Estrogen reduces neuronal generation of Alzheimer β-amyloid peptides

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Alzheimer's disease (AD) is characterized by the accumulation of cerebral plaques composed of 40- and 42-amino acid β-amyloid (Aβ) peptides, and autosomal dominant forms of AD appear to cause disease by promoting brain Aβ accumulation. Recent studies indicate that postmenopausal estrogen replacement therapy may prevent or delay the onset of AD. Here we present evidence that physiological levels of 17β-estradiol reduce the generation of Aβ by neuroblastoma cells and by primary cultures of rat, mouse and human embryonic cerebrocortical neurons. These results suggest a mechanism by which estrogen replacement therapy can delay or prevent AD.

Alzheimer's disease is associated with neuronal cell loss, neurofibrillary tangle formation and deposition of β-amyloid (Aβ) plaques in susceptible brain regions. The main constituents of these plaques are 40–42 amino acid Aβ peptides[1,2] which are derived from a ~700 amino acid Aβ precursor protein (βAPP). Full length βAPP can undergo proteolytic cleavage at the N-terminus of the Aβ region by an enzymatic activity designated "β-secretase". Further cleavages, by "γ-secretases", lead to the production of 40 and 42 amino acid Aβ peptides. In addition to this amyloidogenic pathway for βAPP metabolism, full length βAPP molecules can also be proteolyzed by an enzyme termed "α-secretase", which cleaves βAPP within its Aβ region, releasing a soluble fragment ($sβAPP_α$) from the βAPP extracellular domain and precluding the formation of $Aβ^{1-40}$ and $Aβ^{1-42}$ peptides. In certain cell types (e.g., HEK293 cells[3]), successive cleavage by both α- and γ- secretases results in the release of a 3 kD peptide, designated "p3" ($Aβ^{17-40/42}$) (see ref. 4 for review of βAPP metabolism).

The relative utilization of βAPP processing pathways is controlled by certain signal transduction systems. In an earlier study we observed that treatment of a breast carcinoma-derived cell line with physiological concentrations of 17β-estradiol (17β-E_2) led to increased $sβAPP_α$ release[5]. Here we have investigated the potential ability of estrogen to regulate Aβ release by neuroblastoma cells and by primary cultures of rat, mouse and human embryonic cerebrocortical neurons. We demonstrate that treatment with 17β-E_2 not only increases $sβAPP_α$ release but also significantly decreases the production of the Aβ peptides which are believed to play a major role in the etiology of all forms of AD. Of therapeutic relevance, physiological levels of estrogen diminished Aβ release by primary cultures derived from rodent and human fetal cerebral cortex.

Estrogen decreases $Aβ^{1-40}$ and $Aβ^{1-42}$ release

Treatment of N2a cells with 17β-E_2 resulted in increased $sβAPP_α$ release and decreased $Aβ^{1-40}$ release in a dose-dependent manner (Fig. 1*a–c*). A protein of 3 kD also decreased in response to 17β-E_2 (Fig. 1*a*). Immunoprecipitation-mass spectrometry (IP-MS) analysis[6] (Fig. 1*b*), and radiosequencing of the peptide (Gouras *et al.*, manuscript in preparation), indicated that the 3 kD peptide was composed of two species; a major component was $Aβ^{11-40}$ while a minor component was $Aβ^{17-40}$ (conventional "p3"; ref. 3). In response to 17β-E_2, $Aβ^{11-40}$ release decreased, and $Aβ^{17-40}$ appeared to increase (Fig. 1*b*). Thus, the novel 3 kDa($Aβ^{11-40}$)-generating pathway is regulated in a fashion similar to that of the conventional β-secretase pathway and, as expected, the $Aβ^{17-40}$-generating pathway seems to be regulated in a fashion similar to that of the $sβAPP_α$-generating α-secretase pathway.

The effects of 17β-E_2 were apparently not due to a change in the transcription of βAPP, since βAPP protein levels were unaffected by treatment with 17β-E_2 using either a 4 hour or a 10 minute metabolic labelling procedure (data not shown). In support of this interpretation, the level of $βAPP_{695}$ mRNA was unaltered (102 ± 6%) by 17β-E_2, as demonstrated by RNAse protection assay[7] (data not shown).

The responses of $sβAPP_α$ and $Aβ^{1-40}$ to 17β-E_2 were studied as a function of duration of hormone treatment. Maximal increases in $sβAPP_α$ and decreases in Aβ generation were observed when cells were treated with 17β-E_2 for seven to ten days (Fig. 1*d*).

