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17 β -Estradiol regulates insulin-degrading enzyme expression via an ER β /PI3-K pathway in hippocampus: relevance to Alzheimer's prevention

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Abstract

Insulin-degrading enzyme (IDE), an enzyme that primarily degrades insulin, has recently been demonstrated to play a significant role in the catabolism of amyloid β (A β) protein in the brain. Reduced IDE expression and/or activity have been associated with the etiology and development of Alzheimer's disease (AD). Using three model systems, the present investigation provides the first documentation indicating that estrogen robustly regulates the expression of IDE in normal, menopausal and early-stage AD brains. *In vitro* analyses in primary cultures of rat hippocampal neurons revealed that 17 β -estradiol (17 β -E2) increased IDE in both mRNA and protein levels in a time-dependent manner. Further pharmacological analyses indicated that 17 β -E2-induced IDE expression was dependent upon estrogen receptor (ER) β and required activation of phosphatidylinositol 3-kinase (PI3-K). *In vivo* analyses in adult female rats revealed a brain region-specific responsive profile. Ovariectomy (OVX) induced a significant decline in IDE expression in the hippocampus, which was prevented by 17 β -E2. However, both OVX and 17 β -E2 did not exert a significant effect in the cerebellum. *In vivo* analyses in triple transgenic AD (3xTg-AD) female mice revealed an inverse correlation between the age-related increase in A β load and the decrease in IDE expression in the hippocampal formation. Treatment with 17 β -E2 attenuated A β accumulation/plaque formation and elevated hippocampal IDE expression in 12-month-old 3xTg-AD OVX mice. Collectively, these findings indicate that 17 β -E2 regulates IDE expression in a brain region-specific manner and such a regulatory role in the hippocampus, mediated by an ER β /PI3-K pathway, could serve as a direct mechanism underlying estrogen-mediated preventative effect against AD when timely initiated at the onset of menopause.

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Disclosure statement

The authors have no conflicts of interest to disclose. The use of animals was approved by the Institutional Animal Care and Use Committee at the University of Southern California.

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Keywords

17 β -estradiol (17 β -E2); Alzheimer's disease (AD); amyloid β (A β); insulin-degrading enzyme (IDE); estrogen receptor β (ER β); phosphatidylinositol 3-kinase (PI3-K); hippocampus; ovariectomy (OVX); triple transgenic Alzheimer's disease (3xTg-AD) mouse model

1. Introduction

Amyloid β (A β) protein, which is abnormally accumulated in human brains afflicted with Alzheimer's disease (AD), has long been proposed as the most likely culprit in the pathogenesis of the disease (Hardy and Selkoe, 2002; Tanzi and Bertram, 2005). In a healthy brain, A β remains at a steady-state level as a result of the metabolic balance between production of A β from intramembranous processing of amyloid precursor protein (APP) and removal of A β from cellular uptake and proteolytic degradation (Saido, 1998; Selkoe, 2000). Such a dynamic equilibrium, however, could be altered by genetic variations or environmental factors that further lead to the etiology of AD. In brains with early-onset familial AD, increased A β accumulation is evidenced to result largely from hyperactive anabolism of APP caused by genetic mutations in APP, presenilin-1 (PS-1) and PS-2 genes (Gandy, 2005; Hardy, 2004). Whereas in brains with late-onset sporadic AD that constitutes more than 90% of AD cases, increased A β accumulation has been associated with defective A β clearance and/or degradation (Selkoe, 2001; Tanzi, et al., 2004).

A number of proteases involved in A β degradation have been identified (Eckman and Eckman, 2005; Nalivaeva, et al., 2008). Insulin-degrading enzyme (IDE), a zinc metalloprotease with a molecular weight of ~110 kDa, was first discovered as the primary mechanism responsible for insulin degradation (Duckworth, et al., 1998; Kuo, et al., 1991). Recent research indicates that IDE is also highly expressed (Bernstein, et al., 1999; Kuo, et al., 1993) and critically involved in the degradation of A β in the brain (Kurochkin, 2001; Kurochkin and Goto, 1994; Qiu, et al., 1998; Selkoe, 2001). Reduced IDE expression and/or activity have been associated with the etiology and development of AD (Perez, et al., 2000; Zhao, et al., 2007).

Estrogen therapy (ET), when initiated at the onset of menopause, has been reported to reduce the risk or delay the onset of AD in women (Zhao and Brinton, 2007b; Zhao, et al., 2005). In agreement with clinical observations, basic science indicates that estrogen is neuroprotective and efficacious in preventing or slowing the development of AD pathology in particular A β accumulation and plaque formation in diverse animal models of AD (Carroll, et al., 2007; Petanceska, et al., 2000; Zheng, et al., 2002). Mechanistically, multiple lines of evidence have suggested that estrogen may regulate the production of A β and in turn sustain an improved A β homeostasis by increasing the secretory metabolism of APP (Greenfield, et al., 2002; Jaffe, et al., 1994). Estrogen may also regulate mitochondrial bioenergetic capacity such as aerobic glycolysis that is predicted to prevent conversion of the brain to using alternative sources of fuel such as the ketone body pathway characteristics of AD (Brinton, 2008; Brinton, 2009).

Past research indicates that estrogen regulates the expression and activity of IDE in female reproductive system, where IDE is involved in cellular growth and differentiation (Udrisar, et al., 2005). In the present study, using three *in vitro* and *in vivo* model systems, we investigated the impact of estrogen on IDE expression in the brain as well as the underlying signaling cascade and functional significance in an AD brain. Findings presented herein suggest a novel mechanism of estrogen anti-A β effect and provide insights into the therapeutic window of estrogen intervention in AD prevention in menopausal women.

2. Methods

2.1. Chemicals

17 β -Estradiol (17 β -E2) was purchased from Steraloids (Newport, RI). The estrogen receptor (ER) α agonist, PPT (Stauffer, et al., 2000), the ER β agonist, DPN (Meyers, et al., 2001), and the ER α agonist/ER β antagonist, (R,R)-THC (Sun, et al., 1999), were purchased from Tocris Cookson (Ellisville, MO). The phosphatidylinositol 3-kinase (PI3-K) inhibitors, wortmannin and LY-294002 were purchased from Calbiochem (San Diego, CA).

