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Estrogen receptor KO mice study on rapid modulation of spines and long-term depression in the hippocampus

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ABSTRACT

Rapid modulation of hippocampal synaptic plasticity through synaptic estrogen receptors is an essential topic. We analyzed estradiol-induced modulation of CA1 dendritic spines using adult male ERαKO and ERβKO mice. A 2 h treatment of estradiol particularly increased the density of middle-head spines (diameter 0.3-0.4 μm) in wild type mouse hippocampal slices. The enhancement of spinogenesis was completely suppressed by MAP kinase inhibitor. Estradiol-induced increase in middle-head spines was observed in ERβKO mice (which express ERα), but not in ERαKO, indicating that ERα is necessary for the spinogenesis. Direct observation of the dynamic estradiol-induced enhancing effect on rapid spinogenesis was performed using time-lapse imaging of spines in hippocampal live slices from yellow fluorescent protein expressed mice. Both appearance and disappearance of spines occurred, and the number of newly appeared spines was significantly greater than that of disappeared spines, resulting in the net increase of the spine density within 2 h. As another type of synaptic modulation, we observed that estradiol rapidly enhanced

Abbreviations: used: ACSF, artificial cerebrospinal fluid; AMPA, 2-amino-3-(5-methyl-3-oxo-1, 2- oxazol-4-yl)propanoic acid; DG, dentate gyrus; DPN, (4-hydroxyphenyl)-propionitrile; EPSP, excitatory postsynaptic potential; ER, estrogen receptor; E2, estradiol; KO., Knockout; LTD, long-term depression; LTP, long-term potentiation; PBS, phosphate-buffered saline; PPT, (propyl-pyrazole-trinyl)tris-phenol; PRE, presynaptic membrane-rich fraction; PSD, postsynaptic density; SDS, sodium dodecyl sulfate.

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N-methyl-D-aspartate (NMDA)-induced long-term depression (LTD) in CA1 of the wild type mouse hippocampus. In contrast, estradiol did not enhance NMDA-LTD in ERαKO mice, indicating the involvement of ERα in the estrogen signaling.

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1. Introduction

In addition to slow/genomic (within days) functions of 17β-estradiol, rapid (within 2 h) modulation of synaptic plasticity by estradiol has been attracted much attention in relation to memory performance (Bi et al., 2000; Foy et al., 1999; Ooishi et al., 2012; Vouimba et al., 2000). Estradiol plays an essential role not only in the female but also in the male hippocampus, because the male rat hippocampus endogenously synthesizes several-fold more estradiol than the female hippocampus does (Higo et al., 2009; Hojo et al., 2004; Hojo et al., 2008; Kato et al., 2013; Kawato et al., 2002; Kretz et al., 2004). To clarify rapid synaptic modulation, the molecular mechanisms of estrogen signaling should be identified. Analysis of rapid spinogenesis suggests participation of synaptic estrogen receptors such as estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Estradiol rapidly increased the spine density of male rat hippocampal glutamatergic neurons in CA1 (Mukai et al., 2007; Murakami et al., 2006). ERα agonist, propyl-pyrazole triol (PPT), but not ERβ agonist, diarylpropionitrile (DPN), showed the increase in the spine density. Synaptic localization of ERα was demonstrated in rat hippocampal glutamatergic neurons by immunoelectron microscopic analysis (Mukai et al., 2007). Localization of ERβ at synapses is also observed in rat hippocampal glutamatergic neurons by immunoelectron microscopic analysis (Milner et al., 2005). These synaptic ERα and ERβ are good candidates which mediate rapid modulation by estrogen. Different from rats, however, only a few studies have successfully showed the estradiol effect on spinogenesis in mouse slices, partly due to difficulty in the staining of mouse spines with a suitable dye (Li et al., 2004).

Electrophysiological investigation is another sensitive method to examine rapid effects of estradiol at synapses within 2 h. Estradiol exerts a rapid influence on hippocampal long-term potentiation (LTP) in CA1 (Bi et al., 2000; Foy et al., 1999; Ooishi et al., 2012), long-term depression (LTD) in CA1 (Mukai et al., 2007; Vouimba et al., 2000) or kainate current in CA1 (Gu and Moss, 1996; Gu et al., 1999). ERα agonist but not ERβ agonist induces rapid enhancement of the NMDA-induced LTD, indicating that ERα drives the enhancement of LTD (Mukai et al., 2007).