In view of recent evidence indicating that mutations in the presenilin proteins (PS1, PS2) can influence Aβ metabolism[8–11], presenilin metabolism was examined in the absence and presence of 17β-E_2 . The levels of the N- and C-terminal fragments of

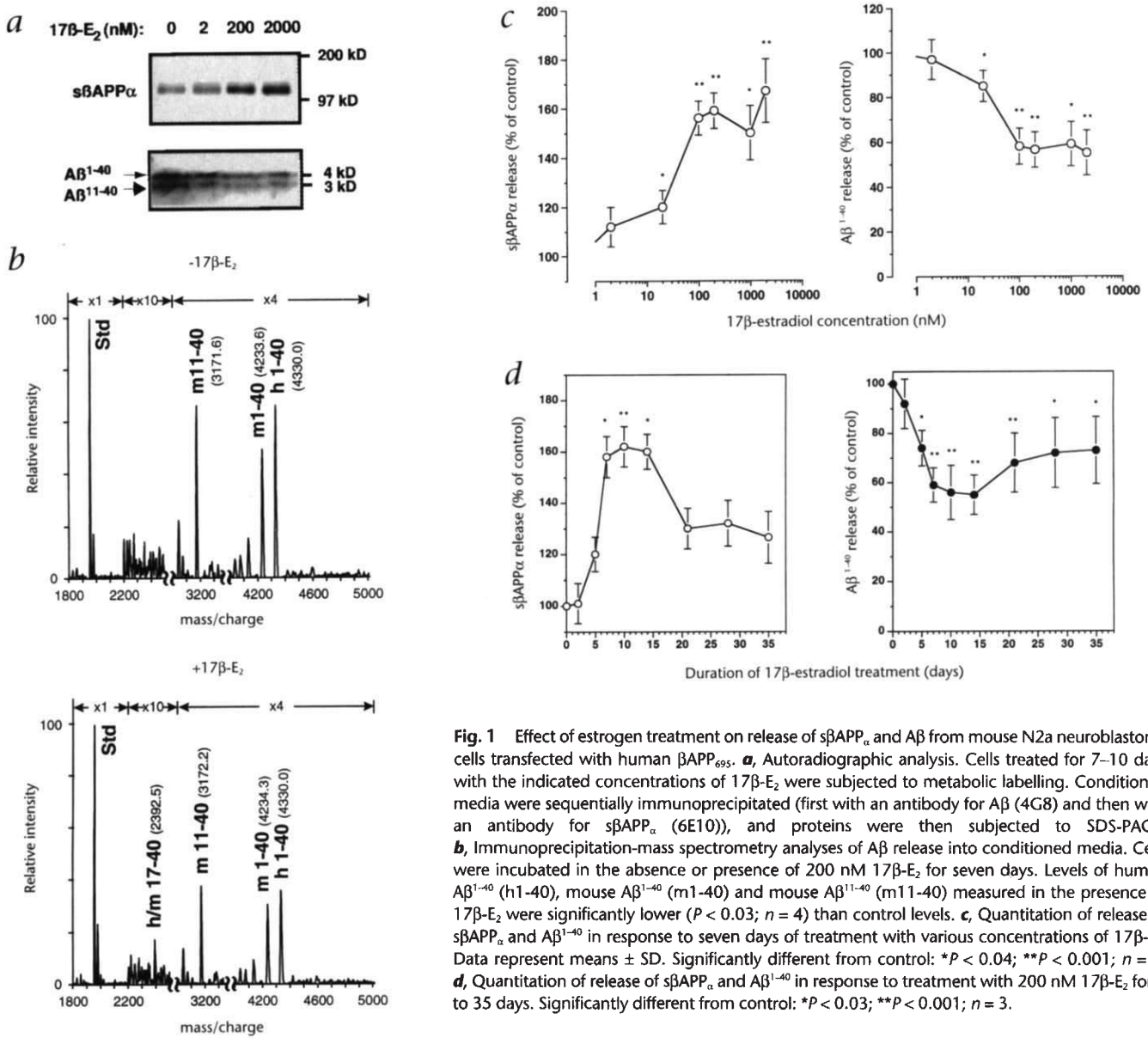

Fig. 1 Effect of estrogen treatment on release of $s\beta APP_{\alpha}$ and Aβ from mouse N2a neuroblastoma cells transfected with human βAPP_{695}. ***a***, Autoradiographic analysis. Cells treated for 7–10 days with the indicated concentrations of 17β-E_2 were subjected to metabolic labelling. Conditioned media were sequentially immunoprecipitated (first with an antibody for Aβ (4G8) and then with an antibody for $s\beta APP_{\alpha}$ (6E10)), and proteins were then subjected to SDS-PAGE. ***b***, Immunoprecipitation-mass spectrometry analyses of Aβ release into conditioned media. Cells were incubated in the absence or presence of 200 nM 17β-E_2 for seven days. Levels of human $A\beta^{1-40}$ (h1-40), mouse $A\beta^{1-40}$ (m1-40) and mouse $A\beta^{11-40}$ (m11-40) measured in the presence of 17β-E_2 were significantly lower ($P < 0.03$; $n = 4$) than control levels. ***c***, Quantitation of release of $s\beta APP_{\alpha}$ and $A\beta^{1-40}$ in response to seven days of treatment with various concentrations of 17β-E_2. Data represent means ± SD. Significantly different from control: $^{*}P < 0.04$; $^{**}P < 0.001$; $n = 4$. ***d***, Quantitation of release of $s\beta APP_{\alpha}$ and $A\beta^{1-40}$ in response to treatment with 200 nM 17β-E_2 for 1 to 35 days. Significantly different from control: $^{*}P < 0.03$; $^{**}P < 0.001$; $n = 3$.

