

©2004, Acta Pharmacologica Sinica  
Chinese Pharmacological Society  
Shanghai Institute of Materia Medica  
Chinese Academy of Sciences  
<http://www.ChinaPhar.com>

# Disruption of estrogen receptor beta in mice brain results in pathological alterations resembling Alzheimer disease<sup>1</sup>

Qing-hong ZHANG<sup>2</sup>, Yan-hong HUANG<sup>3</sup>, Yu-zhen HU, Geng-ze WEI,  
Xue-feng HAN, Shun-yan LU, Yu-feng ZHAO

<sup>2</sup>*Department of Physiology, Institute of Basic Medical Science, <sup>3</sup>Department of Gynaecology and obstetrics, Xijing Hospital, The Fourth Military Medical University, Xi-an 710032, Shaanxi, China*

**KEY WORDS** beta-amyloid peptide; apolipoprotein E; estrogen receptor beta; Alzheimer disease

## ABSTRACT

**AIM:** To study the pathological characteristics of the mice with estrogen receptor  $\beta$  (ER $\beta$ ) disruption in brain. **METHODS:** Immunohistochemistry method was applied in the study. **RESULTS:**  $\beta$ -Amyloid peptide (A $\beta_{42}$ ) and apolipoprotein E (ApoE) immunoreactive substances were accumulated notably in cortex and limbic structures such as the hippocampus and amygdala in brain, resembling the pathological changes of human Alzheimer disease (AD). A $\beta$  formed cloudy-like deposits in parenchyma of brain, while apoE also deposited along or surrounding the blood vessels. **CONCLUSIONS:** ER $\beta$  is crucial to the development of neural degenerative disease, so modulation of A $\beta$  metabolism via ER $\beta$  signal pathway might be beneficial for AD prevention or therapy.

## INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease prevalent among the elderly people, involving a number of genetic components, risk factors, and other poorly defined elements<sup>[1]</sup>. The synapse loss and neuronal cell death, characteristics of AD, are believed to result in large part from the neurotoxic effects of beta-amyloid peptide (A $\beta$ ), a 40-42 amino acid peptide (s) derived proteolytically from beta-amyloid precursor protein (APP)<sup>[2,3]</sup>. The deposition of amyloid peptide resulting in the formation of extracellular neuritic plaques in the brain are also diagnostic neuropathological fea-

tures of this neurodegenerative disorder<sup>[4]</sup>.

Apolipoprotein E is an important constituent of plasma lipoproteins and a ligand for several lipoprotein receptors. Three major forms of apoE (E2, E3, and apoE4) have been described in the plasma where they take part in the transport and cellular uptake of lipids<sup>[5]</sup>. Next to the liver, the brain is the second richest in the content of apoE mRNA. Genetic-epidemiological studies have also identified a highly reproducible association between the three common apoE gene polymorphisms and the relative risk for development of AD<sup>[6]</sup>. Furthermore, amyloid deposition as well as neuritic plaque formation are dependent on ApoE expression<sup>[7]</sup>.

It has been long recognized that estrogen is a potent neurotrophic and neuroprotective factor during embryonic and neonatal development. Its role in adult brain is more appealing because of the potential therapeutic importance in neurodegenerative disease<sup>[8]</sup>. Evidence from epidemiological studies supports enhanced cognitive function in women with AD taking estrogen

<sup>1</sup> Supported by the Scientific Innovation Project of 4th Military Medical University, No CX02A024.

<sup>2</sup> Correspondence to Dr Qing-hong ZHANG.

Phn 86-29-337-4521, ext 803. Fax 86-29-337-4552.

E-mail [z\\_qinghong@hotmail.com](mailto:z_qinghong@hotmail.com).

Received 2003-07-21

Accepted 2003-12-03

replacement therapy (ERT) as well as a reduced risk for developing AD in healthy women receiving ERT. Estrogen also reduces A $\beta$  burden in animal models of AD and secretion of A $\beta$  from neuronal cultures<sup>[9,10]</sup>.