To directly examine different roles of ERα and ERβ in modulating synaptic plasticity, we here use ERαKO and ERβKO mice. Two representative and different types of synaptic modulation (spinogenesis and LTD) by estradiol are investigated. Selective ERKO mice are key tools to identify the role of each estrogen receptor, because a widely used inhibitor ICI is not selective for ERα or ERβ, and ICI cannot suppress estradiol-induced rapid modulation of LTD or kainate currents (Gu et al., 1999; Mukai et al., 2007). Using male ERKO mice, we successfully demonstrate that both spinogenesis and LTD are enhanced through ERα (not through ERβ). Time-lapse imaging enables us to confirm the estradiol-induced increase in spine density in the live hippocampus by using mice which express yellow fluorescent protein (YFP). This analysis also reveals dynamic nature of spinogenesis.

2. Results

2.1. *Rapid effect of estradiol on spinogenesis in hippocampal slices from estrogen receptor KO mice*

We analyzed the mechanisms of estradiol for modulation of spine density and morphology. Following a 2 h treatment with 10 nM estradiol, we compared spines with those in control samples (i.e. with no estradiol). To investigate different contributions of ERα and ERβ to spinogenesis, spines of glutamatergic neurons in CA1 of hippocampal slices prepared from ERαKO and ERβKO mice were compared with those in wild type mice. From dose-dependent and time-dependent analysis of spines, a 2 h treatment with 10 nM estradiol was most effective on spinogenesis in the hippocampal CA1 region (Mukai et al., 2007).

In wild type mice, a 2 h treatment with 10 nM estradiol increased the total spine density from 1.34 to 1.61 spines/μm (Figs. 1A and 2A). Blocking phosphorylation of Erk MAP kinase (MAPK) by 25 μM U0126 (Favata et al., 1998; Ooishi et al., 2012) completely prevented estradiol-induced spinogenesis. The significant increase in spine density was not observed in ERαKO mice (1.28 spines/μm for control and 1.39 spines/μm for estradiol) (Figs. 1B and 2B). We also observed the estradiol-induced increase of total spine density from 1.26 to 1.56 spines/μm in ERβKO mice (Figs. 1C and 2C). There was no significant difference of spine density between all types of mice (wild type, ERαKO and ERβKO mice) without estradiol treatments.

To assess morphology of the increased spines, we analyzed the distribution of spine head diameter with and without a 2 h estradiol treatment. In wild type mice, a significant increase from 0.63 to 0.88 spines/μm occurred only in the middle-head spines, having head diameter between 0.3 and 0.4 μm (Fig. 3A). Blocking Erk MAPK by U0126 prevented this estradiol-induced increase of middle-head spines. In ERαKO mice, there was no increase in the density of the middle-head spine (0.56 spines/μm for control and 0.58 spines/μm for estradiol) (Fig. 3B). The density of the middle-head spine was also increased significantly in ERβKO mice from 0.53 to 0.78 spines/μm (Fig. 3C). On the other hand, the density of small-head (< 0.3 μm in diameter) and large-head (> 0.4 μm) spines were not significantly changed by the presence of estradiol in all types of mice (wild type, ERαKO and ERβKO).

