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Estradiol rapidly modulates spinogenesis in hippocampal dentate gyrus: Involvement of kinase networks

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ABSTRACT

Estradiol (E2) is locally synthesized within the hippocampus and the gonads. Rapid modulation of hippocampal synaptic plasticity by E2 is essential for synaptic regulation. The molecular mechanisms of modulation through the synaptic estrogen receptor (ER) and its downstream signaling, however, are largely unknown in the dentate gyrus (DG). We investigated the E2-induced modulation of dendritic spines in male adult rat hippocampal slices by imaging Lucifer Yellow-injected DG granule cells. Treatments with 1 nM E2 increased the density of spines by approximately 1.4-fold within 2 h. Spine head diameter analysis showed that the density of middle-head spines (0.4–0.5 μm) was significantly increased. The E2-induced spine density increase was suppressed by blocking Erk MAPK, PKA, PKC and LIMK. These suppressive effects by kinase inhibitors are not non-specific ones because the GSK-3β antagonist did not inhibit E2-induced spine increase. The ER antagonist ICI 182,780 also blocked the E2-induced spine increase. Taken together, these results suggest that E2 rapidly increases the density of spines through kinase networks that are driven by synaptic ER.

Introduction

The hippocampus is heavily involved in learning and memory processes. Findings that estrogen and androgen are synthesized in the adult male/female hippocampus have opened a new field of study regarding estrogen function as it relates to the regulation of daily memory formation (Kimoto et al., 2001; Kawato et al., 2002, 2003; Hojo et al., 2004, 2008; Kretz et al., 2004; Mukai et al., 2006a, 2010; Murakami et al., 2006a; Munetsuna et al., 2009).

The level of adult hippocampal 17β-estradiol (E2) is higher (~8 nM in male, 0.5–4 nM in female) than that of plasma E2 (0.02–0.1 nM), as determined by mass spectrometric analysis (Hojo et al., 2009, 2014; Kato et al., 2013).

Hippocampal glutamatergic neurons express estrogen receptors (ERs). Both forms, ERα and ERβ, are located not only in the cytoplasm and the nuclei but also within dendritic spines (Milner et al., 2005; Mukai et al., 2007, 2010; Hojo et al., 2008; Murakami et al., 2014). Therefore, E2 could produce rapid non-genomic effects (occurring within 2 h of exposure) through synaptic ERs. Of course, chronic and genomic effects (occurring over 1–5 days) can also occur through nuclear/cytoplasmic ERα/ERβ.

We should seriously consider the modulatory actions of hippocampus-derived E2 (Tsurugizawa et al., 2005; Mukai et al., 2007, 2010; Tanaka and Sokabe, 2012; Pettorossi et al., 2013; Luine, 2014; Hasegawa et al., 2015) in addition to the functions of gonadal E2 (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992; Leranth et al., 2003). Accumulating evidence suggests that E2 has acute modulatory effects on spinogenesis, long-term potentiation (LTP) and long-term depression (LTD) in the CA1 and CA3 regions. In previous studies, we showed that 2 h incubation in 1 nM E2 increased dendritic spine density in the CA1 stratum radiatum and decreased the density of dendritic thorns in the CA3 stratum lucidum. From mechanistic analyses, these rapid E2 effects have been shown to be mediated by various kinase networks, including Erk MAPK, PI3K, PKA, PKC and LIMK (Tsurugizawa et al., 2005; Murakami et al., 2006b; Ishii et al., 2007; Hojo et al., 2008; Ogiue-Ikeda et al., 2008; Hasegawa et al., 2015).

The dentate gyrus (DG) receives excitatory inputs from the lateral and medial entorhinal cortex through the perforant path into the molecular layer (Thomas et al., 1994; Dolorfo and Amaral, 1998). The perforant path terminates in synaptic contacts with dendritic spines of granule neurons. The DG plays an essential role in several cognitive functions, including pattern separation (McHugh et al., 2007; Aimone

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et al., 2011) and the formation of contextual fear-conditioned memories (Liu et al., 2014). The DG has also been studied extensively as a region of adult neurogenesis (Kuiper et al., 1997; Perez-Martin et al., 2003a; Olariu et al., 2005; Tanapat et al., 2005). LTP in the DG has also been investigated (Trommald et al., 1996), and it has been found that this phenomenon correlates with spatial pattern separation (Clelland et al., 2009; Nakashiba et al., 2012).

However, only a few studies have been reported concerning the effects of estrogen on spinogenesis in the DG (Miranda et al., 1999). We therefore investigated whether E2 can rapidly increase the presence of spines on granule neurons in the adult male hippocampal DG region. Furthermore, we investigated whether multiple kinases might be involved in E2 signaling.

Materials and methods

Animals

Twelve-week-old adult male Wistar rats were purchased from Tokyo Experimental Animal Supply. All experimental procedures used for this research were approved by the Committee for Animal Research of Univ. of Tokyo.

Chemicals

Lucifer Yellow and LY294002 were purchased from Sigma (USA). Chelerythrine, H-89, GSK-3β Inhibitor 8 (I8), and LIMK inhibitor (LIMKi) were purchased from Calbiochem (USA). 17β-estradiol (E2), U0126 and ICI 182,780 (ICI) were purchased from Wako Pure Chemical Industries (Japan).