Despite an important role of estrogen in determining the risk for AD, little is known about the mechanism for estrogen deficiency that subserves in AD pathogenesis. It is increasingly accepted that physiological levels of estrogen requires the involvement of estrogen receptors, while pharmacological levels of estrogen appear to by-pass receptors via cross-talk with other second messenger signaling molecules<sup>[11]</sup>. So the traditional signaling via ER is more appealing if implement of long-term estrogen therapy to reduce the neurodegenerative disease.

Although estrogen receptor was discovered in the 1980s, the more recent finding of its subtype, ER $\beta$ , has added the complexity to the study of estrogen signaling in the brain. ER $\beta$  was diversely distributed in a different pattern with ER $\alpha$  in brain<sup>[12]</sup>, implying a special role of ER $\beta$  in signaling the protective action of estrogen in brain. ER $\beta$  knockout mice (BERKO) provide us a reasonable and practical way to approach the purpose quickly. Previous study reported neuronal deficit in BERKO brain<sup>[13]</sup>, speculating ER $\beta$ 's important role in the development of neural degenerative disease. In the present study, we verified the hypothesis by providing strong evidence of diversely deposition of A $\beta$  and ApoE in BERKO brain.

## MATERIALS AND METHODS

**Animals and brain preparation** BERKO mice were provided by Department of Medical Nutrition of Karolinska Institute. Heterozygote mice were used for breeding. C57BL/6J strain mice from whom BERKO were created, were set as wild-type (WT) control. The mice were free to tap water and rodent row. Female BERKO (2-4 months,  $n=8$ ) were ovariectomized 2 weeks before anesthetized with an over-dose of pentobarbital sodium, and perfused through the heart with phosphate buffer (0.1 mol/L PBS), pH 7.4, followed by ice cold 40 g/L paraformaldehyde fixation in 0.1 mol/L phosphate buffer. The brains were carefully removed and postfixed in the fixative on 4 °C overnight, then were transferred into 30 % sucrose in 0.1 mol/L PBS until it settled. Serial sections (30  $\mu$ m thick) were cut with a cryostat.

**Immunohistochemistry** Floating sections were incubated in 50 % (v/v) methanol with 1 % (v/v) H<sub>2</sub>O<sub>2</sub>

