Estrogen induces rapid decrease in dendritic thorns of CA3 pyramidal neurons in adult male rat hippocampus

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Abstract

Modulation of hippocampal synaptic plasticity by estrogen has been attracting much attention. Thorns of thorny excrescences of CA3 hippocampal neurons are post-synaptic regions whose presynaptic partners are mossy fiber terminals. Here we demonstrated the rapid effect of estradiol on the density of thorns of thorny excrescences, by imaging Lucifer Yellow-injected CA3 neurons in adult male rat hippocampal slices. The application of 1 nM estradiol induced rapid decrease in the density of thorns on pyramidal neurons within 2 h. The estradiol-mediated decrease in the density of thorns was blocked by CNQX (AMPA receptor antagonist) and PD98059 (MAP kinase inhibitor), but not by MK-801 (NMDA receptor antagonist). ERα agonist PPT induced the same suppressive effect as that induced by estradiol on the density of thorns, but ERβ agonist DPN did not affect the density of thorns. Note that a 1 nM estradiol treatment did not affect the density of spines in the stratum radiatum and stratum oriens. A search for synaptic ERα was performed using purified RC-19 antibody. The localization of ERα (67 kDa) in the CA3 mossy fiber terminals and thorns was demonstrated using immunogold electron microscopy. These results imply that estradiol drives the signaling pathway including ERα and MAP kinase.
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The hippocampus is essentially involved in learning and memory processes, and is known to be a target for the modulatory actions of estrogens and androgens produced in the gonads, reaching the brain via blood circulation [1–4]. Extensive studies have been performed to investigate their role in modulating hippocampal plasticity and function, slowly and genomically (over 1–5 days). The density of dendritic spines of pyramidal neurons in the CA1 region of the hippocampus is modulated in vivo by the depletion and replacement of estrogens [1,3]. On the other hand, little is known regarding estrogen effect on CA3 pyramidal neurons, which consist of four regions, i.e., the stratum lucidum, the stratum radiatum, the stratum lacunosum-moleculare, and the stratum oriens. In three regions (stratum radiatum, stratum lacunosum-moleculare, and stratum oriens), CA3 pyramidal neurons have separated spines which have the same appearance as spines in CA1 neurons. In the stratum oriens and stratum lacunosum-moleculare region, estrogen has been shown to induce no

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significant effect on the CA3 spine density across the estrous cycle [1,2]. In the stratum lucidum of the CA3, pyramidal neurons have huge and complex post-synaptic structures, named thorny excrescences, instead of separated spine structures. One thorny excrescence consists of multiple heads named thorns with one neck along a dendritic branch [5,6]. One mossy fiber terminal of dentate granule cells contacts multiple thorns of thorny excrescences of CA3 neuron. Thorny excrescences may play essential roles on hippocampal function. Chronic restraint stress has induced the retraction of thorny excrescences, which has subsequently been reversed after water maze training. On the other hand, water maze training alone has increased the volume of thorny excrescence as well as the number of thorns per thorny excrescence [7].

In addition to slow effect, estradiol exerts a rapid (within 2 h) influence on the synaptic plasticity of rat hippocampal neurons, as has been demonstrated by a number of electrophysiological investigations in male rat [8–10]. To explain these rapid modulations, clarification of cellular and subcellular localization (particularly synaptic localization) of estrogen receptors in glutamatergic neurons is essential. To date several ultrastructural investigations have suggested their presence in GABAergic interneurons and cholinergic neurons however, pyramidal neurons have not been suggested to contain significant amount of estrogen receptors [11–13]. So far, two distinct types of estrogen receptors have been identified in the mammalian brain: ERα, which mediates many of the transcriptional actions of estrogen [14], and ERβ, whose role in the nervous system is less well defined [15]. Molecular type and localization of estrogen receptors have been a matter of hot debate for more than a decade in adult rat hippocampus.

In the present study, we analyzed the rapid modulation of density of thorns and spines in CA3 neurons by 17β-estradiol as well as agonists of ERα and ERβ.

Materials and methods

Animals

Male adult Wistar rats were used in the present study. Rats were purchased from Saitama Experimental Animal Supply. All experiments using animals in this study were conducted according to the Institutional Guidelines.

