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# A priming role of local estrogen on exogenous estrogen-mediated synaptic plasticity and neuroprotection

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Abbreviations: Arc, activity-regulated cytoskeleton associated protein; Bcl2, B cell lymphoma 2; DPN, estrogen receptor β agonist; E2, estrogen; ER, estrogen receptor; GnRH, gonadotrophin releasing hormone; $H_2O_2$, hydrogen peroxide; OVX, ovarectomized; PI-3K, phosphoinositide kinase-3; PPT, estrogen receptor α agonist; PSD-95, postsynaptic density protein-95; Wort, wortmannin

## Abstract

**The localization of estrogen (E2) has been clearly shown in hippocampus, called local hippocampal E2. It enhanced neuronal synaptic plasticity and protected neuron form cerebral ischemia, similar to those effects of exogenous E2. However, the interactive function of hippocampal and exogenous E2 on synaptic plasticity activation and neuroprotection is still elusive. By using hippocampal H19-7 cells, we demonstrated the local hippocampal E2 that totally suppressed by aromatase inhibitor anastrozole. Anastrozole also suppressed estrogen receptor (ER)β, but not ERα, expression. Specific agonist of ERα (PPT) and ERβ (DPN) restored ERβ expression in anastrozole-treated cells. In combinatorial treatment with anastrozole and phosphoinositide kinase-3 (PI-3K) signaling inhibitor wortmannin, PPT could not improve hippocampal ERβ expression. On the other hand, DPN induced basal ERβ translocalization into nucleus of anastrozole-treated cells. Exogenous E2 increased synaptic plasticity markers expression in H19-7 cells. However, exogenous E2 could not enhance synaptic plasticity in anastrozole-treated group. Exogenous E2 also increased cell viability and B-cell lymphoma 2 (Bcl2) expression in $H_2O_2$-treated cells. In combined treatment of anastrozole and $H_2O_2$, exogenous E2 failed to enhance cell viability and Bcl2 expression in hippocampal H19-7 cells. Our results provided the evidence of the priming role of local hippocampal E2 on exogenous E2-enhanced synaptic plasticity and viability of hippocampal neurons.**

**Keywords:** estrogen receptor beta; estrogens; hippocampus; neuronal plasticity; neurons; neuroprotective agents

## Introduction

E2 is mainly synthesized in the gonad, called gonadal or exogenous E2, and reaches its target organ *via* blood circulation. It has been reported to influence memory function (Henderson, 2010; Gorenstein *et al*., 2011) and neuroprotection (McCullough *et al*., 2003) through estrogen receptor (ER), which highly expressed in cerebral cortex and hippocampus (Henderson, 2010). In menopausal women, E2 replacement therapy relieved memory impairment (Gorenstein *et al*., 2011). Previous study demonstrated the decreasing of hippocampal dendritic spine in ovarectomized (OVX) rats that restored by an administration of exogenous E2 (Gould *et al*., 1990). During estrous cycle, the hippocampal spine density is varied in response to fluctuating level of E2 in female rats (Woolley and McEwen, 1992). Moreover, our previous studies demonstrated that a variety of synaptic marker proteins is up-regulated after exogenous E2 application (Chamniansawat and Chongthammakun, 2009; 2010), confirming the positive role of exogenous E2 on synaptic plasticity and memory consolidation. E2 also prevents neuronal death from ischemic brain injury (Dubal *et al*., 1999). It regulated neuronal viability through Bcl2 expression, but not other members of Bcl2 family (Dubal *et al*., 1999).