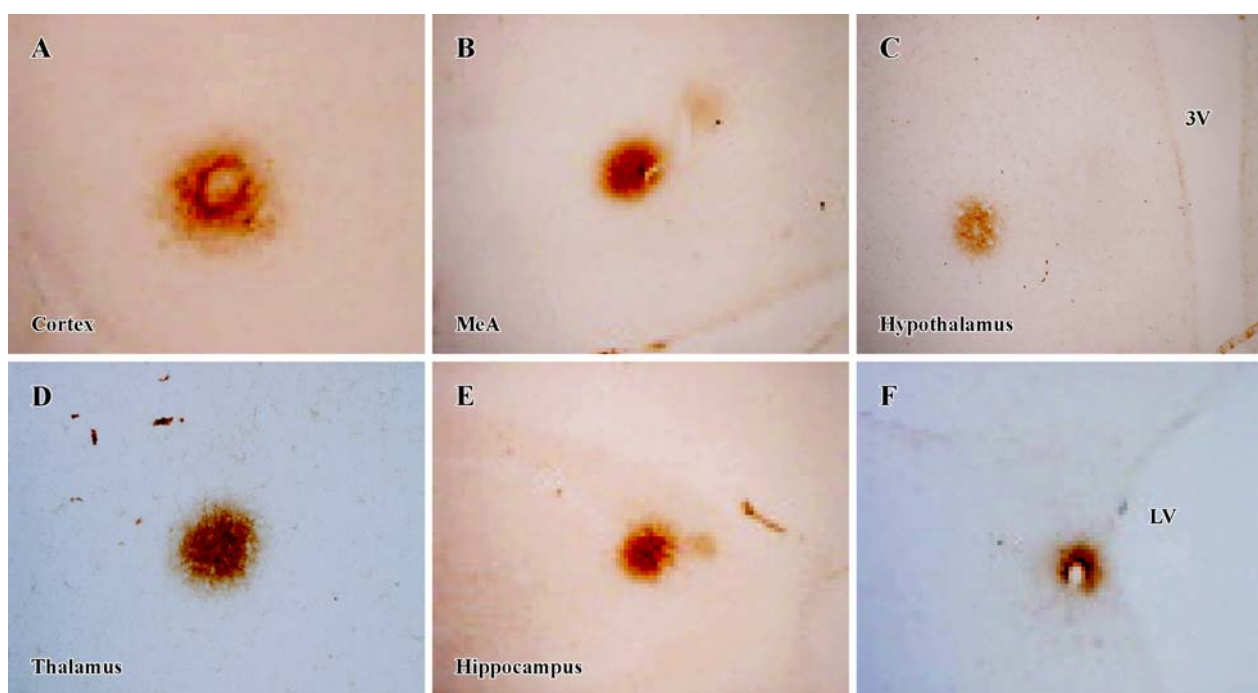
for 30 min to quench the endogenous peroxidases, followed by 100 g/L normal horse serum, 0.5 % (v/v) Triton X-100, 10 g/L BSA in 0.01 mol/L PBS for 30 min to block nonspecific binding. Sections were then incubated with anti-human A $\beta$ <sub>42</sub> (1:50, Transduction Laboratories, UK) or apoE (1:100, Transduction Laboratories), both with 100 g/L normal horse serum, 0.5 % Triton X-100, 30 g/L BSA in 0.01 mol/L PBS for 36 h at 4 °C. Immunohistochemistry method with ABC kit (Vector Laboratories) was applied afterwards. In brief, biotinylated rabbit anti-goat IgG (1:200) and avidin-biotin-complex (1:200) incubated for 2 h respectively. Staining was developed with 3,3'-diaminobenzidine (Sigma) catalyzed by H<sub>2</sub>O<sub>2</sub> for 10 min. Then the sections were completely washed in 0.01 mol/L PBS and finally mounted. The slides were observed under a microscopy (Olympus Optical Co, Japan) and the pictures were taken at a magnificent of  $\times 200$  by an attached digital camera.