PS1 were unaffected by 17β-E_2, indicating that PS1 metabolism is not an important target for estrogen action (data not shown).

Although $A\beta^{1-40}$ accounts for most of the Aβ generated by cells[8], $A\beta^{1-42/x-42}$ is apparently crucial in initiating Aβ deposition[12]. Therefore, it was important to determine specifically whether $A\beta^{1-42/x-42}$ production was altered by 17β-E_2. Since most cells (including N2a cells transfected with human βAPP_{695}) generate extremely low levels of $A\beta^{1-42/x-42}$ (refs. 8–11), we used N2a cells coexpressing human βAPP_{695} and the familial AD-linked PS1 variant which lacks amino acids 290–319 encoded by exon 10 of the PS1 gene (PS1ΔE10) (ref. 13). These PS1ΔE10 cells have been documented to generate excessive (and therefore readily detectable) levels of $A\beta^{1-42}$ (ref. 9).

These doubly-transfected cells were maintained for seven days in the absence or presence of 17β-E_2. $s\beta APP_{\alpha}$ and Aβ peptide release were determined by sequential immunoprecipitation, using antibodies of defined specificities[14,15] (see Methods). These cells released $s\beta APP_{\alpha}$, $A\beta^{1-40}$, $A\beta^{11-40}$ and $A\beta^{1-42}$, but no $A\beta^{11-42}$ was detected. Treatment with 17β-E_2 caused an increase in $s\beta APP_{\alpha}$ release and a decrease in $A\beta^{1-40}$, $A\beta^{11-40}$ and $A\beta^{1-42}$ release (Fig. 2). Notably, $A\beta^{1-42}$ generation was reduced by approximately 50% at 200 nM 17β-E_2, roughly equivalent to that observed for $A\beta^{1-40}$ (Fig. 2*b*). The ratio of $A\beta^{1-42}/A\beta^{1-40}$ was not altered by estrogen treatment at any concentration (Fig. 2*b*). IP-MS analysis (Fig. 2*c*) corroborated the metabolic labeling data, confirming that generation of $A\beta^{1-40}$, $A\beta^{11-40}$ and $A\beta^{1-42}$ was diminished in response to 17β-E_2 treatment.

Decreased Aβ release by rodent and human neurons

Based on the observation that 17β-E_2 reduces Aβ generation by N2a cells, we examined primary cultures of rodent and human embryonic neurons. Primary cultures were prepared from E17 rat cerebral cortical tissue[16] and incubated either in standard medium or in 17β-E_2-supplemented medium. Following metabolic labeling, neuronal conditioned media were subjected to immunoprecipitation with antibody 4G8. Release of both 4 kD and 3 kD Aβ-like species were reduced by approximately 50% in the presence of 17β-E_2 (Fig. 3*a*). IP-MS analysis (Fig. 3*b*) and ra-