2.2. Animals

Embryonic day-18 fetuses derived from Sprague-Dawley pregnant-timed rats (Harlan, Indianapolis, IN) were used to obtain primary hippocampal neurons for *in vitro* experiments. Sprague-Dawley adult female rats (14 to 16-week-old, weighing 270–290 g) (Harlan) were used for *in vivo* experiments. A triple transgenic AD (3xTg-AD) mouse model developed by Dr. Frank Laferla, which harbors three human AD-relevant genetic alterations: APP_{swe}, PS1_{M146V} and tau_{p301L} (Oddo, et al., 2003), were used for A β -related pathological analyses. Animals were housed under controlled conditions of temperature (22°C), humidity and light (14 h light, 10 h dark) with water and food available *ad libitum*. .3. Primary hippocampal neuronal culture and treatment Primary culture of rat hippocampal neurons was prepared as previously described (Zhao, et al., 2004b). Test chemicals were first dissolved in analytically pure DMSO and diluted in culture medium. The working concentrations of 17 β -E2 (10 nM), PPT (10 nM) and DPN (10 nM) have been previously shown to be neuroprotective (Zhao, et al., 2004b). The working concentrations of (R,R)-THC (100 nM), wortmannin (50 nM) and LY-294002 (25 μ M) were based on the literature (Mannella and Brinton, 2006; Sun, et al., 1999; Zhao, et al., 2004a). After grown for 7 d *in vitro* (DIV), neurons were incubated with test chemicals for 48 h, followed by total RNA or protein isolation.

2.3. Primary hippocampal neuronal culture and treatment

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2.4. Animal treatment and tissue collection

Animals were ovariectomized (OVX) or underwent a sham-OVX operation and placed on a phytoestrogen-reduced diet, TD.96155 (Harlan Teklad) for 2 w before treatments. 17 β -E2 was first dissolved in analytically pure DMSO and diluted in corn oil (50 μ l of DMSO in 950 μ l of corn oil). Rats were treated, once daily for 2 d, with a subcutaneous injection of vehicle alone or 17 β -E2 (30, 70 or 300 μ g/kg body weight (BW)). Mice were treated, once daily for 4 d, with a subcutaneous injection of vehicle alone or 17 β -E2 (125 μ g/kg BW; surface area equivalent to the dosage of 70 μ g/kg BW in rats). The duration of treatment in rats was in accordance with that used *in vitro*. The duration of treatment in mice was based on a preliminary time-course response analysis (data not shown). Animals were sacrificed 24 h following the last injection. At the time of sacrifice, uteri were removed and the wet uterine weight was used as an indicator of estrogenic response. One hemisphere was fixed in

4% paraformaldehyde for histochemical analyses. The other hemisphere was dissected into hippocampus, cortex and cerebellum and frozen for Western blot and ELISA analyses.

2.5. RT-PCR

Total RNA samples (0.1 µg) were analyzed using semi-quantitative RT-PCR on an AccessQuick RT-PCR system (Promega, Madison, WI). The primer sequence for IDE was 5'-cctcaagactcactcaacg-3', 5'-tagcaaaattggctgtttgt-3'. The RT-PCR procedure consisted of 1) reverse transcription incubation at 45°C for 45 min, 2) initial denaturation at 95°C for 2 min, 3) denaturation at 95°C for 30 s, annealing at 57°C for 45 s, extension at 72°C for 30 s, for 25 cycles, and final extension at 72°C for 10 min. 5 µl of the products derived from each reaction were loaded and separated on a 2% agarose gel. DNA was detected by ethidium bromide staining. The 18s rRNA in the same RNA samples was also analyzed and used as the loading control.

2.6. Western blot

Total protein samples (20–40 µg) were loaded and separated on a 10% SDS-PAGE. Proteins were then transferred to PVDF membranes. Membranes were incubated with the primary antibody, anti-IDE (1:1000, Calbiochem), at 4°C overnight, followed by incubation with the HRP-conjugated secondary antibody (1:10,000, Vector Laboratories, Burlingame, CA) at room temperature (RT) for 1 h. Bands were visualized with a TMB peroxidase kit (Vector Laboratories) or by chemiluminescence using an ECL detection kit (Amersham, Piscataway, NJ). Relative intensities of the immunoreactive bands were quantified using an image digitizing software, Un-Scan-It Version 5.1 (Silk Scientific, Orem, UT). β-tubulin in the same protein samples was also analyzed and used as the loading control.

2.7. Immunocytochemistry

Neurons grown on chamber slides for 7 DIV were fixed in 70% methanol. Neurons were permeabilized in PBS + 0.01% triton X-100 for 5 min prior to incubation with the primary antibody, anti-IDE (1:1000; Calbiochem), at RT for 2 hr, followed by incubation with the FITC-conjugated secondary antibody (1:250; vector Laboratories) at RT for 45 min. Slides were mounted with mounting medium with DAPI and coverslipped. The immunoreactivity was observed under a Zeiss Axiovert 200M Marianas digital microscopy workstation equipped with 3I Slidebook imaging software (Intelligent Imaging Innovations, Denver, CO).

2.8. MultiBrain sectioning

Rat (or mouse) hemispheres were sectioned using MultiBrain processing at NeuroScience Associates (NSA; Knoxville, TN). Briefly, hemispheres were treated with 20% glycerol and 2% DMSO to prevent freeze-artifacts and subsequently embedded together in a gelatin matrix. After curing, the block of embedded hemispheres was rapidly frozen by immersion in isopentane chilled to -70°C with crushed dry ice and mounted on a freezing stage of an AO 860 sliding microtome. The block of rat hemispheres was sectioned at 40 µm in the coronal plane through the hippocampus (Bregma -1.5 to -6.5 mm). The block of mouse hemispheres was sectioned at 35 µm in the coronal plane through the hippocampus (Bregma -1.0 to -4.0 mm). Cut sections were collected sequentially into 12 containers that were filled with antigen preserve solution (50% PBS, 50% ethylene glycol and 1% polyvinyl pyrrolidone, pH 7.0). At the completion of sectioning, each container held a serial set of one-of-every-12th section. Each of the large sections cut from the block is a composite section holding individual sections from each of the hemispheres embedded in each block. With such composite sections, uniformity of staining was achieved across treatment groups.