## RESULTS

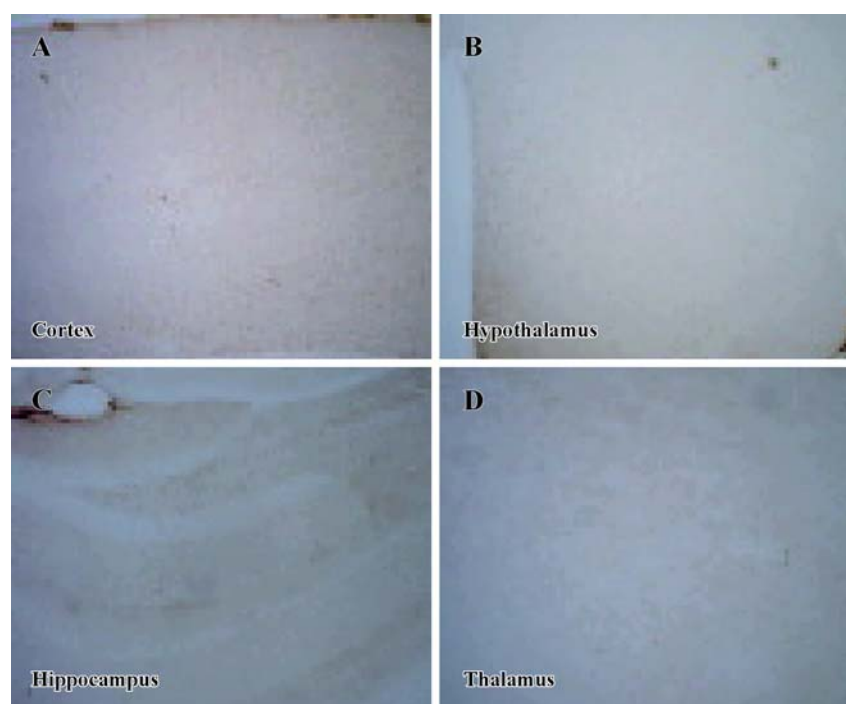
**Deposits of A $\beta$ <sub>42</sub> in BERKO brain** Amyloid plaques were found in great numbers in BERKO brain and had a wide distribution, most notably in cortex and limbic structures such as the hippocampus and medial amygdala (Fig 1). All the BERKO mice developed extracellular A $\beta$ <sub>42</sub> deposits as early as 2 months old. In contrast, the deposits never occurred in age- and sex-matched WT mice (Fig 2). This could be described as "non or all" phenomenon. Micrographs of BERKO brain sections showed A $\beta$ <sub>42</sub>-immunoreactive (ir) deposits in representative sections. The deposits were predominantly diffuse with minimal A $\beta$ <sub>42</sub>-ir cellular staining, forming cloudy-like senile plaques. The plaques differed in size and intensity. Some had solid cores with thread-like A $\beta$ <sub>42</sub> in the periphery, the others with radical formation.

The cerebral cortex contained a substantial density of deposits. A $\beta$ <sub>42</sub>-ir plaques were distributed predominantly in association with cortical zones while with a lower density in paralimbic cortical areas. Detailed examination of the whole sections clearly showed that the deposits within vessels were most frequent seen in neocortex, where A $\beta$ <sub>42</sub> was present in plaques.

**Accumulation of ApoE in brain** ApoE positive staining was found primarily in the parenchyma as well as peri/para blood vessels in cortex, hippocampus, and hypothalamus (Fig 3). Some ApoE vascular deposits lined along the blood vessels so well that gave prominence to



