons with ^{35}S-methionine for five hours and immunoprecipitation with 4G8 antisera showed a major band (~4 kD) corresponding to Aβ, and a band (~3.2 kD) corresponding to Aβ11–40/42 and Aβ17–40 (p3) in control culture expressing APPswe (Fig. 1c). However, although Aβ17–40 is readily secreted, no Aβ1–40/42 or Aβ11–40/42 accumulated in conditioned media from *BACE1*$^{-/-}$ cultures expressing APPswe (Fig. 1c). We confirmed this result by IP-MS of conditioned media from the same cultures as in Fig. 1c. As expected, a major peak corresponding to human Aβ1–40, and minor peaks corresponding to human Aβ1–42 and murine Aβ11–40 were detected in control cells (Fig. 1d). Whereas little Aβ17–40 peptide was observed in control cells, only the Aβ17–40 peptide was detected in *BACE1*$^{-/-}$ neurons (Fig. 1d), confirming that the band corresponding to p3 in control cells (Fig. 1c) was mainly Aβ17–40. This result is consistent with the view that secretion of Aβ17–40 increases in *BACE1*$^{-/-}$ neurons. Moreover, immunoprecipitation analysis using CT15, an antibody specific for the carboxyl-terminal 15 residues of APP, revealed the accumulation of full-length APP and APP α-CTF in *BACE1*$^{-/-}$ detergent lysates, but failed to detect APP β-CTF in control lysates (Fig. 1e).

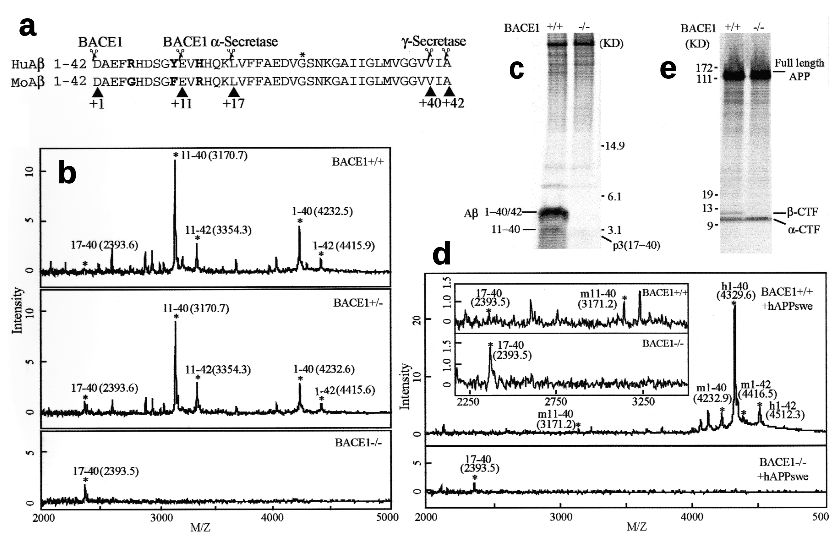

Fig. 1. BACE1-deficient neurons fail to secrete Aβ peptides or generate β-CTF. (**a**) Sequence alignment of human and murine Aβ1-42 peptides. BACE1, α- and γ-secretase cleavage sites are marked and numbered. Asterisk, start of transmembrane domain. (**b**) IP-MS analysis of secreted Aβ peptides from primary cultured cortical neurons derived from wild-type (+/+), heterozygous (+/–), and homozygous (–/–) *BACE*1 knockout E16.5 embryos. Asterisks, peaks corresponding to murine Aβ peptides. In parentheses, mass of each peptide. (**c–e**) Conditioned media (**c**, **d**) from *BACE*1$^{+/+}$ and *BACE*1$^{-/-}$ neuronal cell cultures expressing APPswe radiolabeled with ^{35}S-methionine were immunoprecipitated with 4G8 (**c**), or subjected to IP - MS analysis (**d**); asterisks, peaks corresponding to murine and human Aβ peptides (mass). Inset, enlargement of region between M/Z 2000 and 3500. Detergent lysates from these cultures (**e**) were immunoprecipitated with CT15.

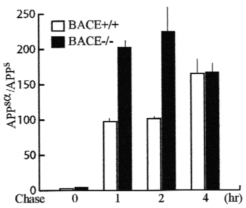

Fig. 2. Altered APP metabolism in *BACE1*$^{-/-}$ neurons. The $APP^{s\alpha}$ and APP^{s} signals at each point of the pulse-chase experiments were quantified by phosphoimaging analysis and the mean ratios of $APP^{s\alpha}$ to APP^{s} (from two experiments) calculated. For detailed methods, see *Nature Neuroscience* web site.

Because β- and α-secretases compete for the same substrate, APP soluble ectodomain (APPsα) produced by α-secretase might be increased without BACE1. Pulse-chase studies showed an approximately twofold increase in the initial rate of secretion of APPsα in *BACE1*$^{-/-}$ neurons as compared to controls; this rate reached a plateau after four hours (Fig. 2). Furthermore, although we failed to detect any accumulation of β-CTF or secretion of Aβ, the rate of secretion of Aβ17-40 was increased in the *BACE1*$^{-/-}$ cultures (supplementary data on the *Nature Neuroscience* web site). These results establish that BACE1 competes with α-secretase in APP processing.