2.9. Immunohistochemistry

For the triple staining, free-floating rat hemisphere sections were washed with PBS containing 0.1% Triton X-100 (PBST). Nonspecific binding was blocked by incubation in blocking buffer (5% normal goat serum + 0.3% Triton X-100 in PBS) at RT for 1 h. Sections were incubated overnight at 4°C with a mixture of primary antibodies containing monoclonal Anti-MAP2 (1:1000, Sigma) and rabbit polyclonal anti-IDE (1:1000, Calbiochem) in PBST. Sections were then incubated at RT for 1 h with a mixture of secondary antibodies containing FITC-conjugated horse anti-mouse IgG (1:500, Chemicon) and Cy3-conjugated goat anti-rabbit IgG (1:1000, GE Healthcare, Pittsburgh, PA) in PBST. Subsequently, sections were incubated overnight at 4°C with the third primary antibody monoclonal anti-GFAP (1:1000, Chemicon). Sections were then incubated at RT for 1 h with secondary antibody Cy5-conjugated goat anti-mouse IgG (1:1000, GE Healthcare). For the double staining, free-floating mouse hemisphere sections were washed with PBS containing 0.1% Triton X-100 (PBST) and incubated with 88% formic acid for 10 min. Nonspecific binding was blocked by incubation in blocking buffer (3% normal goat serum + 3% normal horse serum + 0.3% Triton X-100 in PBS) at RT for 1 h. Sections were incubated overnight at 4°C with a mixture of primary antibodies containing mouse monoclonal human anti-A β protein clone 6E10 (1:1000, Signet Laboratories, Dedham, MA) and rabbit polyclonal anti-IDE (1:1000, Calbiochem) in PBST. Sections were then incubated at RT for 1 h with a mixture of secondary antibodies containing FITC-conjugated horse anti-mouse IgG (1:500, Vector Laboratories) and Cy3-conjugated goat anti-rabbit IgG (1:500, GE Healthcare) in PBST. Sections were mounted with mounting medium with DAPI and coverslipped. The immunoreactivity was observed under a Zeiss Axiovert 200M Marianas digital microscopy workstation equipped with 3I Slidebook imaging software.

2.10. Campbell-Switzer silver staining of A β plaques

Campbell-Switzer silver staining that labels A β plaques (Campbell, et al., 1987) was performed on a serial set of one-of-every-6th (210 μ m interval) sections (20 sections per animal) of mouse hemispheres. Briefly, the sections were placed in freshly prepared 2% ammonium hydroxide for 5 min. The sections were next placed in a silver-pyridine-carbonate solution for 40 min, 1% citric acid for 3 min, and 0.5% acetic acid until ready for development. The sections were developed in Physical Developer ABC solution (after Gallyas; containing Na carbonate citric acid, tungstosilicic acid and formaldehyde) with the development time being visually assessed. The development was stopped by briefly placing the sections in 0.5% acetic acid. The stained sections were then mounted on gelatinized glass slides and viewed under a Zeiss Axiovert 200M Marianas digital microscopy workstation equipped with 3I Slidebook imaging software. The depth and width/density of plaque load were quantitated and compared between treatment groups. As an indicator of the depth of plaque load, the number of sections affected by plaques was counted for each animal. The percentage of plaques-affected sections out of the total sections was calculated for each treatment group. As an indicator of the width/density of plaque load, 5 representative sections (sections 13–17 for all animals), which covered the fraction of sections loaded with the most of plaques across animals, were chosen, and the area occupied by plaques on these sections were measured using the image processing and analysis software ImageJ (<http://rsbweb.nih.gov/ij/>; developed by Wayne Rasband at NIH, Bethesda, MD). The percentage of plaques-occupied area out of the total area of interest (for instance, the subicular region) was calculated for each treatment group.

2.11. ELISA analyses of A β _{1–42}

A β _{1–42} was quantitated using a solid phase sandwich ELISA system (Biosource, Camarillo, CA). Briefly, brain tissues collected from treated mice were weighed and homogenized in 4x wet mass of cold PBS supplemented with 1x Protease Inhibitor Cocktail (Calbiochem). The

homogenates were mixed with 10 M guanidine HCl/100 mM Tris HCl (pH 8.0) to yield a solution with 5 M final guanidine concentration, and incubated at RT for 4 h. Mixed samples were diluted at 1:10 with cold reaction buffer BSAT-DPBS (Dulbecco's phosphate buffered saline with 5% BSA and 0.03% Tween-20, pH 7.4)) and centrifuged at 16,000 x g at 4°C for 10 min. Supernatants were collected and diluted at 1:5 with sample diluent buffer. 50 µL/well of human Aβ₁₋₄₂ standards and diluted samples were added to a pre-coated 96-well plate with an antibody specific for the NH₂-terminus of human Aβ and incubated with 50 µL/well of detection antibody solution (specific for the COOH-terminus of human Aβ₁₋₄₂) at RT for 3 h. Plate was washed with wash buffer and incubated with 100 µL/well of anti-rabbit Ig's-HRP solution at RT for 30 min. After washing, 100 µL/well of stabilized chromogen were added to the plate and incubated at RT in the dark for 10–30 min. 100 µL/well of stop solution were added to the plate and mixed gently. Optical density was measured at 450 nm. Results were calculated from the standard curve.

2.12. LC/MS/MS Analyses of 17β-Estradiol

17β-estradiol was quantitated using LC/MS/MS at NMS Labs (Willow Grove, PA). Briefly, D4-estradiol was added as the internal standard in acetonitrile to 0.2 mL aliquots of plasma. Samples were mixed and centrifuged to yield clear supernatants that were transferred and analyzed by LC/MS/MS. Instrumental analyses were performed on a Shimadzu HPLC system with a Supelco LC-8, 3.3 cm × 3.0 mm, 3 µm particles analytical column using a gradient mobile phase program. The detector was an Applied Biosystems API 5000 triple quadrupole mass spectrometer, equipped with an ElectroSpray ion source operated in the negative mode. One ion transition was monitored for the internal standard and analyte. Each analytical run was independently calibrated at concentrations of 2, 5, 10, 50, 100 and 500 pg/mL. Three levels of control were run in each analytical batch. This LC/MS/MS method had a LLOQ of 2.0 pg/mL and between run % CV's of 11.53, 4.91, 5.59 % at 20, 100 and 200 pg/mL, respectively.