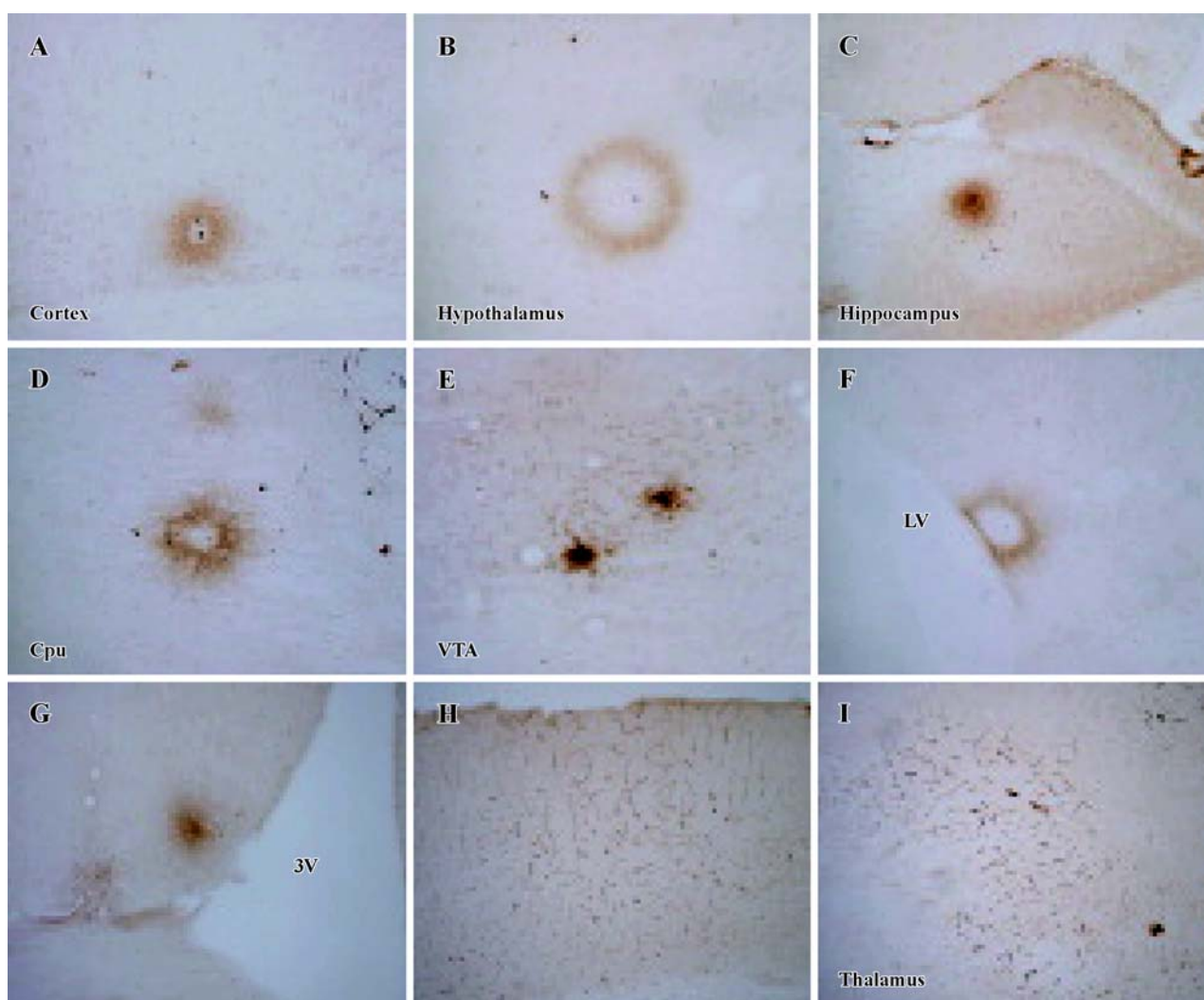
**Fig 1.** A $\beta$  immunoreactive staining in cortex (A), medial amygdala (MeA,B), hypothalamus (C), thalamus (D), hippocampus (E), close to lateral ventricle (F).



**Fig 2.** No A $\beta$ -immunoreactive staining in cortex (A), hypothalamus (B), hippocampus (C), thalamus (D) in WT.

the vessels. The other positive staining were similar to A $\beta_{42}$  deposits, also forming the spots in the parenchyma. It was interesting to see some ApoE-ir substances surrounding the tiny vessels that were so close to the brain

ventricles (Fig 3F, G), suggesting ApoE's role as the cholesterol transmitter between brain and cerebrospinal fluid. In every section of WT brain, no positive material could be seen in both the parenchyma or along



**Fig 3.** ApoE immunoreactive staining in parenchyma (A-G) and blood vessels or perivascular areas (H, I). Typical changes were represented in cortex (A), hypothalamus(B), hippocampus (C), caudate putamen (Cpu, D), ventral tegmentum area (VTA, E) and the area close to lateral (LV, F) or 3rd ventricle (3V, G).

the blood vessels (data not shown), confirming that ER $\beta$  disruption is the only explanation for A $\beta$ <sub>42</sub> or apoE accumulation in brain. Obliterating the primary A $\beta$  for A $\beta$ <sub>42</sub> or apoE also yielded negative data, verifying the specificity of above results.

## DISCUSSION

In this study, we found widespread  $\beta$ -amyloid deposits and apoE in the brain of BERKO mice, indicating that alteration in ER $\beta$  signaling pathway of functional neurons directly involved in neurodegenerative disease, supporting the hypothesis that ER $\beta$  plays a critical role in the neuroprotective effect of estrogen in brain.