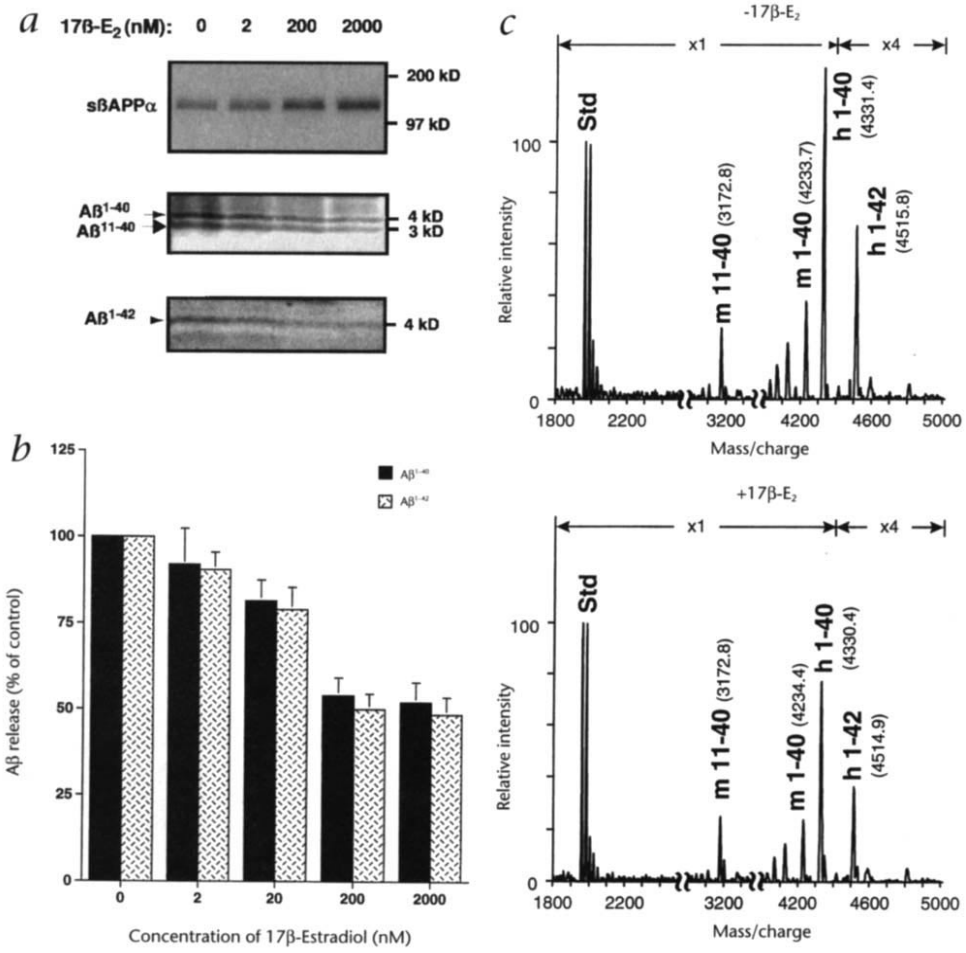

Fig. 2 Effect of estrogen treatment on release of sβAPP$_\alpha$ and Aβ from mouse N2a neuroblastoma cells doubly transfected with human βAPP$_{695}$ and exon-10-deleted PS1. ***a***, Autoradiographic analysis. Cells treated for 7–10 days with the indicated concentrations of 17β-E$_2$ were subjected to metabolic labelling. Conditioned media were sequentially treated, first with an antibody to immunoprecipitate Aβ$^{1-42}$ (FCA3542), then with an antibody to immunoprecipitate Aβ$^{1-40}$ and Aβ$^{11-40}$ (FCA3340), and finally with an antibody to immuoprecipitate sβAPP$_\alpha$ (6E10). The immunoprecipitated proteins were subjected to SDS-PAGE. ***b***, Quantitation of Aβ$^{1-40}$ and Aβ$^{1-42}$ release as a function of estrogen concentration. Data were normalized to the amount of Aβ$^{1-40}$ or Aβ$^{1-42}$, respectively, generated by untreated cells, and represent means ± SD. The average ratio of Aβ$^{1-40}$/Aβ$^{1-42}$ was 8.35 ± 1.5 in untreated cells and was not changed by 17β-E$_2$ treatment. ***c***, Immunoprecipitation-mass spectrometry analyses of Aβ release into conditioned media. Experimental procedures were as described in the legend to Fig. 1*b*. Human Aβ$^{1-40}$ (h1–40) and Aβ$^{1-42}$ (h1–42), and mouse Aβ$^{1-40}$ (m1–40) and Aβ$^{11-40}$ (m11–40) levels measured in the presence of 17β-E$_2$ were significantly lower ($P < 0.02$; $n = 4$) than control levels.

diosequencing (Gouras *et al.*, manuscript in preparation) indicated that the 4 kD species was "conventional" Aβ, bearing an aspartyl residue at position 1. As had been the case for the conditioned media of neuroblastoma cells, the more abundant species migrating at ~3 kD was an Aβ variant bearing Glu[11] at its amino-terminus. This Aβ (Glu[11]) generated by rat neurons may be derived from an ~100 amino acid intermediate bearing the corresponding glutamate at its N-terminus[17]. The estrogen-related diminution in Aβ generation was accompanied by an approximate 30–40% increase in sβAPP release (Fig. 3*a*). IP-MS analysis confirmed that levels of both Aβ$^{1-40}$ and Aβ$^{11-40}$ were reduced by 17β-E$_2$ (Fig. 3*b*). No changes in protein levels of neuronal βAPP, PS1 or their derivatives, occurred in association with 17β-E$_2$ treatment (data not shown).

The reduction in levels of Aβ42 derived from neurons may be central to estrogen's effects on amyloid accumulation in the brain. Since Aβ42 species were readily detectable in primary cultures of mouse neurons but not rat neurons, we studied the effect of 17β-E$_2$ treatment on Aβ42 generation in cortical cultures derived from E14 mouse cortex. In mouse cortical cultures treated for five days with 200 nM 17β-E$_2$, Aβ$^{1-40}$ and Aβ$^{11-40}$ were decreased by 45% and 40% ($P < 0.01$; $n = 3$), respectively, following 17β-E$_2$ treatment, whereas sβAPP$_\alpha$ release was increased by 55% ($P < 0.01$; $n = 3$) (Fig. 3*c*). Also, in the mouse cortical cultures, released Aβ$^{1-42}$ and Aβ$^{11-42}$ were reduced following 17β-E$_2$ treatment by 52% and 55% ($P < 0.01$; $n = 3$), respectively.