2.13. Data analyses

Data are presented as group mean ± S.E.M. Statistically significant differences were determined by a one-way analysis of variance followed by a Student-Newman-Keuls post hoc analysis.

3. Results

3.1. 17β-E2 increased IDE mRNA and protein expression in a time-dependent manner in rat primary hippocampal neurons; 17β-E2-induced increase in IDE protein expression was completely abolished in the presence of PI3-K inhibitors

Estrogen regulation of IDE expression was first studied *in vitro* in primary cultures of rat hippocampal neurons. Immunocytochemical staining of neuronal cultures grown for 7 DIV revealed that IDE was abundantly and primarily expressed in the cytoplasm and neural processes of rat hippocampal neurons (Fig. 1A). Results from a time-course analysis demonstrated that neurons treated with 17β-E2 exhibited a time-dependent increase in IDE expression at both the mRNA (Fig. 1B) and protein level (Fig. 1C). In detail, a significant increase in IDE mRNA expression was evident at 6 h, which continued to rise at 12 h and reached the maximum at 24 to 48 h (Fig. 1B; * $P < 0.05$ compared to 0 h). In comparison with the mRNA expression profile, a significant increase in IDE protein expression was evident at a later time point of 12 h, which continued to rise at 24 and 48 h (Fig. 1C; * $P < 0.05$ compared to 0 h). Mechanistic analyses from a 48-h treatment indicated that the presence of either one of two PI3-K inhibitors, wortmannin or LY-294002, was able to completely block 17β-E2-induced increase in IDE protein expression in hippocampal neurons (Fig. 1D; ** $P < 0.01$ compared to vehicle alone-treated control cultures).

3.2. The ER β agonist, DPN, not the ER α agonist, PPT, increased IDE protein expression in rat hippocampal neurons; DPN-induced increase in IDE protein expression was completely abolished in the presence of ER α agonist/ER β antagonist, (R,R)-THC, or PI3-K inhibitors

To determine the role of ER subtypes, ER α /ER β , in mediating estrogen regulation of IDE expression in hippocampal neurons, total protein samples collected from neuronal cultures following a 48-h treatment with the ER α agonist, PPT, or the ER β agonist, DPN, alone or in the presence of PI3-K inhibitors, wortmannin or LY-294002, or the ER α /ER β antagonist, (R,R)-THC, were comparatively analyzed by Western blot analyses. Results shown in Fig. 1E and 1F demonstrated that the ER β agonist DPN induced a significant increase in IDE protein expression in hippocampal neurons (Fig. 1F; * $P < 0.05$ compared to control cultures), whereas the ER α agonist PPT did not show an effect (Fig. 1E). Consistent with the observation in neurons treated with 17 β -E₂, DPN-induced increase in IDE protein expression was completely abolished in the presence of wortmannin or LY-294002 (Fig. 1F). Moreover, DPN-induced increase in IDE protein expression was completely abolished in the presence of the ER α /ER β antagonist, (R,R)-THC (Fig. 1G). Treatment with either PI3-K inhibitors (Fig. 1E, 1F) or (R,R)-THC (Fig. 1G) alone had no effect on the basal level of IDE as exhibited in control cultures.

3.3. Ovariectomy induced a region-specific decline in IDE protein expression in the hippocampus of adult female rats which was prevented by 17 β -E₂

17 β -E₂ regulation of brain IDE expression was further studied *in vivo* in adult female rats. Immunohistochemical labeling of intact rat brain sections revealed that IDE (red) was co-localized with the neuronal marker, MAP2 (green), and not with the glial marker, GFAP (blue), indicating that IDE was expressed in neurons and not in glial cells of adult female rat brain (Fig. 2A). Consistent with the *in vitro* observation, IDE was abundantly expressed in the cytoplasm and neural processes of neurons (Fig. 2A). In the following experiment, rats were ovariectomized (OVX) and treated with vehicle alone or 17 β -E₂ for 2 d. Total protein samples collected from the hippocampus and cerebellum of treated rats were comparatively analyzed by Western blot analyses. Compared to the sham-OVX group, OVX induced a significant reduction in IDE protein expression in the hippocampus (Fig. 2B; ** $P < 0.01$), and not in the cerebellum (Fig. 2C), of adult female rats. Treatment with 17 β -E₂ reversed OVX-induced decline in hippocampal IDE protein expression in a dose-dependent manner (Fig. 2D). A significant increase was observed in OVX rats treated with 17 β -E₂ at 70 and 300 μ g/kg BW, but not at 30 μ g/kg BW (Fig. 2D; ** $P < 0.01$ compared to vehicle alone-treated OVX control rats). Furthermore, results shown in Fig. 2E indicated that 17 β -E₂ regulation of IDE expression is brain region selective, with a significant increase detected in the hippocampus (Fig. 2E; ** $P < 0.01$ compared to OVX control rats), and not in the cerebellum (Fig. 2F), of OVX mice treated with 17 β -E₂ at 70 μ g/kg BW. Table 1 shows the uterine weights of treated rats that are consistent with estrogenic responses in 17 β -E₂-present compared to 17 β -E₂-deprived animals. OVX induced a nearly 50% reduction in uterine weight compared to sham-OVX rats (Table 1; * $P < 0.05$). There was no significant difference between sham-OVX and 17 β -E₂-treated OVX rats, indicating that 17 β -E₂ at 70 μ g/kg BW mimicked the endogenous level of estrogen as seen in sham-OVX rats (Table 1; ** $P < 0.01$ compared to OVX rats).