Imaging and analysis of dendritic spine morphology

Current injection of Lucifer Yellow. Adult male rats were anesthetized with ethyl ether and decapitated. The brains were removed and placed in artificial cerebrospinal fluid (ACSF) at 4 °C. The hippocampus was dissected and 400 μm transverse slices to the long axis from the middle third of the hippocampus were cut with a vibratome (Dosaka, Japan). 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. Hippocampal slices were transferred into an incubating chamber containing ACSF held at 25 °C for 2 h. Slices were then incubated with 0.1–10 nM estradiol or other drugs such as 50 μM MK-801, 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 100 nM (propyl-pyrazole-triyl)tris-phenol (PPT), 100 nM hydroxyphenyl-propionitrile (DPN) or

20 μM PD98059 from Sigma (USA). Slices were then prefixed with 4% paraformaldehyde in PBS at 4 °C for 2–4 h. Neurons within slices were visualized by an injection of Lucifer Yellow (Sigma, USA) under E600FN microscope (Nikon, Japan) equipped with infrared camera C2400-79H (Hamamatsu Photonics, Japan) and a 40× water immersion lens, NA 0.8 (Nikon, Japan). Dye injection was performed with a glass electrode whose tip was filled with 5% Lucifer Yellow in distilled water under a negative DC current of 10 nA for 15 min, using Axopatch 200B (Axon Instruments, USA). Approximately five neurons within a depth of 100–200 μm from the surface of a slice were injected with Lucifer Yellow [16]. After the injection, slices were fixed again with 4% paraformaldehyde overnight at 4 °C and stored in PBS at 4 °C.

Confocal laser microscopy and analysis. The imaging and analysis were performed from sequential *z*-series scans with an MRC-1024 confocal microscope (Bio-Rad, USA) [17]. For Lucifer Yellow, the excitation and emission wavelengths were 488s and 515 nm, respectively. For the analysis of thorns or spines, three-dimensional images were constructed from approximately 40 sequential *z*-series sections of neurons scanned every 0.5 μm at high zoom (1.5–3.0) with a 60× oil immersion lens, NA 1.4 (Nikon, Japan). The applied zoom factor (1.5–3.0) yielded 9.1–18 pixels per 1 μm. The *z*-axis resolution was approximately 0.34 μm. The confocal lateral resolution was approximately 0.18 μm. Our resolution limits were regarded to be sufficient to allow the determination of the density of thorns or spines. Confocal images were then deconvolved using AutoDeblur software (AutoQuant Imaging, USA) which uses an estimated and iteratively refined theoretical point spread function (PSF) that can be adjusted by wavelength and refractive index of lens [18]. In each slice, 2–3 neurons with more than 100 thorns or spines were analyzed, and at least 90 thorns or 50 spines were counted on each frame. In total, $N = 5$–9 neurons and $N = 300$–1000 total thorns or spines (300–1000 μm total dendritic branch) were analyzed for each drug treatment.

The density of thorns or spines was analyzed by tracing neurons with Neurolucida software (MicroBrightField, USA). The single apical dendrite which had thorns was analyzed separately. These dendrites were present within the stratum lucidum, within 100 μm from the soma. The density of thorns was calculated from the number of thorns along both primary and secondary dendrites having a total length of 20–100 μm. While counting the thorns in reconstructed images, the position and verification of thorns were aided by three-dimensional reconstructions and by observation of the images in consecutive single planes. The density of spines was analyzed in the stratum oriens (within 150 μm from the soma) and stratum radiatum (between 100 and 200 μm from the soma) in CA3 neurons. In these regions, the density of spines was calculated from the number of thorns along secondary dendrites having a total length of 50–100 μm.

Post-embedding immunogold method for electron microscopy

Hippocampal slices were prepared from rat deeply anesthetized with pentobarbital and perfused transcardially with a phosphate-buffered saline (PBS; 0.1 M phosphate buffer and 0.14 M NaCl (pH 7.3)), followed by fixative solution of 4% paraformaldehyde. The hippocampi were postfixed and slicing was performed at 4 °C using a vibratome (Leica VT1000S). A coronal section including the dorsal hippocampus was processed for ultrathin sectioning. Freeze substitution and low-temperature embedding of the specimens were performed as described previously [11]. Briefly, slices were plunged into liquid propane in a Universal Cryofixation System KF80 (Reichert-Jung, Vienna, Austria). The samples were immersed in uranyl acetate in anhydrous methanol in a cryosubstitution AFS unit (Leica, Vienna, Austria). The samples were washed and infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences, Ft. Washington, PA) at −45 °C with a progressive increase in the ratio of resin to methanol. Polymerization was performed with ultraviolet light. Ultrathin sections (approximately 80 nm in thickness) were cut and mounted on nickel mesh grids. For immunolabeling, sections were incubated with primary antibody RC-19 (1/1000 dilution) in the above diluent overnight, and incubated with secondary gold-tagged (10 nm) Fab fragment in TBS. RC-19 antibody was prepared as peptide antibody for most