Multiple levels of evidence implicate a central role of A $\beta$  in the pathophysiology of AD. It is indicated that

the neuritic dystrophy, neurofibrillary tangle formation, gliosis, microglial reactivity, and other degenerative changes seen in AD brains are results of altered metabolism of A $\beta$  peptides<sup>[14]</sup>.

Estrogen is identified as a potential modulator of A $\beta$  precursor metabolism, it might also modify other factors contributing to A $\beta$  deposition and fibril formation<sup>[15]</sup>. Our results implied the crucial point of ER $\beta$  in modulating A $\beta$  metabolism in brain. It is in line with previous report of neural loss and gliosis in BERKO which are also pathological characteristics of AD<sup>[13]</sup>.

The cognitive function loss is the hallmark of AD patients, which has much to do with the malfunction of limbic system and cortex<sup>[16]</sup>. The distribution of A $\beta$  deposits in these vulnerable areas such as hippocampus, amygdala, and cortex of BERKO brain contribute di-

rectly to the disfunction.

Neurodegeneration in AD is a pathological condition of neurons rather than an accelerated way of ageing<sup>[17]</sup>. So it was not surprised to see A $\beta$  deposits in the brain of BERKO mice as young as 2 months old. It also suggests that disruption of ER $\beta$  signaling of estrogen in brain may lead to A $\beta$  deposition directly.

Phytoestrogen such as kaempferol protects PC12 neuroblastoma and T47D human breast cancer cells from beta-amyloid-induced toxicity. The effects of the weak estrogen receptor agonists alpha-estradiol and kaempferol were comparable to that of the strong estrogen receptor agonist 17 $\beta$ -estradiol, suggesting a mode of action independent from the nuclear estrogen receptor<sup>[18]</sup>. It is a pity that the author ignored ER $\beta$  and drew a wrong conclusion. Phytoestrogen kaempferol is a potent agonist of ER $\beta$  stronger than ER $\alpha$ <sup>[19]</sup>, so it may act via ER $\beta$  instead of ER $\alpha$  to exert the same effect as ER $\alpha$  agonist beta-estradiol. The seemed-negative data conversely supported the significant role for ER $\beta$  in protecting the PC12 cells from beta-amyloid-induced toxicity.

The accumulation of apoE in BERKO also suggested a consequent event resulted from ER $\beta$  disruption. ApoE is a plasma cholesterol and phospholipid transporter which plays a central role in lipoprotein metabolism in the brain. The apoE epsilon4 allele (ApoEepsilon4) is associated with a selective increase in deposition of the 40-amino acid form of A $\beta$  (A $\beta$ 40) in endstage AD. As to the mechanism of apoE involvement in AD, several possibilities have been put forward that invoke the binding to tau protein affecting its hyperphosphorylation and therefore the microtubular stability, a differential rate of clearance of neurotoxic A $\beta$  to which apoE binds<sup>[20]</sup>. Moreover, apoE forms stable complexes with recombinant beta-amyloid precursor protein, so A $\beta$  can be conformationally modified by apoE *in vitro*.

Although we found some AD-related pathological alterations in the brain of BERKO mice, it deserves further investigation to determine the first event elicited by the disruption of ER $\beta$  pathway in brain. It was reported that ApoE may promote hypertension and contribute to AD pathogenesis by enhancement of vasoconstriction<sup>[21]</sup>. So it is reasonable to speculate a link between ER $\beta$ , hypertension, ApoE, cerebral amyloid angiopathy and AD. Anyhow, from the therapeutic point of view, modulation of A $\beta$  metabolism via ER $\beta$  signal pathway might be beneficial in individuals with, or at risk for AD.