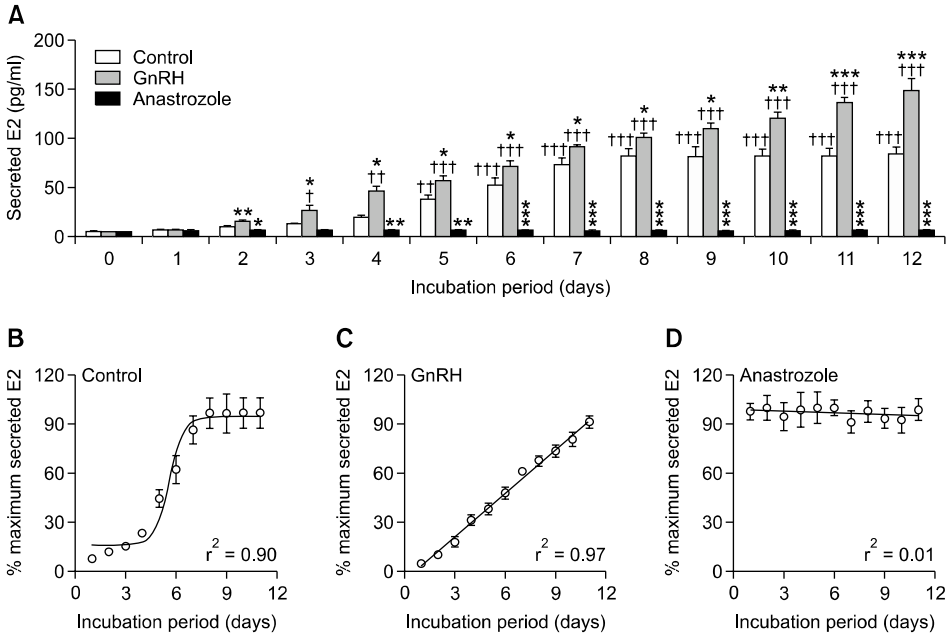


**Figure 1.** Secreted local E2 in culture media of hippocampal H19-7 neurons. Secreted E2 of control (open bars), GnRH-treated (gray bars) and anastrozole-treated (black bars) in H19-7 hippocampal cells (A). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs* day-matched control group, †*P* < 0.05, ††*P* < 0.01, †††*P* < 0.001 *vs* corresponding day 0 group. Percent maximum secreted E2 of control (B), GnRH-treated (C) and anastrozole-treated (D) in H19-7 hippocampal cells. Dark line represented the best-fitted sigmoid line (non-liner regression) in B and straight line (linear regression) in C and D (*n* = 5).

In addition to gonad, endogenous E2 production and secretion in hippocampus were well demonstrated (Prange-Kiel and Rune, 2006). The presence of all enzymes responsible for endogenous E2 biosynthesis and a sixfold higher E2 concentration in hippocampus than that in plasma (Hojo *et al*., 2004) strongly indicate the potential roles of local hippocampal E2 on hippocampal functions. Previous studies revealed that inhibition of the local E2 biosynthesis by aromatase inhibitor significantly reduced the density of hippocampal synapses and down-regulated synaptic proteins, including spinopholin and synaptophysin (Kretz *et al*., 2004; Mukai *et al*., 2010; Zhou *et al*., 2010). Local hippocampal E2 also regulated the expression of ER (Murata *et al*., 2003; Prange-Kiel *et al*., 2003; Oliveira *et al*., 2004). In addition to synaptic plasticity, the neuroprotective role of local hippocampal E2 had been demonstrated by using aromatase knockout mice (McCullough *et al*., 2003). Loss of endogenous or local E2 exhibited an increase in the severity of ischemic injury compared with the normal as well as OVX mice. These evidences indicated that the neuronal functions are mainly affected by local hippocampal E2. However, the decreased in synaptic plasticity was presented in OVX rats (Woolley and McEwen, 1992), which local hippocampal E2 should be intact. Therefore, hippocampal neuronal function is depended on both endogenous and exogenous E2. We hypothesized in the present study that the mechanism of exogenous E2 action is an endogenous E2-dependent manner. The aims of this study were to investigate the effects and mechanisms of endogenous E2 action on the modulation of synaptic plasticity and neuroprotection in hippocampal H19-7 cells.

## Results

### Local E2 production in hippocampal H19-7 cells

To determine the production and secretion of E2 in hippocampal H19-7 cells, we measured E2 concentration in culture media of untreated, gonadotrophin releasing hormone (GnRH)-treated or anastrozole-treated hippocampal H19-7 cells by using ELISA kit. At day 0, basal E2 levels of untreated, GnRH-treated, and anastrozole-treated cells were 5.46 ± 0.51, 5.11 ± 0.25, and 4.89 ± 0.25 pg/ml, respectively (Figure 1A). In untreated hippocampal H19-7 cells, secreted E2 significantly increased from days 5 through 12 (37.52 ± 4.58 pg/ml to 80.04 ± 7.40 pg/ml) (Figure 1A). Maximum secreted E2 level in untreated hippocampal H19-7 cells was comparable to those report in hippocampal slice cultures (Kretz *et al*., 2004). GnRH increased hippocampal E2 secretion from days 3 through 12 (26.81 ± 4.55 to 148.72 ± 12.47 pg/ml) (Figure 1A). Moreover, when compared with the untreated group, GnRH significantly increased E2 levels from days 2 through 12 (Figure 1A). However, when the activity of aromatase was inhibited by anastrozole, the E2 levels were not changed throughout 12 days of experimental period.

The hippocampal E2 secretion profile of control, GnRH-treated, and anastrozole-treated hippocampal H19-7 cells were demonstrated by the percent