C-terminal 19 amino acids of rat ERa, and purified by affinity chromatography. Sections were washed, counterstained with 1% uranyl acetate and Reynolds lead citrate, and viewed on a JEOL 1200EX electron microscope (Japan). Images were captured using an Advantage CCD camera (Advanced Microscopy Techniques, USA). Controls omitting the primary antibody were performed and no immunogold labeling was observed. A search for immunogold-labeled ERα proteins was performed for at least 30 synapses per CA3 region from more than 30 independent pictures.

Results

The Lucifer Yellow-injected neurons of the CA3 region were imaged using confocal laser microscopy. Figs. 1, 2A, 3A and C show the maximal intensity projection onto the XY plane. Thorny excrescences were located on apical dendrites within 100 μm from the soma, on which mossy fiber terminals attached. Thorny excrescences had 2–12 bulbous-shaped huge heads (thorns), which connected with the dendrite via a slender neck (Fig. 1) [6]. The structure of thorny excrescences was distinctly different from that of spines in other regions (Fig. 1). Only separated single spines were observed in other regions of CA3 neurons such as the stratum radiatum and stratum oriens.

Following a 2 h treatment with estradiol, treated dendrites exhibited fewer thorns than those receiving no estradiol treatment, with the density of thorns decreasing from 2.19 ± 0.17 thorns/μm (control) to 1.53 ± 0.13 thorns/μm (1 nM estradiol) (Figs. 2A and B). In contrast, in the stratum oriens and stratum radiatum, estradiol treatment had no effect of the estradiol treatment on the density of spines (Figs. 3A–D).

To investigate whether estradiol effect was mediated by ERα or ERβ, slices were incubated with 100 nM PPT (ERα selective agonist) or 100 nM DPN (ERβ selective agonist). PPT significantly decreased the density of thorns to 1.66 ± 0.15 thorns/μm (Fig. 2B). In contrast, DPN did not significantly change the density of thorns (2.27 ± 0.25 thorns/μm) (Fig. 2B).

Blocking of AMPA receptors by CNQX, which is an antagonist of AMPA/kainate receptor, completely blocked the estradiol-induced decrease of thorns (2.20 ± 0.28 thorns/μm) (Fig. 2B). On the other hand, blocking of NMDA receptors by MK-801, which is an antagonist of NMDA receptor, did not prevent the estradiol-induced decrease of thorns (observed reduction to 1.50 ± 0.11 thorns/μm) (Fig. 2B). We investigated whether extracellular Ca^{2+} contributed to the change in the density of thorns. When slices were incubated with 1 nM estradiol in ACSF without Ca^{2+}, the density of thorns did not decrease (2.20 ± 0.08 thorns/μm) (Fig. 2B). Application of PD98059, which is an inhibitor of MAP kinase, completely blocked the estradiol-induced decrease of thorns (2.14 ± 0.19 thorns/μm) (Fig. 2B).