## REFERENCES

- Jacobsen JS. Alzheimer's disease: an overview of current and emerging therapeutic strategies. *Curr Top Med Chem* 2002; 2: 343-52.
- Galvan V, Chen S, Lu D, Logvinova A, Goldsmith P, Koo EH, *et al*. Caspase cleavage of members of the amyloid precursor family of proteins. *J Neurochem* 2002; 82: 283-94.
- Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 2002; 19: 353-6.
- Selkoe DJ. Cell biology of the amyloid beta-protein precursor and the mechanism of Alzheimer's disease. *Ann Rev Cell Biol* 1994: 373-403.
- Mahley RW. Apolipoprotein E :cholesterol transport protein with expanding role in cell biology. *Science* 1988; 240: 622-30.
- Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, *et al*. Gene dose of Apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 1993; 261: 921-23.
- Holtzman DM, Bales KR, Tenkova T, Fagan AM, Parsadanian M, Sartorius LJ, *et al*. Apolipoprotein E isoform-dependent amyloid deposition and neuritic degeneration in a mouse model of Alzheimer's disease. *Proc Natl Acad Sci USA* 2000; 97: 2892-7.
- Cholerton B, Gleason CE, Baker LD, Asthana S. Estrogen and Alzheimer's disease: the story so far. *Drugs Aging* 2002; 19: 405-27.
- Petanceska SS, Nagy V, Frail D, Gandy S. Ovariectomy and 17beta-estradiol modulate the levels of Alzheimer's amyloid beta peptides in brain. *Neurology* 2000; 54: 2212-7.
- Greenfield JP, Leung LW, Cai D, Kaasik K, Gross RS, Rodriguez-Boulan E, *et al*. Estrogen lowers Alzheimer beta-amyloid generation by stimulating trans-Golgi network vesicle biogenesis. *J Biol Chem* 2002; 5: 12128-36.
- Wise PM, Dubal DB, Wilson ME, Rau SW, Bottner M. Minireview: neuroprotective effects of estrogen-new insights into mechanisms of action. *Endocrinology* 2001; 142: 969-73.
- Greco B, Allegretto EA, Tetel MJ, Blaustein JD. Coexpression of ER beta with ER alpha and progesterin receptor proteins in the female rat forebrain: effects of estradiol treatment. *Endocrinology* 2001; 142: 5172-81.
- Wang L, Andersson S, Warner M, Gustafsson JA. Morphological abnormalities in the brains of estrogen receptor beta knockout mice. *Proc Natl Acad Sci USA*. 2001; 98: 2792-6.
- Selkoe DJ. The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. *Trends Cell Biol* 1998; 8: 447-53.
- Gandy S, Petanceska S. Regulation of Alzheimer beta-amyloid precursor trafficking and metabolism. *Biochim Biophys Acta* 2000; 1502: 44-52.
- Fama R, Shear PK, Marsh L, Yesavage JA, Tinklenberg JR, Lim KO, *et al*. Remote memory for public figures in Alzheimer's disease: relationships to regional cortical and

- limbic brain volumes. *J Int Neuropsychol Soc* 2001; 7: 384-90.
- 17 Maccioni RB, Munoz JP, Barbeito L. The molecular bases of Alzheimer's disease and other neurodegenerative disorders. *Arch Med Res* 2001; 32: 367-81.
- 18 Roth A, Schaffner W, Hertel CJ. Phytoestrogen kaempferol (3,4',5,7-tetra hydroxyflavone) protects PC12 and T47D cells from beta-amyloid-induced toxicity. *Neurosci Res* 1999; 57: 399-404.
- 19 Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, *et al*. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* 1998; 139: 4252-63.
- 20 Strittmatter WJ, Roses AD. Apolipoprotein E and Alzheimer's disease. *Annu Rev Neurosci* 1996; 19: 53-77.
- 21 Paris D, Town T, Parker TA, *et al*. Isoform-specific vasoconstriction induced by apolipoprotein E and modulation of the effect by Alzheimer's beta-amyloid peptide. *Neurosci Lett* 1998; 256: 73-6.