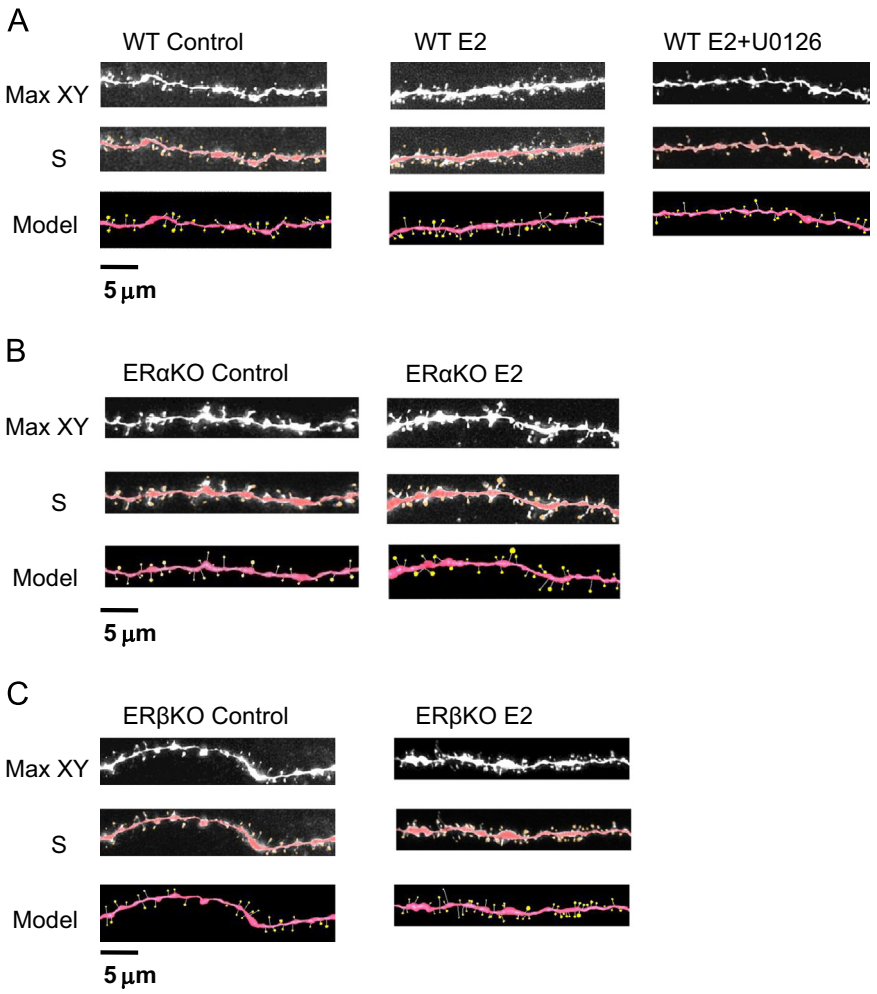

Fig. 1 – Representative images of confocal micrographs; showing spines along the secondary dendrites of hippocampal CA1 pyramidal neurons. (A) wild type mice (WT), (B) ERαKO mice (ERαKO), and (C) ERβKO mice (ERβKO). Spines without drug-treatments (Cont) and spines along dendrite after estradiol treatment for 2 h (estradiol, E2), and treatments with estradiol plus U0126 (E2+U0126). Maximal intensity projection onto XY plane from z-series confocal micrographs (Max XY), image analyzed by Spiso-3D (S) and 3 dimensional model (Model) are shown for all the cases. Bar, 5 μm.

2.2. *Time lapse imaging analysis of estradiol-induced spinogenesis using live slices of YFP mice*

In the former section, we statistically analyzed the data of spines after fixation of slices treated with or without estradiol. To obtain a dynamic nature of the acute estradiol effect on spinogenesis, we performed time-lapse imaging of spines in live hippocampal slices prepared from adult male YFP mice. Recurrent images from the same dendrite were collected every 30 min during 10 nM estradiol perfusion for 2 h. Appearance of new spines (red arrows) as well as disappearance of spines (blue arrows) were observed (Fig. 4A and B).

During the perfusion of estradiol for 2 h, the number of newly appeared spines (0.24 spines/μm/2 h) was significantly larger than that of disappeared spines (0.05 spines/μm/2 h), resulting in the net increase of the spine density (Fig. 4C). In case of appearance of spines, first, spine protrusions, without distinct head structure, grew up to spines with distinct head. In opposite cases, spines lost head structure and became protrusions, and finally disappeared. As another example of fluctuation, complex deformations of spine shapes were observed (green arrows). In the absence of estradiol, no difference in spine numbers was observed between appeared and disappeared spines. From these observations, these dynamic changes of individual spines also might occur in slices shown in Figs. 2 and 3, during the estradiol treatments before fixation, resulting in the net increase of spine density.

2.3. *Rapid effect of estradiol on LTD*

Electrophysiological investigations were performed to analyze the effect of estradiol on LTD. LTD was investigated in CA1 of adult male hippocampal slices. Recordings were performed using novel 64 multi-electrodes particularly arranged to stimulate the Schaffer collaterals in the stratum radiatum of CA1. LTD was induced pharmacologically by the transient application (3 min) of NMDA. Upon application of 30 μM NMDA, the maximal amplitude of excitatory postsynaptic potential (EPSP) was transiently decreased to a minimal value, and then recovered to reach an approximate plateau level (< 100%) within 50–60 min after