It should be noted that 2 h treatment with 1 nM estradiol was most effective in decrease of thorns, when we changed the incubation conditions. The time dependency was examined by treating slices for 0.5, 1, and 2 h with 1 nM estradiol. The decreasing effect on the density of thorns was approximately proportional to the incubation time, showing 2.05 ± 0.10 (0.5 h), 1.81 ± 0.17 (1 h), and 1.53 ± 0.13 thorns/μm (2 h) (Fig. 4A). The dose dependency was also examined after 2 h incubation. The enhancing effect
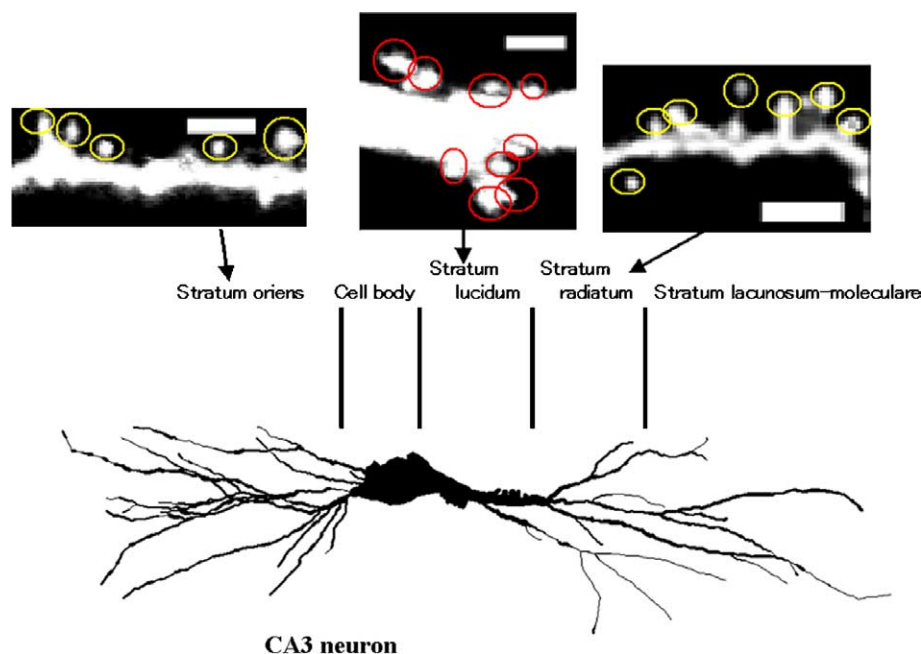

Fig. 1. Maximal intensity projections onto XY plane from z-series confocal micrograph showing thorns and spines along the primary and secondary dendrites of hippocampal CA3 pyramidal neurons. Upper left shows distribution of spines along the basal dendrite in stratum oriens, bar 2 μm. Upper middle shows the distribution of thorny excrescences along the apical dendrite in stratum lucidum, bar 2 μm. Upper right shows the distribution of spines along the apical dendrite in stratum radiatum, bar 2 μm. Thorny excrescences have bulbous-shaped huge heads named "thorns" (red circles) which are considerably different from spines with separated distribution (yellow circles). Lower shows a traced whole image of Lucifer Yellow-injected CA3 neuron.