Imaging and analysis of dendritic spine density and morphology

Hippocampal slice preparation and current injection of Lucifer Yellow

Male rats aged 12 weeks were deeply anesthetized and decapitated. The brains were removed and placed in artificial cerebrospinal fluid (ACSF) at 4 °C. ACSF consisted of (mM) 124 NaCl, 5.0 KCl, 1.25 NaH_2PO_4, 2.0 $MgSO_4$, 2.0 $CaCl_2$, 22 $NaHCO_3$, and 10 glucose and was equilibrated with 95% O_2/5% CO_2. The hippocampus was dissected, and 300-μm slices transverse to the long axis from the middle third of the hippocampus were cut using a vibratome (Dosaka, Japan). These 'fresh' hippocampal slices were transferred into an incubating chamber containing ACSF held at 25 °C and kept there for 2 h for slice recovery. The resulting 'acute' slices were then incubated in 0.1–10 nM E2 together with ER antagonist (ICI 182,780) or inhibitors of protein kinases. All steroids, antagonist and inhibitors were diluted with 1/1000 dimethylsulfoxide (DMSO) in ASCF. Slices were then fixed with 4% paraformaldehyde in PBS at 4 °C overnight. Neurons within slices were visualized by injecting the tissue with Lucifer Yellow (Molecular Probes, USA) and placing it in a Nikon E600FN microscope (Japan) equipped with a C2400-79H infrared camera (Hamamatsu Photonics, Japan) and a 40× water immersion lens (Nikon, Japan). Dye injection was performed using a glass electrode filled with 5% Lucifer Yellow for 15 min, using Axopatch 200B (Axon Instruments, USA). Approximately five neurons within a depth of 100 μm from the surface of a slice were injected with Lucifer Yellow (Duan et al., 2003).

Confocal laser microscopy and morphological analysis

Imaging was performed by taking sequential z-series scans with a confocal laser scanning microscope (LSM5; Carl Zeiss, Germany), at high zoom (3.0×) with a 63× water immersion lens, NA 1.2. For Lucifer Yellow, the excitation and emission wavelengths were 488 nm and 515 nm, respectively. For analysis of spines, three-dimensional images were reconstructed from approximately 40 sequential z-series sections taken 0.45 μm apart with a 63× water immersion lens, NA 1.2. The applied zoom factor (3.0×) yielded 23 pixels per μm. The z-axis resolution was approximately 0.71 μm per section. The confocal lateral resolution was approximately 0.26 μm per pixel. Our resolution limits were considered to be sufficient to allow for the determination of spine density. Confocal images were then deconvolved using AutoDeblur software (AutoQuant, USA).

The density of spines and spine head diameters were analyzed using Spiso-3D (automated software that calculates the geometrical parameters of spines), which was developed as part of the Kawato group's Bioinformatics Project (Mukai et al., 2011). Spiso-3D has functionality that is equivalent to Neurolucida (MicroBrightField, USA); furthermore, Spiso-3D considerably reduces the number of human errors and the amount of experimenter labor required for analysis. Each dendrite was analyzed separately. Spine density was calculated from the numbers of spines along dendrites having a total length of 50 to 100 μm. These dendrites were present within the outer molecular layer, between 50 and 150 μm from the soma. Spine shapes were classified into three categories, as follows: (1) Small-head spines, whose head diameters are between 0.2 and 0.4 μm. (2) Middle-head spines, which have spine heads measuring 0.4–0.5 μm. (3) Large-head spines, whose head diameters are between 0.5 and 1.0 μm. These three categories were useful for distinguishing between responses to different kinase inhibitor applications. Because the majority of spines observed (>95%) had a distinct head and neck and because stubby spines and filopodium did not considerably contribute to the overall changes, we only analyzed spines with a distinct head.

Post-embedding immunogold method for electron microscopy

Immunoelectron microscopy analysis was performed as described elsewhere (Mukai et al., 2007). Rat hippocampus was coronally sliced at 4 °C using a vibratome. Freeze substitution and low-temperature embedding of the specimens were performed as described previously (Adams et al., 2002). The samples were immersed in uranyl acetate in anhydrous methanol (−90 °C). They were then infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences, USA), and polymerization was performed using ultraviolet light. Ultrathin sections were prepared using a Reichert-Jung ultramicrotome. For immunolabeling, sections were incubated overnight in either RC-19 (Mukai et al., 2007), purified primary antibody for ERα (diluted to 1/1000), or anti-human cytochrome P450arom IgG (Hojo et al., 2004) (diluted to 1/500), followed by incubation with secondary gold-tagged (10 nm) Fab fragments in Tris-buffered saline (TBS). Sections were counterstained with 1% uranyl acetate and viewed on a JEOL 1200EX electron microscope (Japan). Images were captured using a CCD camera (Advanced Microscopy Techniques, USA).

Statistical analysis

The significance of the effects of incubating in E2 or other drugs was examined via statistical analysis using Tukey–Kramer post-hoc multiple comparisons test when one-way ANOVA tests yielded $P < 0.05$.

Results

We investigated the effect of E2 on modulation of the dendritic spine density in the hippocampus dentate gyrus molecular layer. Lucifer Yellow-injected neurons in hippocampal slices from 12-week-old male rats were imaged using confocal laser scanning microscopy and analyzed using Spiso-3D software (Fig. 1A, B). Spines within 50 μm to 150 μm from the soma where the perforant path from entorhinal cortex attached were studied (Fig. 1C).

Estrogen-related increase in spine density in DG molecular layer

The level of E2 in control slices was below 0.5 nM because E2 was leaked to the outer medium during the 2-h recovery incubation in

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