Fetal human cerebrocortical neurons were cultured in the absence or presence of 17β-E$_2$ for seven to ten days. In three experiments, cells were metabolically labeled and Aβ release was analyzed by immunoprecipitation with antibody 4G8. As compared to untreated neurons, Aβ generation was reduced by approximately 45% in human primary neuronal cultures by incubation with 17β-E$_2$ (Fig. 4). Importantly, the half-maximal effect for diminution of Aβ generation by 17β-E$_2$ was observed at approximately 3–4 nM, a level within the physiological range (2–10 nM) of circulating estrogen in premenopausal women[18]. In two other experiments, ELISA assays of human neuronal culture media confirmed these results: the levels of β-amyloid released by cells treated with 17β-E$_2$ for eight days were 48% and 56% of the respective untreated control values.

Discussion

The observation that postmenopausal estrogen replacement therapy is associated with a decreased risk of AD (ref. 19) provides an important lead for understanding the basis of AD and for developing improved medications for treating the disease. It should now be possible to dissect the mechanism(s) through which estrogen exerts its anti-neurodegenerative effect. The central role of Aβ in AD pathogenesis, together with the indication that estrogen replacement therapy may prevent AD, suggests to us that one mechanism of action of estrogen in the brain might be to modulate Aβ generation. The data reported here demonstrate that physiological concentrations of estrogen can reduce neuronal Aβ generation, revealing an attractive mechanism through which es-