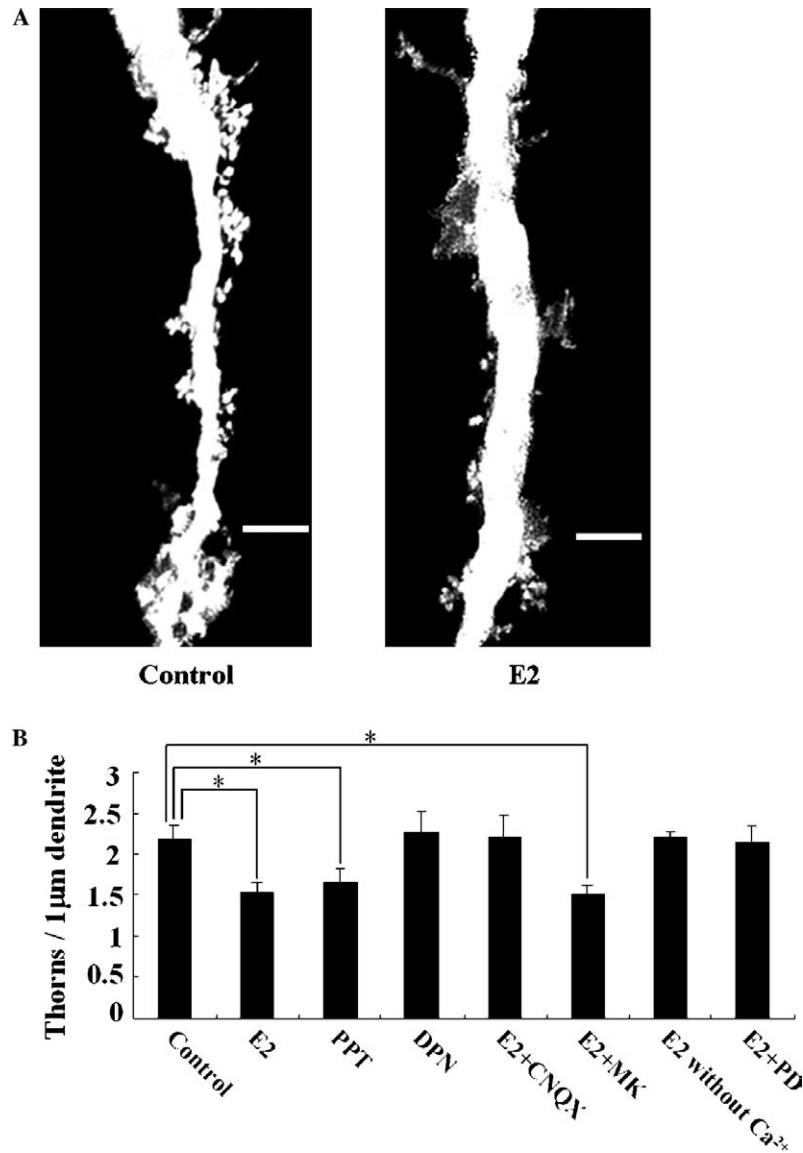
Fig. 2. Effect of estradiol treatments on the density of thorns in hippocampal CA3 neurons. (A) Maximal intensity projections onto XY plane from z-series confocal micrographs, showing control without drug-treatments (control) and thorns after estradiol treatments (E2). Bar 5 μm. (B) Vertical axis is the average number of thorns per 1 μm dendritic segment. A 2 h treatment in ACSF without estradiol (control, $N = 5$), with 1 nM estradiol (E2, $N = 6$), with 100 nM PPT (PPT, $N = 5$), with 100 nM DPN (DPN, $N = 5$), with 1 nM estradiol and 20 μM CNQX (E2 + CNQX, $N = 6$), with 1 nM estradiol and 50 μM MK-801 (E2 + MK, $N = 4$), with 1 nM estradiol and 20 μM PD98059 (E2 + PD, $N = 5$), and a 2 h treatment in ACSF not containing Ca^{2+} with 1 nM estradiol (E2 without Ca^{2+}, $N = 4$). Results are reported as means ± SEM. The significance of the drug effects was confirmed by using ANOVAs (*$p < 0.05$ to control). N is the number of neurons, and for each neuron 90–200 thorns were analyzed.