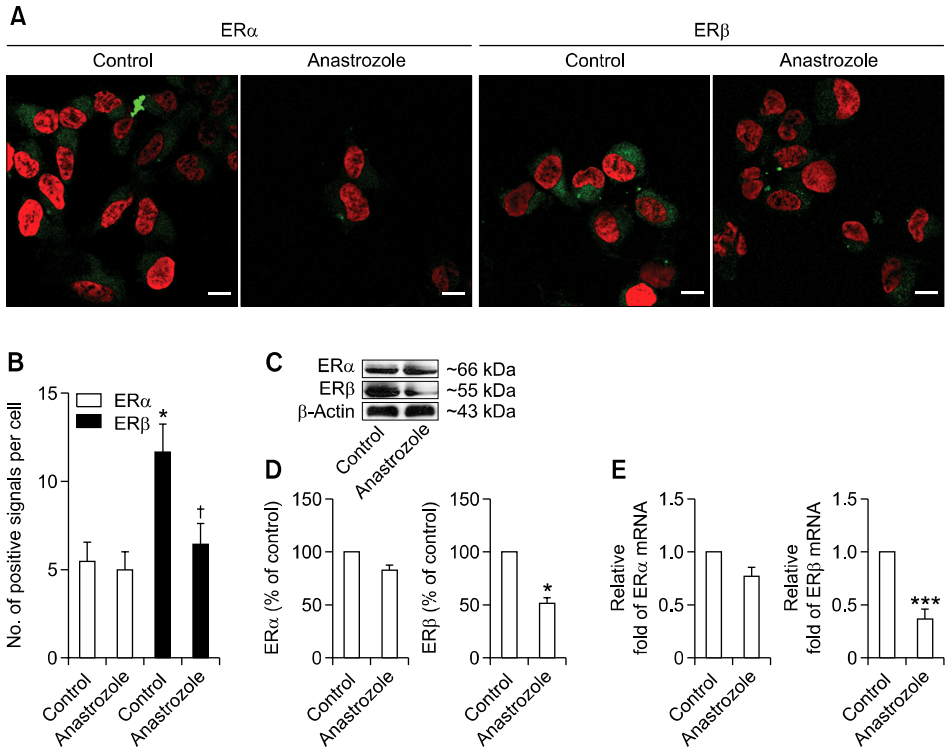


**Figure 2.** Endogenous H19-7 hippocacampal E2 regulates ERβ expression. Representative immunofluorescent images of ERα and ERβ expression of control, and anastrozole-treated H19-7 hippocampal cells (A). Red signal represented nuclear-staining and green signal represented ERα or ERβ (scale bars = 10 μm). The number of ERα or ERβ positive green signal was counted and presented (B). Quantitative immunoblot analysis of ERα and ERβ proteins expression in control and anastorzole-treated H19-7 hippocampal cells (C, D). Representative qRT-PCR of *ERα* and *ERβ* mRNA expression in H19-7 hippocampal neurons (E). β-actin was the housekeeping protein. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs* control group, $^{\dagger}P < 0.05$ *vs* anastrozole-treated group ($n = 5$).

maximum secreted E2-experimental period relationship. The data of control cells were best fitted with the non-liner regression equation that revealed the sigmoidal line ($r^2 = 0.90$, Figure 1B). In the first three days, E2 levels slowly increased, while it rapidly increased from 4$^{th}$ day and to the maximum level in 8$^{th}$ day (Figure 1B). The half maximum E2 secretion was presented in 5$^{th}$ day to 6$^{th}$ day and the maximum E2 secretion with the plateau phase was showed in day 8 though 12 (Figure 1B). Therefore, we selected 8$^{th}$ day as a suitable culture condition in next experiments for studied the effect of local E2 on hippocampal H19-7 neuron. GnRH changed the secretory profile form sigmoid function to linear function ($r^2 = 0.97$, Figure 1C). The E2 levels of GnRH-treated group rapidly increased from day 1 through 12 (Figure 1C), whereas E2 levels of anastrozole-treated group showed relatively constant throughout 12 days of experimental period (Figure 1D).

## Local E2 regulates ERβ expression in hippocampal H19-7 cells

It is well known that ER, including ERα and ERβ, expression is upregulated by E2 (Murata *et al*., 2003; Oliveira *et al*., 2004). We therefore investigated the effects of the local E2 on ER expression by using the confocal immunocytochemistry, qRT-PCR, and Western blot techniques. To determine those effects, hippocampal H19-7 cells were cultured in either steroid-deprived media with or without anastrozole for 8 days. In control group, ERβ expression was significantly higher than ERα (Figures 2A-2D). In the presence of anastrozole, the level of ERβ expression was significantly decreased, whereas ERα expression was not changed (Figures 2A-2E). These results indicated that the local E2 up-regulated ERβ, but not ERα, expression in hippocampal H19-7 cells.

## Local E2-increased ERβ expression is a basal ERα- and ERβ-dependent mechanism

We further examined the underlying mechanism of local E2-induced ERβ expression by using qRT-PCR and Western blotting analysis. Hippocampal H19-7 cells exposed to anastrozole for 8 days had significantly lower ERβ expression than control group (Figures 3A-3C). The level of ERβ expression could be restored by administration of specific agonist of ERα (100 nM PTT) and ERβ (100 nM DPN) in day 7$^{th}$ of experimental period. This result indicates that the local E2-induced ERβ expression is a basal ERα and ERβ-dependent mechanisms. Wortmannin (200 nM Wort.), a specific PI-3K inhibitor, had no additive effect on anastrozole-suppressed ERβ expression (Figures 3A-3C). Wortmannin attenuated PPT-, but not DPN-, induced ERβ expression. Therefore, local hippocampal E2