To confirm that BACE1 cleaves APP at both the +1 and +11 sites of Aβ, we infected neuronal cultures with adenovirus expressing either humanized wild-type APP (hAPPwt) or its variants (hAPPswe or hAPP717). As expected, IP-MS analysis of conditioned media using 4G8 antibody showed that the human and murine Aβ1–40 and Aβ1–42 were secreted (Fig. 3b). However, the human Aβ11–40 peptide was not secreted, although the murine Aβ11–40 was readily detected (Fig. 3c). This apparent discrepancy raised the possibility that the cleavage site at +11 of Aβ is species specific, that is, human or murine BACE1 cleaves respectively, human or murine APP at +11 site of Aβ, whereas no species selectivity occurs at the +1 site. Thus, we co-infected murine neuronal cultures with adenovirus expressing both human BACE1 and hAPPwt or its variants. IP-MS analysis of conditioned media using 4G8 antibody now revealed the secretion of human Aβ11–40 peptide in addition to the murine Aβ11–40 peptide (Fig. 3d).

As human BACE1 cleaves human APP at the +11 site of Aβ, we also examined accumulation of the +11 derived β-CTF in neurons co-expressing human BACE1 and human APPwt or its variants. As expected, whereas α-CTFs were readily immunoprecipitated using the CT15 antibody from control, hAPPwt, hAPPswe or hAPP717 lysates, the +1-derived β-CTF was observed only in the hAPPswe lysate (Fig. 3e, lanes 1–4). However, when neurons co-expressed human BACE1 and hAPPwt or its variants, accumulation of a peptide corresponding to the +11-derived β-CTF (+11-CTF) was observed in addition to the +1-derived β-CTF (Fig. 3e, lanes 5–8). To verify the accumulation of these +11-CTFs in neurons co-expressing human BACE1 and hAPPwt or its variants, analyses of lysates on a 16% Tris tricine gel revealed the diminution of bands corresponding to α-CTFs and the appearance of the slower-moving bands corresponding to human +11-CTFs (Fig. 3f, lanes 6–8, compare with lanes 2–4). In the amino acid sequences of Aβ in humans and mice, a sequence divergence occurs around the +11 site, whereas there is absolute conservation at the +1 site (Fig. 1a).

Our finding that secretion of Aβ1–40/42 and Aβ11–40/42 is abolished in *BACE1*$^{-/-}$ neurons establishes that BACE1 is the principal β-secretase in neurons. Although Aβ11–40/42 peptides have been observed in neuronal cultures[11] and in the brains of patients with Alzheimer's disease[12], the involvement of these peptides in its pathogenesis remains elusive. Aβ beginning at +11 is a major species in rodents *in vivo*[13], and this peptide is more fibrillogenic and neurotoxic than full-length Aβ *in vitro*[14]. Because of our finding that the +11 site is a major cleavage site for BACE1, it will be important to re-examine the involvement of Aβ11–40/42 in pathogenesis of Alzheimer's disease. Whereas both β-and γ-secretase activities represent therapeutic targets for the development of protease inhibitors for Alzheimer's disease, our finding that BACE1 is the principal β-secretase in neurons suggests focusing on the design of therapeutics to inhibit BACE1 activity. Because BACE1 null mice are viable, it may be possible to develop BACE1 inhibitors that ameliorate β-amyloid deposition without profound adverse effects. The crystal structure of the protease domain of BACE1 associated with an eight-residue peptide inhibitor may aid in developing drugs to inhibit BACE1 activity[15].

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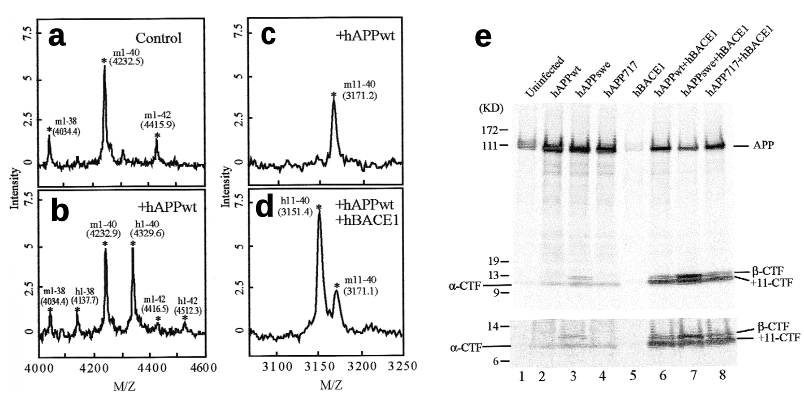

Fig. 3. Species-specific cleavage of APP at the +11 site of Aβ. (**a–d**) Conditioned media of cultured neurons uninfected (**a**), or infected with adenovirus expressing hAPPwt alone (**b**, **c**) or co-expressing hAPPwt and human BACE1 (**d**) were subjected to IP-MS analysis; asterisks, peaks corresponding to murine or human Aβ peptides (mass). (**e**, **f**) Cultured neurons expressing either hAPPwt or its variants (lanes 2–4) or co-expressing hBACE1 and hAPPwt or its variants (lanes 6–8) were pulse labeled for 5 h and lysates from these cultures were immunoprecipitated with CT15.