was most significant at 1 nM estradiol (1.49 ± 0.19 thorns/μm) as compared with 0.1 nM (1.73 ± 0.29 thorns/μm) and 10 nM (1.63 ± 0.13 thorns/μm) (Fig. 4B).

The subcellular localization of ERα in CA3 pyramidal neurons and mossy fibers of granule cells in the dentate was investigated via ultrastructural investigations using RC-19 (1:1000). An immunoelectron microscopic analysis using post-embedded immunogold was performed to determine the localization of ERα-immunoreactivity in the hippocampal neurons. ERα was localized in both the mossy fiber terminals and dendritic thorns of principal neurons (Fig. 5A). Gold particles were clustered in the post-synaptic and presynaptic compartments, as well as

in the nuclei (Fig. 5B). At post-synapses, gold particles were distributed within the thorn. Multiple labeling (3 or more) with immunogold in the pre- and post-synaptic compartments was confirmed to ensure the specific labeling. It should be noted that ERα-immunoreactivity in CA3 pyramidal neurons at light microscopic level has already been demonstrated by immunohistochemical examination of slices using RC-19 [19].

Discussion

The current study demonstrated that the activation of ERα by estradiol induced a rapid decrease of thorns of
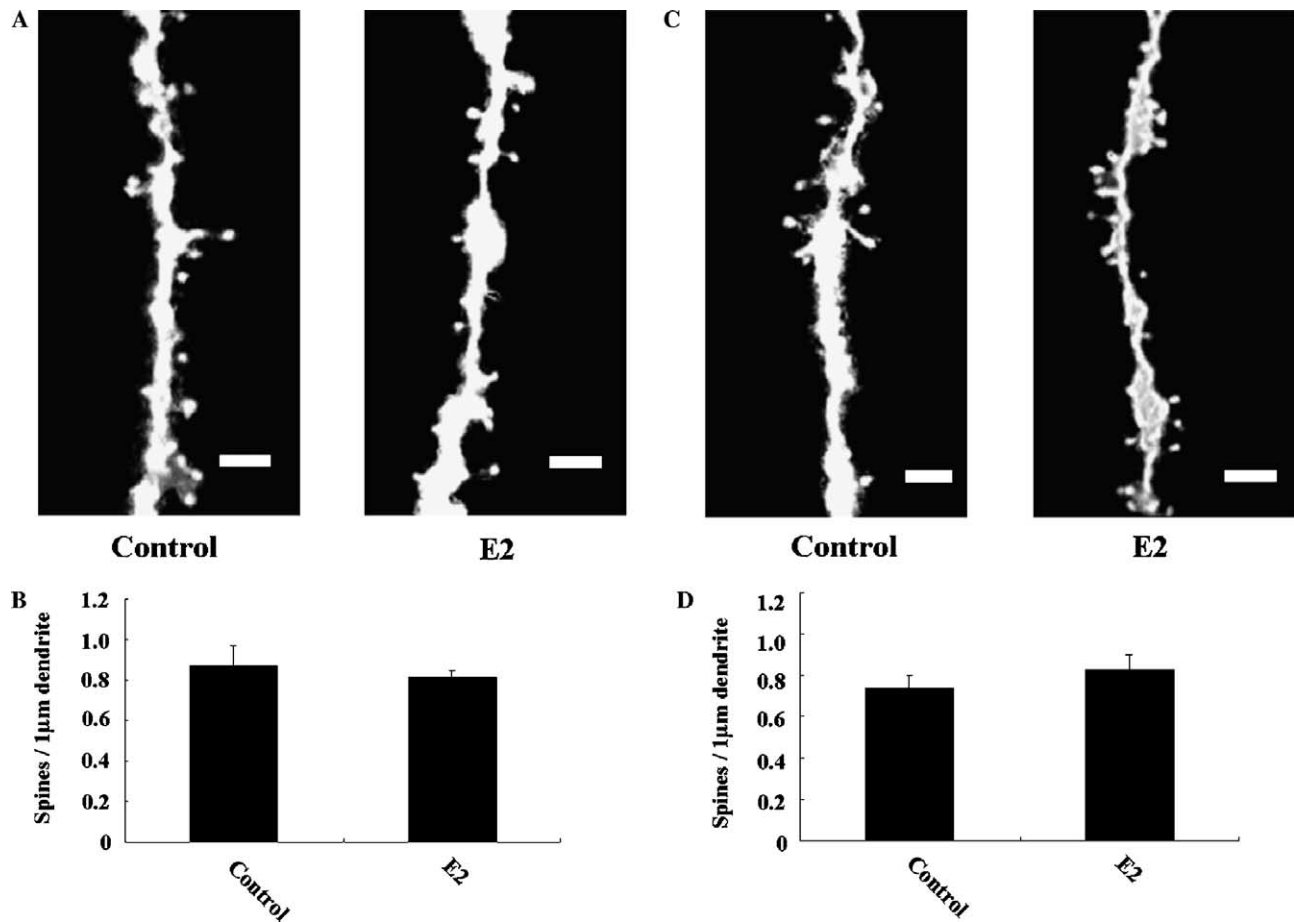
Fig. 3. Effect of estradiol treatments on the density of spines in stratum oriens (A,B) and stratum radiatum (C,D) of hippocampal CA3 neurons. (A,C) Maximum intensity projections onto XY plane from *z*-series confocal micrographs in stratum oriens (A) and stratum radiatum (C). (B,D) A 2 h treatment in ACSF without estradiol (control, $N = 5$), with 1 nM estradiol (E2, $N = 5$) in stratum oriens (B), and a 2 h treatment in ACSF without estradiol (control, $N = 4$), with 1 nM estradiol (E2, $N = 4$) in stratum radiatum (D). Vertical axis is the average number of spines per 1 μm dendritic segment. Results are reported as means ± SEM. The significance of drug effects was confirmed by using ANOVAs ($p < 0.05$). N is the number of neurons, and for each neuron 100–200 spines were analyzed.