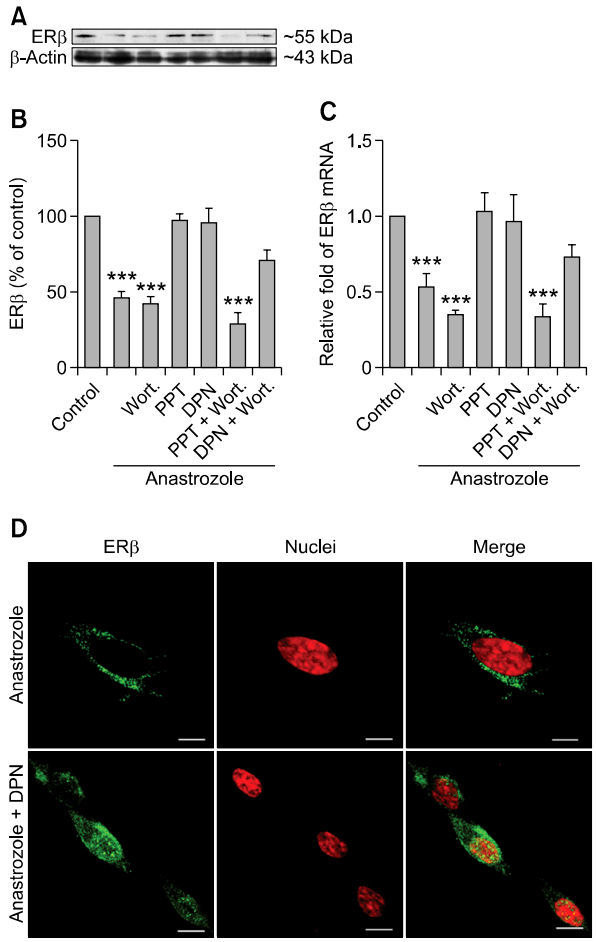


**Figure 3.** The mechanism of local hippocampal E2 regulated ERβ expression. The quantitative immunoblot analysis of ERβ in control and 8 days-anastrozole exposed neuron (A). 100 nM PPT, 100 nM DPN, or 200 nM Wortmannin (Wort.) was added to the culture media of anastrozole-treated neuron in 7-8 days prior to harvested cells. Representative densitometric analysis of ERβ expression in H19-7 cells (B). Representative immunofluorescent image of ERβ (green signal) and nucleus (red signal) of anastrozole and anastrozole plus DPN-treated cells (C). Scale bar = 10 uM. β-actin was the housekeeping protein. ***$P < 0.001$ *vs* control group.

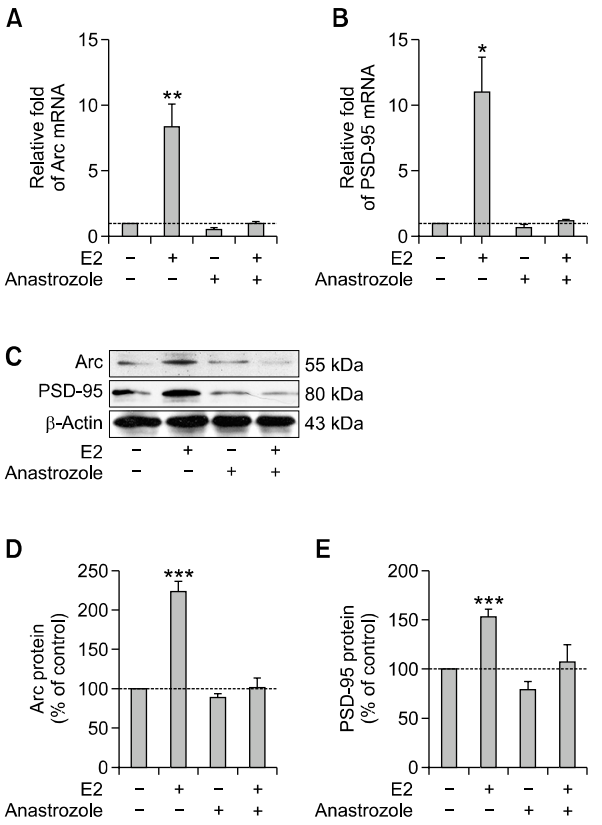


**Figure 4.** The effect of endogenous E2 on exogenous E2-induced H19-7 hippocampal synaptic plasticity. Representative quantitative qRT-PCR (A, B) and Western blotting analysis (C, D, E) of Arc and PSD-95 expression in H19-7 hippocampal neurons. Densitometric analysis of Arc (D) and PSD-95 (E) protein in H19-7 cells. GAPDH was the housekeeping mRNA in real-time PCR. β-actin was the housekeeping protein. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs* control group ($n = 5$).

enhanced ERβ expression in basal ERα-PI-3K dependent mechanism.