3.4. An age-related increase in A β accumulation and plaque formation was accompanied with an overall decrease in IDE protein expression in the hippocampus of 3xTg-AD female mice

To investigate the temporal relationship between brain A β load, plaque formation and IDE protein expression, hippocampal homogenates and hemisphere sections collected from 3xTg-AD female mice at 5 consecutive ages of 3, 6, 9, 12 and 18 months were comparatively analyzed. LC/MS/MS revealed that the endogenous levels of 17 β -E₂ in

plasma of these genetically modified mice were similar to those in age-matching normal mice, with an average level of 10~15 pg/mL at the proestrous stage and a level of < 2 pg/mL at non-proestrous stages of the cycle, in 3 to 12-month-old mice (Table 2). As expected, 18-month-old mice had a much lower high-end level of 17 β -E2 at 5.8 pg/mL and a similar low-end level of < 2 pg/mL (Table 2). Campbell-Switzer silver staining revealed a well-defined temporal-spatial pattern in the formation of plaques in these mice. Specifically, no plaque was formed at 3, 6 and 9 months of ages (Fig. 3A & 3B). At the age of 12 months, plaques began to form in select brain regions. Of 20 sections collected at 210 μ M intervals throughout the hippocampus, plaque formation was primarily concentrated in the subicular region (Fig. 3A). At the age of 18 months, substantial plaques were observed throughout all sections spanning across the entire hippocampus and some regions of the cortex notably within the amygdalar complex (Fig. 3A). In addition, a more neurotoxic form of mature plaques stained in amber (Campbell, et al., 1987) was observed in these aged AD mice (Fig. 3A, high-magnitude image). Consistent with plaque formation, ELISA measurement of total A β ₁₋₄₂ content in the hippocampus confirmed the age-related development of A β pathology in 3xTg-AD female mice. Although no plaque formation was observed at the age of 9 months, there was a moderate increase in intraneuronal A β ₁₋₄₂ content in the hippocampus of mice at this age (Fig. 3B). The hippocampal A β ₁₋₄₂ content continued to increase significantly at ages of 12 months (Fig. 3B; ** P < 0.01 compared to 9 months) and 18 months (Fig. 3B; ** P < 0.01 compared to 9 and 12 months).

Contrary to the age-related increase in A β ₁₋₄₂ accumulation and plaque formation, IDE protein expression in the hippocampus of 3xTg-AD female mice decreased overall with age (Fig. 3C). Western blot analyses of the same protein samples used in ELISA analyses revealed a moderate reduction in IDE protein expression in the hippocampus as early as 6 months, which reached the statistical significance with the maximal reduction at 12 months (Fig. 3C; ** P < 0.01 compared to 3, 6 and 9 months). The unexpected higher expression level of IDE protein observed in the hippocampus of 18-month-old mice than the expression level in 12-month-old mice could be attributed to a potential compensatory response to the drastic elevation in plaque formation occurred at 18 months (Fig. 3C; * P < 0.05). Immunohistochemical analyses of hippocampal sections confirmed the above observations. A β immunoreactivity labeled with 6E10, which indicated both intraneuronal deposition and extraneuronal plaques, increased with age. IDE immunoreactivity was decreased in 12-month-old compared to 9-month-old mice; while it was increased, particularly in the vicinity of plaques, in 18-month-old compared to 12-month-old mice (Fig. 3D; CA1 region is displayed).

3.5. 17 β -E2 attenuated A β accumulation/plaque formation and elevated IDE protein expression in the hippocampus of 12-month-old 3xTg-AD female mice

To determine the impact of estrogen on IDE expression in association with A β accumulation and plaque formation in a human AD-like state, 12-month-old 3xTg-AD female mice were OVX and treated with vehicle alone or 17 β -E2 at 125 μ g/kg BW (surface area equivalent to the dosage of 70 g/kg BW in rats) for 4 d. Hippocampal homogenates and hemisphere sections were comparatively analyzed. Campbell-Switzer silver staining revealed that compared to gonadally intact mice at this age (Fig. 3A), OVX mice exhibited both an elevated plaque formation in the subicular region and further spread of plaques in the hippocampus and amygdalar complex (Fig. 4A). Moreover, OVX mice had a much greater load of A β ₁₋₄₂ in hippocampal homogenates, ~150 pg/mg (Fig. 4A) compared to ~90 pg/mg in intact mice (Fig. 3A). Compared to OVX mice treated with vehicle alone, 17 β -E2 treatment significantly attenuated plaque formation as evidenced by both the fewer sections affected by plaques (Fig. 4B; * P < 0.05) and smaller areas occupied by plaques on each section (Fig. 4C; * P < 0.05). Consistent with the Campbell-Switzer staining, the total

A β ₁₋₄₂ content in hippocampal homogenates was significantly lower in 17 β -E2-treated OVX mice than the vehicle alone group (Fig. 4D; * $P < 0.05$ compared to OVX control mice). Furthermore, Western blot analyses indicated that 17 β -E2-treated OVX mice had a greater level of IDE protein in the hippocampus relative to OVX mice treated with vehicle alone (Fig. 4E; ** $P < 0.01$). Immunohistochemical analyses confirmed the above observations. A β immunoreactivity labeled with 6E10 was decreased, which was accompanied by an increase in IDE immunoreactivity, in hippocampal sections of OVX mice treated with 17 β -E2 compared to OVX control mice (Fig. 4F; CA1 and CA2 regions are displayed). Similar to the effect in rats, 17 β -E2 treatment induced a nearly 2-fold increase in uterine weight compared to vehicle alone-treated OVX control mice (Table 3; * $P < 0.05$).

4. Discussion

The present study in both *in vitro* neuronal cultures and *in vivo* menopausal and AD animal models provides the first documentation that estrogen regulates IDE expression in the hippocampus. Such a role could serve as a direct mechanism underlying estrogen-mediated preventative effect against A β accumulation/plaque formation and further reduction of AD risk.