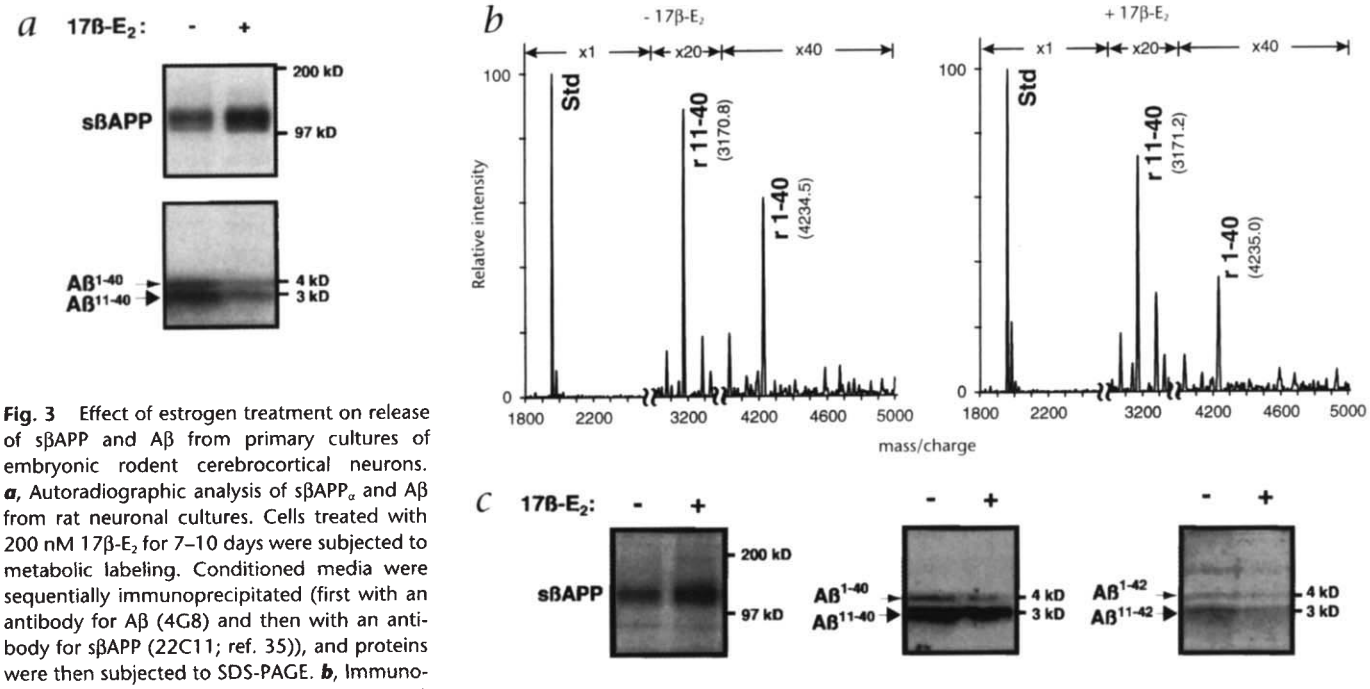

Fig. 3 Effect of estrogen treatment on release of sβAPP and Aβ from primary cultures of embryonic rodent cerebrocortical neurons. ***a***, Autoradiographic analysis of $sβAPP_α$ and Aβ from rat neuronal cultures. Cells treated with 200 nM 17β-E_2 for 7–10 days were subjected to metabolic labeling. Conditioned media were sequentially immunoprecipitated (first with an antibody for Aβ (4G8) and then with an antibody for sβAPP (22C11; ref. 35)), and proteins were then subjected to SDS-PAGE. ***b***, Immunoprecipitation-mass spectrometry analyses of Aβ release into conditioned media of rat neurons cultured in the absence or presence of 17β-E_2. Experimental procedures were as described in the legend to Fig. 1*b*. Rat $Aβ^{1-40}$ (r1–40) and $Aβ^{11-40}$ (r11–40) levels measured in the presence of 17β-E_2 were significantly lower ($P < 0.04$ and $P < 0.01$, respectively; $n = 3$) than control levels. ***c***, Autoradiographic analysis of sβAPP, $Aβ^{1-40/11-40}$ and $Aβ^{1-42/11-42}$ from mouse neuronal cultures. Cells treated with 200 nM 17β-E_2 for five days were subjected to metabolic labeling. Conditioned media were sequentially treated, first with an antibody to immunoprecipitate $Aβ^{1-42}$ and $Aβ^{11-42}$ (FCA3542), then with an antibody to immunoprecipitate $Aβ^{1-40}$ and $Aβ^{11-40}$ (FCA3340), and finally with an antibody to immunoprecipitate sβAPP (22C11). Immunoprecipitated proteins were then subjected to SDS-PAGE.

trogen may protect against AD. We cannot exclude the possibility that additional estrogen activities might also be relevant, including modulation of (i) basal forebrain cholinergic activity and integrity[20], (ii) dendritic plasticity[21], (iii) NMDA receptor density[22], and (iv) neurotrophin signaling[23], as well as prevention of oxidative toxicity due to glutamate, free radicals and Aβ (ref. 24).

The biological basis for the regulation by estrogen of Aβ formation, as reported here, is unknown. It has been shown that estrogen can affect the morphology of the *trans*-Golgi network (TGN), thereby enhancing the biogenesis and trafficking of post-TGN constitutive secretory vesicles and granules[25,26]. Aβ can be generated in the TGN from βAPP (ref. 27). Moreover, formation of Aβ in the TGN and its export from that organelle are regulated by signal transduction pathways[28,29]. We hypothesize that 17β-E_2 promotes egress of βAPP from the TGN and thereby reduces the local concentration of βAPP available as a substrate for Aβ production.

In summary, we have provided evidence that 17β-E_2 can significantly decrease the amount of plaque-forming Aβ released from neuroblastoma cells as well as from cells in primary cultures derived from rodent and human neocortex. These results support the notion that one relevant neuropharmacological activity of estrogen replacement therapy is to reduce Aβ generation and that such an activity contributes to the ability of estrogen replacement therapy to protect against AD.

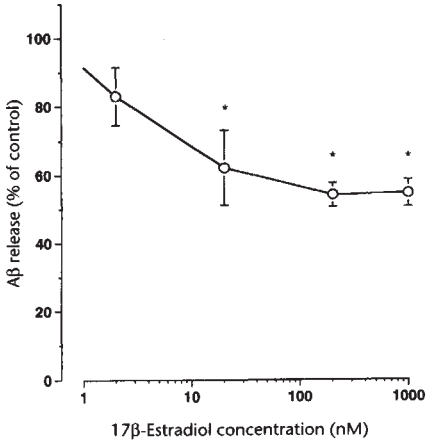

Fig. 4 Estrogen reduces Aβ generation from human embryonic cerebrocortical neurons. Cells treated for 7–10 days with the indicated concentrations of 17β-E_2 were subjected to metabolic labeling. Conditioned media were immunoprecipitated using antibody 4G8, and proteins were subjected to SDS-PAGE. In each experiment, data were normalized to the amount of Aβ generated by untreated cells, and represent means ± SD for individual measurements in each of three separate experiments. Differences in Aβ levels were significant (*$P < 0.01$; $n = 3$) as compared with Aβ levels measured in the absence of 17β-E_2.

Methods

Cell cultures and estrogen treatment. N2a cells, transfected with human $βAPP_{695}$ or doubly transfected with human $βAPP_{695}$ and mutant PS1 (ref. 9), were maintained in medium containing 50% DMEM, 50% Opti-MEM, supplemented with 5% FBS, 200μg/ml G418, and antibiotics (Gibco, BRL), in the absence or presence of water-soluble 17β-E_2 (Sigma). Cells were trypsinized once each week, and medium was replaced every 2 days. The effects of estrogen on βAPP processing were similar when either charcoal-stripped FBS was substituted for FBS or when phenol red-free RPMI was substituted for DMEM/Opti-MEM. Standard conditions for studying N2a cells involved maintaining ~2×10^6 cells per 60mm dish for 7 days in the absence or presence of 17β-E_2. On day 7, the cells were subjected to metabolic labeling for 4 hr with 500 μCi/ml [^{35}S]methionine (NEN/Dupont) in the absence of 17β-E_2 and the media analyzed by immunoprecipitation followed by SDS-PAGE.