We further examined the effect of local E2 on ERβ activation by determining the translocation of basal ERβ in hippocampal H19-7 cells by using laser confocal microscopy (Figure 4D). In anastrozole treated group, the basal ERβ localized in cytoplasmic and membranous regions of hippocampal H19-7 cells. After DPN treatment, basal membranous and cytoplasmic ERβ decreased, but the nuclear ERβ increased. This findings suggest that the membranous and cytoplasmic ERβ translocalize into nucleus in ligand-dependent mechanism, indicating a characteristic of classical genomic action.

## Local E2 mediates exogenous E2-induced synaptic plasticity

Although local hippocampal E2- and exogenous E2-enhanced hippocampal synaptic plasticity had been explained (Prange-Kiel and Rune, 2006), the role of the local E2 on exogenous E2-mediated synaptic plasticity is still unclear. By using synaptic plasticity markers, Arc and PSD-95, we observed the interactive effect of local and exogenous E2 on synaptic plasticity using qRT-PCR and Western blot analysis in hippocampal H19-7 cells. As shown in Figures 3A and 3B, exogenous E2 treatment for 30 min and 6 h significantly increased *Arc* and *PSD-95* mRNA expression, respectively. Anastrozole unaltered the basal *Arc* and *PSD-95* mRNA expression. Interestingly, exogenous E2 could not enhance *Arc* and *PSD-95* mRNA expression when hippocampal H19-7 cells were cultured in anastrozole-containing media (Figures 3A and 3B).

Similar to those mRNA, administration of exogenous

E2 (1 h and 48 h for Arc and PSD-95, respectively) also induced Arc and PSD-95 proteins expression (Figures 3C-3E). Anastrozole had no effect on basal Arc and PSD-95 proteins expression. Exogenous E2 failed to enhance Arc and PSD-95 protein expression in anastrozole-treated neurons (Figures 3D and 3E). These findings indicated that hippocampal E2 was prerequisited for exogenous E2-enhanced hippocampal synaptic plasticity.

### Local E2 mediates neuroprotective effect of exogenous E2

The neuroprotective effects of E2 have been well demonstrated. However, the involvement of local E2 on neuroprotective effect of exogenous E2 is still elusive. As demonstrated in Figure 5A, 1 h of 200 μM $H_2O_2$ exposure significantly decreased hippocampal H19-7 cell viability. $H_2O_2$-induced cell death was enhanced by anastrozole. Preincubation of the cells with exogenous E2 for 24 h prior to addition of $H_2O_2$ attenuated $H_2O_2$-induced cell death. However, therapeutic effect of exogenous E2 on $H_2O_2$-induced H19-7 cell death was absent when cells were exposed to anastrozole (Figure 5A).

We also observed the effect of local E2 and exogenous E2 on anti-apoptotic protein Bcl2, the specific target of E2 (Dubal *et al*., 1999). While 24 h of exogenous E2 preincubation significantly enhanced Bcl2 expression, $H_2O_2$ significantly suppressed its expression (Figures 5B and 5C). Additional effect of anastrozole on $H_2O_2$-supressed Bcl2 expression was demonstrated. Similar to those cell viability studies, exogenous E2 totally rescued Bcl2 expression in $H_2O_2$-treated H19-7 cells. Exogenous E2 could not restore $H_2O_2$-supressed Bcl2 expression in anastrozole treated neurons (Figures 5B and 5C). These findings suggest the prerequisite role of local hippocampal E2 on neuroprotective function of exogenous E2.

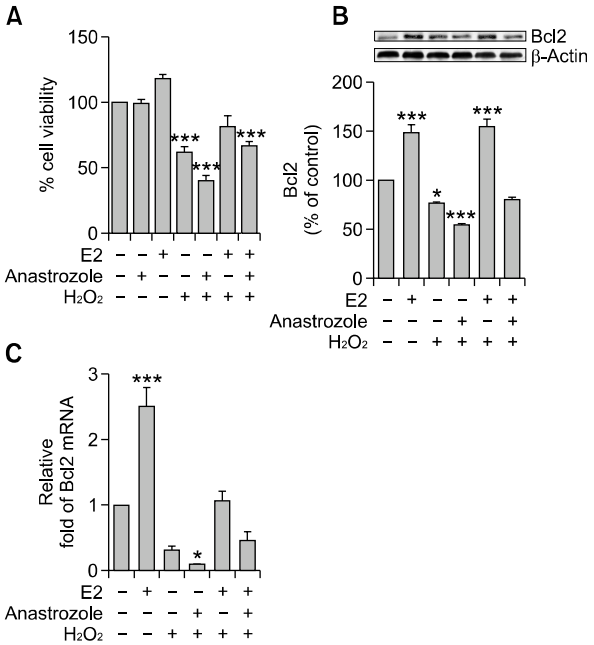


**Figure 5.** The effect of endogenous E2 on neuroprotective effect of exogenous E2. Representative cell viability data from MTT assay (A). Anti apoptotic Bcl2 expression in H19-7 hippocampal neurons (B and C). β-actin was the housekeeping protein. *$P < 0.05$, ***$P < 0.001$ *vs* control group ($n = 5$).