thorny excrescences in CA3 pyramidal neurons of the adult male rat hippocampus. Estrogen-induced changes have not been observed in the stratum lucidum of the CA3, until the current study. An extremely concentrated distribution of thorny excrescences, as compared with separated distribution of spines located in other regions of the CA3 (Fig. 1), may have prevented detailed analysis of thorny excrescences by previous analysis such as low sensitivity Golgi staining methods [1,2]. We were able to analyze the number of thorns due to the high resolution of the presenting method using Lucifer Yellow-injected images, optical deconvolution, and careful three-dimensional analysis. The rapid modulation of thorns observed in the current study is a novel observation and is essential for a consideration of the synaptic plasticity affected by estrogen. We observed no significant change in the spine density occurred within 2 h in the stratum oriens and stratum radiatum (Fig. 3), implying that thorns or spines were selectively sensitive to estradiol. The mossy fiber terminals originated from granule cells in the dentate gyrus provide excitatory inputs to CA3 neurons via thorny excrescences in the

stratum lucidum. There are few other excitatory and many inhibitory inputs in the stratum oriens and stratum radiatum [20–22]. Our data imply that estradiol may significantly reduce the excitatory input to CA3 from dentate gyrus by decreasing the density of thorns.

The abolishment of the estradiol-induced decrease in the density of thorns by CNQX (Fig. 2B) suggests the particular correlation of the estradiol signaling pathway with AMPA receptors. On the other hand, the application of MK-801 did not prevent estradiol effect (Fig. 2B). Previous study has shown that NMDA receptors-mediated calcium influx within thorny excrescences in the stratum lucidum of CA3 neurons is smaller than that in CA1 spines upon subthreshold activation, while the spontaneous Ca^{2+} influx within thorny excrescences has occurred mainly via voltage activated calcium channels during subthreshold activation of CA3 neurons [23–27]. Our results suggest that the decrease of thorns requires spontaneous post-synaptic Ca^{2+} influx via voltage activated calcium channels depending upon AMPA receptor-mediated, spontaneous voltage fluctuations. In addition, application of PD98059 suppressed
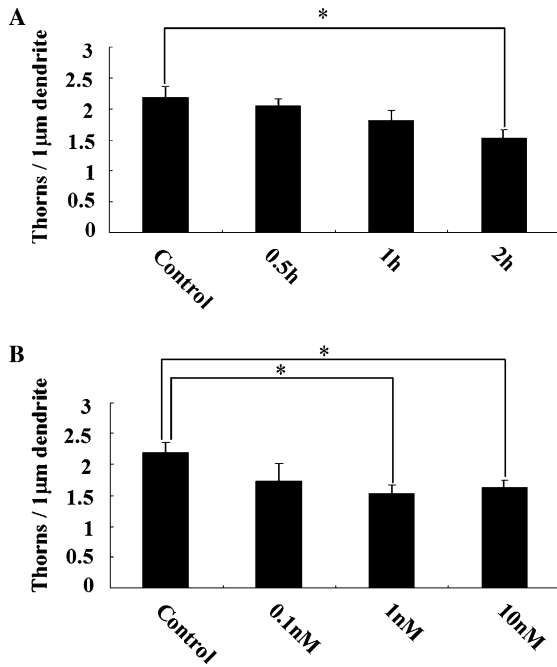
Fig. 4. Time dependency (A) and dose dependency (B) of estradiol. Vertical axis is the average number of thorns per 1 μm dendritic segment. (A) No treatment with estradiol (control, $N = 5$), 0.5 h treatment in ACSF with 1 nM estradiol (0.5 h, $N = 4$), 1 h treatment in ACSF with 1 nM estradiol (1 h, $N = 4$), and 2 h treatment in ACSF with 1 nM estradiol (2 h, $N = 6$). (B) A 2 h treatment in ACSF without estradiol (control, $N = 5$), with 0.1 nM estradiol (0.1 nM, $N = 3$), with 1 nM estradiol (1 nM, $N = 6$), and with 10 nM estradiol (10 nM, $N = 4$). Results are reported as means ± SEM. The significance of the drug effects was confirmed by using ANOVAs (*$p < 0.05$ to control). N is the number of neurons, and for each neuron 60–200 thorns were analyzed.

the effect of estradiol, indicating that the decrease of thorns by estradiol is triggered by MAP kinase pathway.

The decrease of the density of thorns was induced by PPT, but not by DPN. These results indicate for the first time that the decrease of thorns is mediated by ERα not by ERβ. Immunogold electron microscopy demonstrated that a significant distribution of ERα was located in thorns of thorny excrescences in CA3 pyramidal neurons as well as mossy fibers originated from granule cells in the dentate gyrus. Identification of ERα concerning cellular and subcellular distribution has long been a point of heated debate on the hippocampus. We successfully observed the synaptic localization of ERα in mossy fiber terminals and CA3 glutamatergic neurons, due to use of the column-purified antibody RC-19.