4.1. Estrogen regulation of IDE expression involves ER β /PI3-K in hippocampal neurons

Estrogen regulation of brain IDE expression was first evidenced in rat hippocampal neuronal cultures. 17 β -E2 robustly increased IDE in both mRNA and protein expression in a time-dependent manner. Further, the ER β agonist, DPN, but not the ER α agonist, PPT, exerted a similar effect but at a lesser magnitude, a ~25% increase as compared to a ~125% increase induced by 17 β -E2. This difference in potency on IDE between 17 β -E2 and DPN appears to correspond well with their binding affinities for ER β . DPN is an ER β -selective agonist, however, its binding affinity for ER β is only about 20% of 17 β -E2 (Meyers, et al., 2001). These data indicate that ER subtypes may play a differential role in IDE metabolic cascades; ER β , but not ER α , appears to be required for estrogenic regulation of IDE in hippocampal neurons. This finding is further supported by the data on (*R,R*)-THC, an ER α agonist and ER β antagonist that has an ER β -binding affinity of 25% of 17 β -E2 (Sun, et al., 1999). Consistent with the data on PPT, (*R,R*)-THC alone did not exert a significant effect on IDE, confirming that ER α is not involved in estrogenic regulation of IDE in hippocampal neurons. When co-administered with DPN, (*R,R*)-THC completely abolished DPN's effect, providing additional support for a crucial role played by ER β in mediating estrogenic regulation of IDE in hippocampal neurons. Further investigation of the downstream signaling events revealed that the presence of PI3-K inhibitors completely blocked both 17 β -E2 and DPN-induced effect, suggesting that ER β -mediated regulation of IDE expression requires activation of PI3-K in hippocampal neurons.

In a larger context, these findings parallel other studies demonstrating the coupling of estrogen-mediated actions with ER β /PI3-K signaling pathways in the brain. First, the abundant expression of ER β in the brain is in line with its functional significance in mediating, exclusively in some instances, estrogen regulation of a variety of neural functions (Zhao and Brinton, 2006; Zhao and Brinton, 2007a; Zhao, et al., 2004b). Functional significance of ER β is also supported by findings from clinical studies in which genetic variations of ER β were found to increase the risk of AD in women but not in men (Pirkanen, et al., 2005). Furthermore, 17 β -E2 activation of PI3-K leads to the coordinated activation of Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) (Mannella and Brinton, 2006). Both pathways contribute to estrogen-induced neuronal survival against toxic insults from A β ₁₋₄₂ (Brinton, et al., 2000; Honda, et al., 2000; Zhang, et al., 2001). Additionally, 17 β -E2 increases the brain content of insulin-like growth factor 1 (IGF-1) via

activation of PI3-K/Akt (Cheng, et al., 2001; Garcia-Segura, et al., 2007). Increased IGF-1 can further lead to the accelerated clearance of A β out of the brain (Carro, et al., 2002).

4.2. Estrogen regulation of brain IDE expression is “regional” rather than “global” which embodies a “demand” and “supply” relationship

Estrogen regulation of brain IDE was further demonstrated *in vivo* in adult female rats. Results indicated that compared to sham-OVX animals, OVX induced a significant decline in IDE protein expression in select brain regions, providing indirect support for a crucial role of estrogen on IDE expression in brain. A similar deficiency in brain IDE expression associated with loss of estrogen was also found in APP23/Ar^{+/-} mice, a mouse line derived from the crossing of APP23 mice and estrogen-synthesizing enzyme aromatase gene knockout mice (Yue, et al., 2005).

Moreover, the data revealed that OVX-induced decline in IDE expression occurred in the hippocampus and not in the cerebellum of adult female rats. Treatment of OVX rats with 17 β -E2 exerted a similar brain region-specific effect on IDE. These observations are of particular interest because they may suggest that estrogen regulation of brain IDE expression is a “regional” rather than a “global” effect. This brain region-selective profile in response to OVX is to a large extent consistent with clinical observations that also indicate a greater decline in IDE expression and/or activity as a function of age or AD in the hippocampus as compared to other brain regions (Caccamo, et al., 2005; Zhao, et al., 2007). It is well known that in both rodent and human brains, hippocampus is the most critical region responsible for a variety of brain functions including learning and memory, it is also the most vulnerable region that suffers from AD. In contrast, cerebellum is usually refractory to AD. Therefore, the dichotomy in regional regulation of IDE expression by estrogen that parallels AD pathology, underlies a “demand” and “supply” relationship.

If the *in vitro* signaling cascade is replicated *in vivo*, it can be hypothesized that the regional variation in estrogenic regulation of IDE expression between hippocampus and cerebellum could originate from regional variation in ER β expression or downstream events that differ in these brain regions. Both ER α and ER β are expressed in neurons and glial cells in the hippocampus (Shughrue, et al., 1997; Shughrue, et al., 2000). In the cerebellum, despite that several studies have consistently reported ER β and not ER α detected, it remains controversial with respect to ER β localization in cerebellar neurons (Price and Handa, 2000) or primarily in glia (Struble, et al., 2003). If it is true that ER β is also abundantly expressed in cerebellar neurons, the observation that ER β is not coupled with IDE regulation in the cerebellum as it is in the hippocampus may suggest that differences in the downstream signaling may underlay the variation in estrogenic responses in these two largely distinctive brain regions.

4.3. Estrogen regulation of brain IDE expression may serve as a direct mechanism in the prevention of AD

In addition to normal brains, estrogen also regulated IDE expression in brains derived from AD mice. In agreement with an earlier report (Caccamo, et al., 2005), an inverse association between the age-related increase in A β accumulation/plaque formation and the decrease in IDE expression was revealed in the hippocampus of 3xTg-AD female mice. The unexpected observation of an elevated IDE in the hippocampus of 18-month-old compared to 12-month-old AD mice, could result from a compensatory response to the drastically increased formation of plaques that may have recruited more IDE or triggered an increased expression of IDE in order to offset the increased production of A β . A comparable elevation of IDE was found in the brains of aged Tg2576 mice where IDE was overly expressed in the vicinity of plaques (Leal, et al., 2006). Together, these observations support the notion that the gradual

reduction in brain IDE could be a major contributory factor leading to progression of A β deposition in AD.