## Discussion

De novo E2 production and secretion in hippocampus has been demonstrated in both primary hippocampal neurons (Prange-Kiel *et al*., 2003) and hippocampal slice cultures (Kretz *et al*., 2004). In the present study, we showed the production and secretion of local E2 in hippocampal H19-7 cells. Our findings demonstrate the sigmoidal secretory profile of H19-7 hippocampal E2. In first 3 days after seeding the secreted E2 levels relatively constant probably due to the preparation of intracellular machinery that responsible for production and secretion of E2, we named this period as “the preparation state”. In days 4 to 7, secreted E2 rapidly increased form 16.2% to 90.6% of maximum secreted E2; it was named “the active state”. Finally, secreted E2 reached the maximum plateau state in days 8 to 12. However, secreted $E_2$ levels were relatively unaltered when hippocampal H19-7 cells were incubated with anastrozole. This finding indicates that E2 production and secretion in hippocampal H19-7 cells required aromatase activity similar to those reported in primary hippocampal neurons (Prange-Kiel *et al*., 2003) and hippocampal slice cultures (Kretz *et al*., 2004). In addition, the maximum secreted E2 levels in hippocampal H19-7 cells were comparable to that report in hippocampal slice cultures (Kretz *et al*., 2004). Therefore, H19-7 hippocampal cells were a suitable model for studying the effect and mechanism of hippocampal E2 on hippocampal neuronal functions.

It has clearly been demonstrated that GnRH receptor expressed in hippocampus (Chu *et al*., 2008; Schang *et al*., 2011), thus, hippocampal neuron can selectively respond to GnRH treatment. In the present study, we reported the activating effect of GnRH on local hippocampal E2 level, indicating that GnRH enhanced endogenous E2 biosynthesis in hippocampal H19-7 cells. Similar to previous study, that demonstrated the activating

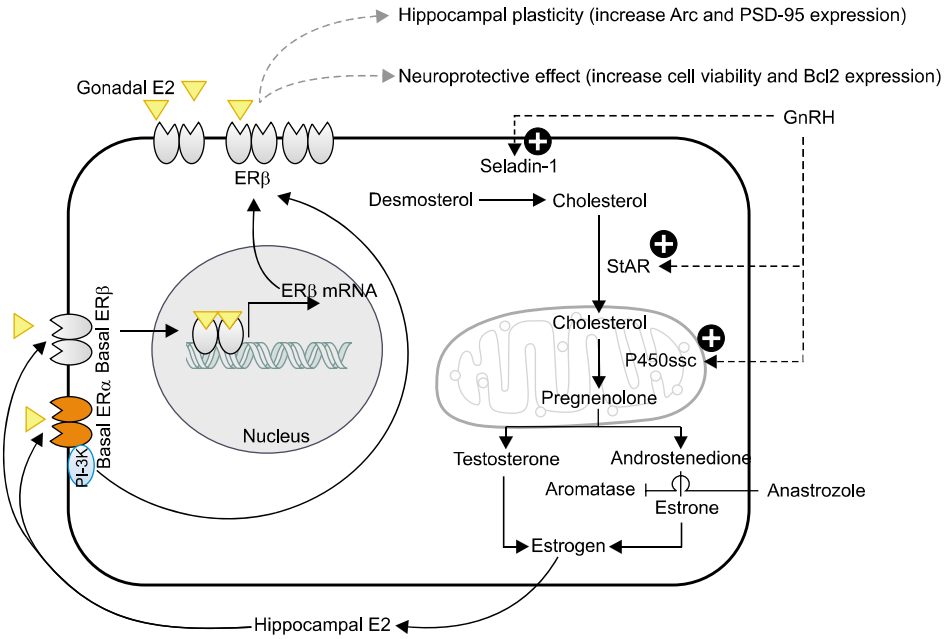


**Figure 6.** Schematic hypothetical model illustrating the prerequisite action of local hippocampal E2 on exogenous E2-induced synaptic plasticity and neuroprotection. GnRH enhanced local hippocampal E2 biosynthesis possibly *via* seladin-1, StAR, and P450scc expression. Hippocampal E2 biosynthesis regulates by endogenous aromatase activity. Secreted hippocampal E2 activates basal ERα and ERβ to further upregulate ERβ expression. Hippocampal synaptic plasticity enhancing and neuroprotective functions of exogenous E2 mediates by ERβ.

effect of GnRH on E2 biosynthesis and secretion in SH-SY5Y neurons (Rosati *et al*., 2011). GnRH enhanced the expression of enzymes involved in E2 biosynthesis, including seladin-1, steroidogenic acute regulatory protein (StAR), and cytochrome P450 (Attar and Bulun, 2006), indicated the feasibility of using hippocampal H19-7 cell for investigating the role of local E2 on hippocampal function.