Over a decade, only a few reports have appeared regarding investigations of the effect of estrogen on CA3 neurons. In vivo studies have shown that in the stratum oriens and stratum lacunosum-moleculare of CA3 pyramidal cells, no significant change has occurred in the CA3 spine density within the estrus cycle [2]. Ovariectomy or estrogen replacement has also not affected the spine density in the stratum oriens and stratum lacunosum-moleculare of CA3 pyramidal neurons [1]. On the other hand, in the CA1 region, a number of investigations have
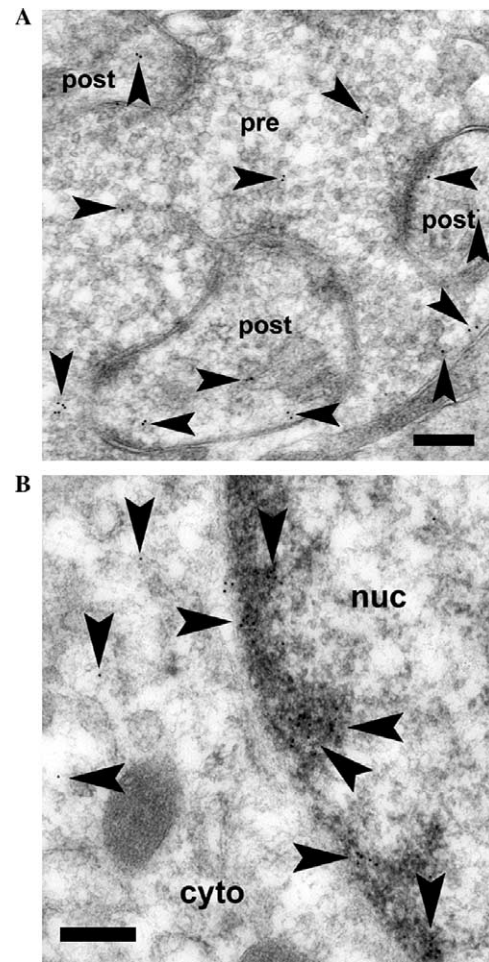

Fig. 5. Immunoelectron microscopic analysis, using RC-19 antibody, of the distribution of ERα within both the mossy fiber terminals and the dendritic thorns in the stratum lucidum (A) and the nuclei (B) of CA3 hippocampal neurons. Representative pictures are shown from ≈50 photographs from 11 independent slices from three animals. (A) Gold particles (arrowheads) were localized in the presynaptic regions (mossy fiber terminals) and postsynaptic regions (thorns). In post-synaptic regions, gold particles were found within the thorns of thorny excrescences. (B) Gold particles were also localized in the nuclei and in the cytoplasmic space. A 1/1000 dilution of RC-19 was used to prevent nonspecific labeling. pre, presynaptic region; post, post-synaptic region; nuc, nuclei; cyto, cytoplasmic space. Scale bar, 200 nm.

been performed concerning the change in spine density induced by estradiol. Woolley and colleagues [2,3] have found that the spine density increases following estrogen replacement in ovariectomized rats. In cultured neonatal hippocampal slices, incubation with letrozole, which is an aromatase inhibitor, has been reported to decrease the spine density of CA1 neurons [28]. CA1 spine density has been observed to increase following several days' treatment of cultured neonatal hippocampal slices with estradiol [29,30]. These results indicate that the estradiol effect is highly heterogeneous between the CA3 and CA1. Therefore, region specific investigations are necessary for the elucidation of the effect of estradiol. Careful attention should be paid to explain the estradiol-induced

spinogenesis of primary cultured hippocampal neurons [30], because dispersed primary cultures are a mixture of CA1, CA3, and dentate gyrus neurons.

In addition to endocrine-derived hormones, we have recently demonstrated the endogenous de novo synthesis of estrogens in male adult rat hippocampal neurons [31–34]. These results imply that hippocampal neurons are exposed to locally synthesized estradiol which might modulate synaptic plasticity rapidly. The observation of a synaptic localization of ERα in glutamatergic CA3 principal neurons is in good accordance with the rapid modulation on the density of thorns by ERα specific agonist PPT as well as estradiol.

Acknowledgments

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