More importantly, the age-related characterization provided the critical guidance for choosing a possibly best window of time for the following experiment on estrogen intervention. At the age of 12 months, 3xTg-AD mice exhibited a substantial accumulation in A β_{1-42} in hippocampal tissues, with a total content of ~90 pg/mg as compared to the base level of < 5 pg/mg at ages of 3 and 6 months and a level of ~20 pg/mg at the age of 9 months. However, since mice at this age just began to form a small amount of plaques in the extracellular space concentrated in the subicular region, it can be speculated that the large percentage of A β_{1-42} deposited in the hippocampal formation could be still intracellular. Based on these analyses, we believed that this age could serve as an effective window of time for investigation of the potential association of estrogen-mediated dual effect on IDE and AD pathology. Not surprisingly, the data indicated that OVX accelerated the progression of A β deposition as demonstrated by both a significant increase in hippocampal A β_{1-42} at a level of ~150 pg/mg and formation of a greater amount of and more spread plaques. Treatment of 12-month-old OVX mice with 17 β -E2 attenuated both markers to the levels as seen in age-matching intact animals. Additionally, a significantly elevated level of IDE was detected in the same hippocampal protein samples used for A β_{1-42} analyses in 17 β -E2-treated mice. Together, these data support that in addition to its role in a normal brain, estrogen also regulates IDE in an at least early-stage AD brain. The inverse changes in IDE expression and A β deposition in response to 17 β -E2 may suggest that estrogen-induced increased IDE could have enhanced degradation of intracellular A β leading to reduced extracellular plaque formation.

It should be noted that the reduction in A β deposition resulted from 17 β -E2 treatment was quite rapid. As demonstrated, a 4-d treatment with 17 β -E2, with one injection daily, induced a ~35% reduction in hippocampal A β_{1-42} content and a ~63% reduction in extracellular plaque formation in the subicular region of the brains of treated 3xTg-AD mice. Such a rapid effect could be tightly coupled with 17 β -E2's rapid effect on IDE and the subsequent IDE-mediated rapid A β degradation. The present investigation in both *in vitro* and *in vivo* models revealed that 17 β -E2 rapidly increased brain IDE protein levels after a 12 to 48-h treatment. A number of recent studies indicate that IDE cleaves APP-derived peptides including A β and APP intracellular domain at multiple sites and that the velocity of the reaction is extremely high (Chesneau, et al., 2000; Edbauer, et al., 2002; Mukherjee, et al., 2000). For instance, in an *in vitro* experiment, purified recombinant wild-type human IDE degraded about 75% of A β following just a 1-h co-incubation (Chesneau, et al., 2000). These findings suggest again the significance of the role played by estrogen on brain IDE, and such a role could serve as a key contributory mechanism underlying estrogen-mediated prevention of AD. Menopausal loss of ovarian estrogens could lead to IDE deficiency, which could further lead to an impaired A β catabolism and increased risk of AD. Moreover, it should be noted that despite the findings presented in this report, the possible impact of estrogen in older mice remains unresolved. Current evidence indicates that IDE preferentially degrades small peptides of about 20–50 amino acids in length (Authier, et al., 1996). Specifically, IDE is selective for monomeric A β and has no effect on aggregated forms such as those deposited onto plaques (Mukherjee, et al., 2000). Therefore, if it holds true that estrogen-mediated anti-A β effect relies heavily on IDE, it can be speculated that in agreement with many predictions stated by others in the field (see our recent reviews (Brinton, 2008; Zhao and Brinton, 2007b)), timing could indeed be a critical issue in order to reap estrogen's beneficial effect against AD. Estrogen could be efficacious when used prior to or at the onset of plaque formation, to accelerate the hydrolysis of monomeric A β before they deposit onto plaques, leading to prevention of the formation and growth of plaques; whereas it could be ineffective in degrading/clearing plaques.

Genetic variants of IDE have been associated with AD in populations with different demographic backgrounds (Bian, et al., 2004; Bjork, et al., 2007; Edland, et al., 2003; Vepsäläinen, et al., 2007). In some instances, the association appears to be dependent upon apolipoprotein E (APOE) genotypic status (Bian, et al., 2004; Edland, et al., 2003). For instance, a genetic association study in the Han Chinese indicated that the association between IDE polymorphism and AD was confined to APOE-ε4 carriers only (Bian, et al., 2004), although opposite results were reported in different populations (Edland, et al., 2003). Future studies to address the question whether estrogen regulation of IDE is modulated by APOE genotype would provide insights into understanding the clinical effect of estrogen therapy on cognitive outcomes in APOE-ε4 negative postmenopausal women (Burkhardt, et al., 2004; Yaffe, et al., 2000). Moreover, recent evidence indicates a positive association between type 2 diabetes and AD (Taubes, 2003; Zhao, et al., 2004a) – both diseases are prevalent in menopausal/postmenopausal women (Revis and Keene, 2007; Yaffe, et al., 2004). Therefore, future studies to address the role of IDE, the key catabolic mechanism for both insulin and Aβ (Bertram, et al., 2000; Ghosh, et al., 2000), in the development of type 2 diabetes and AD, would provide insights into the development of therapeutic strategies against the risk of both diseases in menopausal/postmenopausal women.

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Figure 1.

17 β -E2 increased IDE expression via an ER β /PI3-K pathway in rat hippocampal neurons. (A) Immunocytochemical staining revealed that IDE (green) was abundantly and primarily expressed in the cytoplasm and neural processes of rat hippocampal neurons. (B, C) Treatment with 17 β -E2 (10 nM for 0–48 h) increased IDE expression in both mRNA and protein in a time-dependent manner in rat hippocampal neurons; * $P < 0.05$ compared to 0 h. (D) 17 β -E2 (10 nM for 48 h)-induced increase in IDE protein expression was completely abolished in the presence of PI3-K inhibitors, wortmannin (50 nM) or LY-294002 (25 μ M); ** $P < 0.01$ compared to vehicle alone-treated control cultures. (E, F) Treatment with the ER β agonist, DPN (10 nM for 48 h), not the ER α agonist, PPT (10 nM for 48 h), increased IDE protein expression in rat hippocampal neurons, which was completely abolished in the presence of wortmannin (50 nM) or LY-294002 (25 μ M); * $P < 0.05$ compared to control cultures. (G) Treatment with the ER α agonist/ER β antagonist, (R,R)-THC (100 nM for 48 h), alone did not induce a significant effect; however, when co-administered, (R,R)-THC completely abolished DPN-induced increase in IDE protein expression in rat hippocampal neurons. 18s rRNA and β -tubulin were respectively used as RNA and protein loading controls. Data are presented as (B, C) percent of 0 h and (D–G) percent of vehicle alone-treated control groups and expressed as group mean $\pm$ S.E.M., $n \geq 3$. Wort: wortmannin; LY: LY-294002.

Figure 2.