Cerebrocortical tissue derived from embryonic day 17 rats or day 14 mice was used to prepare primary neuronal cultures as described[16,30] with the fol-

lowing modifications: dissociated cells were plated in 10 cm dishes (~10^7 cells per dish) in fully defined Neurobasal media with N2 supplement (Gibco) for rat neuronal cultures and in F12 with 10%FCS (Gibco) for mouse neuronal cultures. For human mixed brain cultures, cerebrocortical tissue was isolated from gestational age 8–12 week aborted fetuses (Karolinska Hospital, Sweden). Cultures were maintained in the absence or presence of 17β-E_2 for 7 days. Media were replaced every 2 days. For analysis, cells were subjected to metabolic labeling for 4 hrs with 500μCi/ml [^{35}S]methionine in the absence or presence of 17β-E_2, and the media were analyzed by immunoprecipitation followed by SDS-PAGE.

Immunoprecipitation and western analysis. Media were collected and sequentially immunoprecipitated as described in the figure legends. Immunoprecipitates were subjected to SDS-PAGE analysis using 10–20% Tricine gels (for Aβ species) or 4–12% Tris-Glycine gels (for sβAPP$_\alpha$ detection). To assay full-length βAPP, aliquots of metabolically labeled cell lysates were equalized for protein content and subjected to immunoprecipitation using antibody 369 (ref. 31) and SDS-PAGE analysis. For western analysis of PS1 and PS1 fragments, protein content was standardized using aliquots of lysates of nonradioactive cells. These samples were analyzed on 4–20% SDS-PAGE, transferred to PVDF membrane (Millipore) and immunoblotted, using antibody Ab14 for detection of the PS1 N-terminal fragment or antibody "anti-loop" for detection of the PS1 C-terminal fragment[32].

To assay for estrogen receptors in N2a cells or rat neurons, nuclear extracts from these cells were subjected to SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted using an antibody against amino acids 495–595 of the estrogen receptor-α (ERα; ref. 33) (NeoMarker, Inc.). Immunoreactive ERα was readily detectable in 30 μg of total cellular protein as a 67 kD species (data not shown).

For all quantitative analyses, autoradiographic densities were quantified using a Bio-Rad phosphorimager using software 2.0.

Immunoprecipitation-mass spectrometry assay of Aβ peptides. Following various treatments, serum-free media were conditioned by cells for 24 hours in the continued absence or presence of 17β-E_2. Aliquots of conditioned media (1 ml from N2a cells and 5 ml from primary neuronal cultures) were immunoprecipitated by monoclonal antibody 4G8 and Protein G/A agarose[6]. The molecular masses and concentrations of immunoprecipitated Aβ species were measured by MALDI-TOF-mass spectrometry analysis[6]. In each display the various regions of the spectra are shown with the indicated amplification factors. For analyses, $Aβ^{12-28}$ (10 nM, final concentration) was added to samples and served as an internal standard[6].

ELISA detection of Aβ. Sandwich ELISA was performed essentially as described[34]. Aliquots (100 μl) of conditioned media were assayed for Aβ using antibodies 3134C (specific for Aβ residues 32–40) and 6E10 (specific for Aβ residues 5–10). The sandwich ELISA was developed using fluorometric detection. Under the conditions used, the ELISA accurately detected levels of Aβ of 25 pg/ml or greater.