There are two ER subtypes, ERα and ERβ, which are expressed in hippocampus (Bliss and Collingridge, 1993). Expressions of those ERs are regulated by its ligand, E2 (Murata *et al*., 2003; Oliveira *et al*., 2004). In the present study, we showed the direct activating action of local hippocampal E2 on ERβ expression. Suppression of the local E2 by aromatase inhibitor led to downregulation of ERβ expression in hippocampal neurons. Local hippocampal E2 had no effect on ERα expression. In hippocampus, ERβ play an important role in long-term potentiation (LTP) enhancement, memory improvement (Day *et al*., 2005; Liu *et al*., 2008), and neuroprotection (Dubal *et al*., 1999; Zhao *et al*., 2004). The autocrine and paracrine actions of hippocampal E2 on an enhancement of ERβ expression are suggested to prime hippocampal neurons for further activation by exogenous E2.

We also demonstrated that hippocampal E2 acted through basal ERα-PI-3K-dependent pathway to enhance ERβ expression, which classified as a non-genomic pathway. Membrane ERα directly interacted with PI-3K (Simoncini *et al*., 2003), therefore ERα agonist PPT failed to activate wortmannin-treated cells. On the other hand, local hippocampal E2 activated nuclear translocation of basal ERβ in H19-7 hippocampal cells, which was consistent with our previous report (Chamniansawat and Chongthammakun, 2010). Exogenous E2 activated ERβ translocation into nucleus to enhance synaptic plasticity (Chamniansawat and Chongthammakun, 2010), hence local hippocampal and exogenous E2 regulated neuronal function in classical genomic ERβ-dependent mechanism.

Previously, the potential action of hippocampal and exogenous E2 on hippocampal synaptic plasticity has been reported (Gould *et al*., 1990; Prange-Kiel and Rune, 2006). Little is known about the interactive function of local hippocampal and exogenous E2 on synaptic plasticity. Exogenous E2 upregulated synaptic plasticity marker expression in hippocampal H19-7 cells. However, when endogenous E2 synthesis was inhibited by aromatase inhibitor anastrozole, exogenous E2 could not enhance synaptic plasticity in hippocampal H19-7 cells, suggesting that exogenous E2 function is endogenous E2-dependent mechanism. Previously, ERβ knockout mice exhibited attenuation of hippocampal plasticity and memory impairment (Day *et al*., 2005; Liu *et al*., 2008) although exogenous E2 is still present. Since the downregulation of ERβ expression is demonstrated in anastrozole-treated hippocampal H19-7 cells, E2 could not enhance hippocampal synaptic plasticity similar to those transgenic mice. The priming action of local hippocampal E2 on ERβ expression is suggested to

be essential for exogenous E2-enhanced hippocampal synaptic plasticity.

Neuroprotective function of both exogenous E2 and endogenous E2 has been identified (Dubal *et al*., 1999; McCullough *et al*., 2003; Zhao *et al*., 2004). Exogenous E2 exclusively mediated Bcl2 expression, but not other Bcl2 family, including Bax, Bcl-xl, Bcl-xs, and Bad, to prevent neuronal death in ischemic brain injury model (Dubal *et al*., 1999). On the other hand, aromatase knockout mice exhibited higher brain damage than the wild type-OVX mice after cerebral artery occlusion (McCullough *et al*., 2003), indicated an important role of local E2 on neuronal viability. In the present study, we reported the priming action of hippocampal E2 on exogenous E2 enhanced hippocampal H19-7 cell viability and Bcl2 expression. Local hippocampal E2 influenced ERβ expression that required for exogenous E2-enhanced Bcl2 expression and neuronal viability (Dubal *et al*., 1999; Zhao *et al*., 2004). Therefore, neuroprotective action of exogenous E2 requires autocrine and paracrine actions of hippocampal E2-induced ERβ expression.

In conclusions, our study demonstrated the priming action of hippocampal E2 on systemic E2 functions, as depicted in Figure 6. Endogenous aromatase mediates H19-7 hippocampal E2 biosynthesis. GnRH enhances hippocampal E2 biosynthesis and secretion probably *via* StAR and P450scc activation (Mukai *et al*., 2010). Secreted hippocampal E2 upregulates hippocampal ERβ expression through basal ERα and ERβ activations. The basal ERα activation directly mediates PI-3K signaling pathway, whereas basal ERβ activation translocates into nucleus. This mechanism provides the hippocampal ERβ to respond to systemic E2 lead to enhance hippocampal synaptic plasticity and neuroprotection.