17 β -E2 reversed OVX-induced decline in IDE protein expression in the hippocampus of adult female rats. (A) Immunohistochemical triple staining revealed that IDE (red) was co-localized with the neuronal marker, MAP2 (green), and not with the glial marker, GFAP (blue), indicating that IDE was expressed in neurons and not in glial cells of adult female rats. (B, C) OVX induced a significant reduction in IDE protein expression in the hippocampus, and not in the cerebellum, of adult female rats; ** $P < 0.01$ compared to sham-OVX rats. (D) Treatment with 17 β -E2 (30, 70, and 300 μ g/kg BW, once daily for 2 d) increased, in a dose-dependent manner, IDE protein expression in the hippocampus of OVX rats; ** $P < 0.01$ compared to vehicle alone-treated OVX rats. (E, F) Treatment with 17 β -E2 (70 μ g/kg BW, once daily for 2 d) increased IDE protein expression in the hippocampus, and not in the cerebellum, of OVX rats; ** $P < 0.01$ compared to vehicle alone-treated OVX

rats. β -tubulin was used as the loading control. Data are presented as (**B**, **C**) percent of sham-OVX (**D–F**) percent of vehicle alone-treated control groups and expressed as group mean $\pm$ S.E.M., $n \geq 4$.

Figure 3.

The age-related increase in Aβ₁₋₄₂ accumulation and plaque formation was accompanied with the decrease in IDE protein expression in the hippocampus of 3xTg-AD female mice. (A) A total of 20 hippocampal sections collected at 210 μm intervals from one hemisphere of 3, 6, 9, 12 and 18-month-old 3xTg-AD female mice were stained with the Campell-Switzer silver stain, which labels diffuse plaques in black and a more mature form of plaques in amber. (Left) Montage images revealed the temporal-spatial pattern of plaque formation that began and was concentrated in the subicular region in 12-month-old AD mice, which was significantly increased in 18-month-old AD mice. (Right) Upper panel: 10x views of plaque formation in the subicular region; Lower panel: 40x close-up views of a mature form of plaques stained in amber observed in 18-month-old AD mice. (B) ELISA data revealed an age-related increase in Aβ₁₋₄₂ accumulation in the hippocampus of 3xTg-AD female mice; ** $P < 0.01$. (C) Western blot data revealed an age-related overall reduction in IDE protein expression in the hippocampus of 3xTg-AD female mice. Data are presented as the percent of IDE expression in 3-month-old AD mice; # $P < 0.05$ and ## $P < 0.01$ compared to 18-month-old mice; * $P < 0.05$ compared to 12-month-old mice; ** $P < 0.01$ compared to 3, 6, and 9-month-old mice; (D) Immunohistochemical staining revealed changes in immunoreactivity for Aβ (green) and IDE (red) that are consistent with observations from ELISA and Western blot analyses. β-tubulin was used as the loading control. Data are expressed as group mean ± S.E.M., $n \geq 4$.

Figure 4.

17β-E2 attenuated Aβ₁₋₄₂ accumulation and plaque formation, which was accompanied with an increase in IDE protein expression, in the hippocampus of 12-month-old 3xTg-AD female OVX mice. **(A)** A total of 20 hippocampal sections collected at 210 μm intervals were stained with the Campbell-Switzer silver stain. The staining revealed a significant reduction in plaque formation in OVX mice treated with 17β-E2 compared to vehicle alone-treated OVX mice. **(B)** A significantly lower percentage of sections was affected by plaques in OVX mice treated with 17β-E2 compared to vehicle alone-treated OVX mice; * $P < 0.05$. **(C)** A significantly smaller area in the subicular region (defined by box 1) was occupied by plaques in OVX mice treated with 17β-E2 compared to vehicle alone-treated OVX mice; *

$P < 0.05$. **(D)** ELISA data revealed a significant reduction in $A\beta_{1-42}$ accumulation in the hippocampus of OVX mice treated with 17β -E2 compared to vehicle alone-treated OVX mice; * $P < 0.05$. **(E)** Western blot data revealed a significant increase in IDE protein expression in the hippocampus of OVX mice treated with 17β -E2 compared to vehicle alone-treated OVX mice; ** $P < 0.01$. **(F)** Consistent with ELISA and Western blot data, immunohistochemical staining revealed a lower intensity of $A\beta$ immunoreactivity (green) and a higher intensity of IDE immunoreactivity (red) in hippocampal sections of OVX mice treated with 17β -E2 than showed in sections of vehicle alone-treated OVX mice. β -tubulin was used as the loading control. Data are expressed as group mean $\pm$ S.E.M., $n \geq 4$.

Table 1

Treatment effect on uterine weight in adult female rats

Treatment Group	Uterine Weight ^a
Sham-OVX (vehicle)	190.6 ± 26.3 *
OVX (vehicle)	100.0 ± 6.8
OVX_E2 (70 µg/kg BW)	231.9 ± 17.5 **

^aData are presented as percent of vehicle alone-treated OVX rats and expressed as group mean ± S.E.M., n ≥ 4.

* $P < 0.05$ and

** $P < 0.01$ compared to vehicle alone-treated OVX rats. There is no significant difference between sham-OVX (vehicle) and OVX_E2 (70 µg/kg BW) groups.

Table 2Plasma levels of 17 β -E2 in 3xTg-AD female mice

3xTg-AD Female Mice	17 β -E2 (pg/mL) ^a
3-Month	10.2 ^b / _{< 2} ^c
6-Month	13.0/ _{< 2}
9-Month	12.7/ _{< 2}
12-Month	13.3/ _{< 2}
18-Month	5.8/ _{< 2}

^aData are expressed as group mean, n $\geq$ 4.^bThe value reflects the average level at the proestrous stage.^cThe value reflects the average level at non-proestrous stages.

Table 3

Treatment effect on uterine weight in 12-month-old 3xTg-AD female mice

Treatment Group	Uterine Weight ^a
OVX (vehicle)	100.0 ± 26.2
OVX_E2 (125 µg/kg BW)	179.3 ± 16.0 *

^aData are presented as percent of vehicle alone-treated OVX mice and expressed as group mean ± S.E.M., n ≥ 4.

* $P < 0.05$ compared to vehicle alone-treated OVX mice.