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1. Glenner, G. & Wong, C. W. Alzheimer's disease: Initial report of the purification and characteristics of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **122**, 885–890 (1984).
2. Masters, C. L. *et al.* Amyloid plaque core protein in Alzheimer's disease and Down's syndrome. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4245–4249 (1985).
3. Haass, C. Hung, A. Y., Schlossmacher, M. G., Teplow, D. B. & Selkoe, D. J. β-Amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. *J. Biol. Chem.* **268**, 3021–3024 (1993).
4. Haass, C. & Selkoe, D. J. Cellular processing of β-amyloid precursor protein and genesis of the amyloid-β peptide. *Cell* **75**, 1039–42 (1993).
5. Jaffe, A. B., Toran-Allerand, C. D., Greengard, P. & Gandy, S. E. Estrogen regulates metabolism of Alzheimer amyloid β precursor protein. *J. Biol. Chem.* **269**, 13065–13068 (1994).
6. Wang, R., Sweeney, D., Gandy, S. E. & Sisodia, S. S. The profile of soluble amyloid β protein in cultured cell media. *J. Biol. Chem.* **271**, 31894–31902 (1996).
7. Ausubel, F. M. *et al.* in Short Protocols in Molecular Biology, Second Edition, (John Wiley and Sons, New York, 1992).
8. Scheuner, D. *et al.* Secreted amyloid β-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Med.* **2**, 864–870 (1996).
9. Borchelt, D. R. *et al.* Familial Alzheimer's disease-linked presenilin 1 variants elevate Aβ1-42/1-40 ratio *in vitro* and *in vivo*. *Neuron* **17**, 1005–1013. (1996).
10. Duff, K. *et al.* Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. *Nature* **383**, 710–713 (1996).
11. Citron, M. *et al.* Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. *Nature Med.* **3**, 67–72 (1997).
12. Iwatsubo, T. *et al.* Visualization of Aβ 42(43) and Aβ 40 in senile plaques with end-specific Aβ monoclonals: evidence that an initially deposited species is Aβ 42(43). *Neuron* **13**, 45–53 (1994).
13. Perez-Tur, J. *et al.* A mutation in Alzheimer's disease destroying a splice acceptor site in the presenilin-1 gene. *Neuroreport* **7**, 297–301 (1995).
14. Barelli, H. *et al.* Characterization of new polyclonal antibodies specific for 40 and 42 amino acid-long amyloid β peptides: their use to examine the cell biology of presenilins and the immunohistochemistry of sporadic and cerebral amyloid angiopathy cases. *Mol. Med.* **3**, 695–707 (1997).
15. Kim, K. S. *et al.* Detection and quantitation of amyloid β-peptide with monoclonal antibodies. *Neurosci. Res. Commun.* **7**, 113–122 (1990).
16. Lu, B., Yokoyama, M., Dreyfus, C. F., & Black, I.B. Depolarizing stimuli regulate nerve growth factor gene expression in cultured hippocampal neurons. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6289–6292 (1991).
17. Simons, M. *et al.* Amyloidogenic processing of the human amyloid precursor protein in primary cultures of rat hippocampal neurons. *J. Neurosci.* **16**, 899–908 (1996).
18. Feingold, K. R., Galvin, L. A., Schambelan, M. & Sebastian, A. in *Cecil's Essentials of Medicine* (eds. Andreoli, T. E., Carpenter, C. C. J., Plum, F. & Smith, L. H.) 478 (W. B. Saunders Co., Philadelphia, 1990).
19. Tang, M. *et al.* Effect of oestrogen during menopause on risk and age of onset of Alzheimer's disease. *Lancet* **348**, 429–432 (1996).
20. Luine, V. Estradiol increases choline acetyltransferase activity in specific basal forebrain nuclei and projection areas of female rats. *Exp. Neurol.* **89**, 484–490 (1985).
21. Woolley, C. S. & McEwen, B. S. Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *J. Comp. Neurol.* **336**, 293–306 (1993).
22. Woolley, C. S., Weiland, N. G., McEwen, B. S. & Schwartzkroin, P. A. Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. *J. Neurosci.* **17**, 1848–1859 (1997).
23. Toran-Allerand, C. D. The estrogen/neurotrophin connection during neural development: is co-localization of estrogen receptors with the neurotrophins and their receptors biologically relevant? *Dev. Neurosci.* **18**, 36–48 (1996).
24. Behl, C., Widman, M., Trapp, T. & Holsboer, F. 17-beta estradiol protects neurons from oxidative stress-induced cell death in vitro. *Biochem. Biophys. Res. Commun.* **216**, 473–482 (1995).
25. Larson, G. H. & Wise, P. M. Constitutive and regulated prolactin secretion: effects of estradiol. *Biology of Reproduction.* **50**, 357–362 (1994).
26. Scammell, J. G., Burrage, T. G. & Dannies, P. S. Hormonal induction of secretory granules in a pituitary tumor cell line. *Endocrinology* **119**,1543–1548 (1986).
27. Xu, H. *et al.* Generation of Alzheimer β-amyloid protein in the *trans*-Golgi network in the apparent absence of vesicle formation. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3748–3752 (1997).
28. Xu, H., Greengard, P. & Gandy, S. Regulated formation of Golgi secretory vesicles containing Alzheimer β-amyloid precursor protein. *J. Biol. Chem.* **270**, 23243–23255 (1995).
29. Xu, H. Sweeney, D., Greengard, P. & Gandy, S. Metabolism of Alzheimer β-amyloid precursor protein: regulation by protein kinase A in intact cells and in a cell-free system. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4081–4083 (1996).
30. Vincent, B., Beaudet, A., Dauch, P., Vincent, J.P., & Checler, F. Distinct properties of neuronal and astrocytic endopeptidase 3.4.24.16: a study on differentiation, subcellular distribution, and secretion processes. *J. Neurosci.* **16**, 5049–5059 (1996).
31. Buxbaum, J. D. *et al.* Processing of Alzheimer beta/A4 amyloid precursor protein: Modulation by agents that regulate protein phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 6003–6006 (1990).
32. Thinakaran, G. *et al.* Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. *Neuron* **17**, 181–190 (1996).
33. Evans, R. M. The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889–895 (1988).
34. Desdouits, F., Buxbaum, J. D., Desdouits-Magnen, J., Nairn, A. C. & Greengard, P. Amyloid β peptide formation in cell-free preparations. *J. Biol. Chem.* **271**, 24670–24674 (1996).
35. Weidemann, A. *et al.* Identification, biogenesis and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* **57**, 115–126 (1989).