## Methods

### Cell culture

H19-7 cells of hippocampal origin (H19-7 hippocampal cells; ATCC® Number, CRL-2526™) were grown on poly-L-lysine-coated dishes, in Dubecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) in a 5% $CO_2$ humidified atmosphere at 33°C as described previously (Bhargava *et al*., 2000). Hippocampal H19-7 cells are transformed with a temperature-sensitive mutant of simian virus 40 T antigen and are conditionally differentiated. For differentiation, cells were incubated in a 5% $CO_2$ humidified atmosphere at 39°C in DMEM and $N_2$ supplements (Invitrogen, Carlsbad, CA), and the process was accelerated by adding 10 ng/ml basic fibroblast growth factor (Sigma, St. Louis, MO). To find out the optimal level of E2 secretion, hippocampal H19-7 cells were incubated with culture media, 25 nM anastrozole-containing culture, or 0.01 μM GnRH-containing culture media for 12 days. To investigate the effect and mechanisms of local hippocampal E2 on exogenous E2, cells were treated with 10 nM E2, 25 nM anastrozole, 100 nM PPT, 100 nM DPN, or 200 nM wortmannin according to the need of each experiment. All stimulant drugs are purchased from Sigma.

### ELISA

To evaluate secreted E2 levels in culture media, colorimetric rat E2 ELISA kits (TSZ ELISA; Framingham, MA) was performed according to the manufacturer's instructions. Briefly, culture medium were pipetted into micro-assay well which had been precoated with monoclonal antibody against E2, followed by incubation at 37°C for 30 min. And then washing buffer was added to wash an excess binding before added HRP-conjugated reagent to each well. To develop the reaction, chromogen substrate was added and incubated at 37°C for 15 min in light protection. The reaction were stopped by add 50 ml stop solution into each well. Absorbance was read at 540 nm on a microplate reader (Bio-Tek). Samples were quantified by interpolation with standard curve.

### Immunocytochemistry

For immunocytochemistry, hippocampal H19-7 cells were seeded on 12-mm glass coverslips at a density of 10,000 cells/coverslip and cultured overnight as previously described (Chamniansawat and Chongthammakun, 2009). At the end of all treatments, cells were fixed for 15 min in 4% paraformaldehyde at 4°C, followed by permeabilization in 0.2 M glycine. After blocking of nonspecific binding, cells were incubated overnight at 4°C with 1:100 mouse anti-ERα or 1-100 mouse anti-ERβ antibodies (Abcam, Cambridge, UK). After washing away excess primary antibodies, cells were then incubated with 1:1000 fluorescien isothiocyanate (FITC)-coupled anti-mouse IgG secondary antibody (Zymed), and subsequently stained for nuclei with 1:500 TROPO-3 (Molecular Probes, Eugene, OR). We used a confocal laser-scanning microscope (model FV 1000; Olympus, Tokyo, Japan) to analyze the fluorescent signals.

### Western blot analysis

Western blot analysis was performed as described previously (Chamniansawat and Chongthammakun, 2009). H19-7 hippocampal cells were lysed with RIPA buffer. Equal amounts of protein were separated on 10% SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were incubated with 1:1000 mouse anti-ERα, -ERβ, -Arc (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit anti-PSD-95 (Abcam), or -Bcl2 (Abcam) antibodies, and then incubated with a HRP-conjugated secondary antibody (Zymed, San Francisco, CA). The signal was detected with ECL Western blotting substrate (Pierce, Rockford, IL) and captured on Hyperfilm™ (Amersham Pharmacia Biotech, Piscataway, NJ).

### Quantitative real time-PCR (qRT-PCR)

To determine mRNA levels of *Arc, PSD-95, ERα, ERβ,* and *Bcl2* in H19-7 hippocampal neuron, total RNA was extracted by using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol as previously described (Chamniansawat and Chongthammakun, 2009). A 2 μg total RNA was converted to cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qRT-PCR) was performed on the Applied Biosystems PRISM 7700 Sequence Detection System using TaqMan-Gene Expression Assays with FAM™ dye-labeled TaqMan® MGB probes, which contained customized primer design according to the manufacturer's protocol. The positive endogenous control was GAPDH and the negative control was water. The relative expression of mRNA was calculated using the comparative Ct method by using SDS software v.1.3.1 (Applied Biosystems). All data were normalized with the endogenous reference gene GAPDH expression (Applied Biosystems).

### MTT reduction assay

The cell viability was determined by the quantitative colorimetric with MTT assay (3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma). Cells were plated on 96-well culture plates at a density of 10,000 cells per well. Cells were cultured in media or media containing anastrozole for 8 days, then, treated with 200 μM hydrogen peroxide ($H_2O_2$) or culture media for 1 h. The medium was removed and mixed with a solution of 1 mg/ml MTT for 3 h in a 5% $CO_2$ humidified atmosphere at 37°C. The supernatant was then removed, and the formazan crystals in the cells were solubilized with DMSO. Absorbance was read at 570 nm on a microplate reader.

### Data analysis

All data were expressed as mean ± SEM. Two sets of data were compared using the unpaired Student's *t*-test. One-way analysis of variance (ANOVA) with Tukey's posttest was employed for multiple sets of data. The level of significance of all statistical tests was $P < 0.05$. Linear regression and non-linear regression analysis were performed to obtain the % maximum secreted E2-incubation period relationship. All data were analyzed by GraphPad Prism version 5.0 for Window (GraphPad Software Inc., San Diego, CA).

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