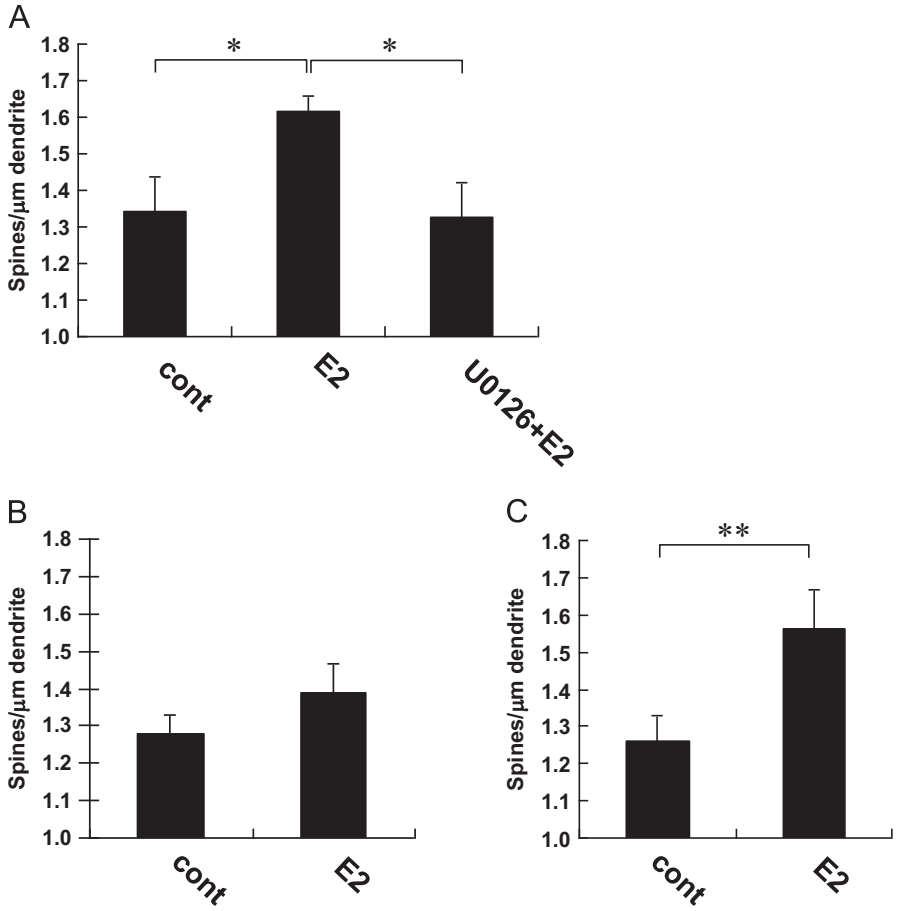

Fig. 2 – Effect of 10 nM E2 treatments on the total spine density in CA1 neurons. Vertical axis is the average number of spines/μm in (A) wild type mice, (B) ERαKO mice, and (C) ERβKO mice. A 2 h treatment in ACSF without drugs (Cont), with 10 nM E2 (E2), or with 25 μM U0126 plus 10 nM E2 (U0126+E2) $^{*}p<0.05$; $^{**}p<0.01$.

NMDA application, indicating LTD establishment. In wild type mice, the plateau EPSP amplitude at 60 min was 78% (Fig. 5A). Perfusion of 10 nM estradiol initiated 30 min before NMDA application significantly enhanced LTD, resulting in the EPSP amplitude of 64%. One nM estradiol had no effect. In ERαKO mice (having ERβ), the EPSP amplitude of LTD was 78%, and 10 nM estradiol perfusion suppressed LTD up to 90% (Fig. 5B), implying that ERα was essential for LTD enhancement in wild type mice. This suppression effect of LTD by estradiol in ERαKO mice was similar to the ERβ agonist-induced suppression of LTD in the rat hippocampus (Mukai et al., 2007), suggesting a possible contribution of ERβ. The effect of estradiol on LTD was also analyzed in hetero knockdown mice, ERα (+/–) and ERβ (+/–) (Fig. S1).

2.4. *Immunohistochemistry of ERα in the hippocampus*

Immunohistochemical staining of ERα proteins with RC-19 antibody was performed to determine the expression and cell-specific localization of ERα in the hippocampus prepared from wild type, ERαKO and ERβKO mice. RC-19 is a home-made affinity column-purified antibody raised against the 19 C-terminal residues of ERα which is identical between rats and mice (Mukai et al., 2007). An intense immunoreactivity was observed in wild type and ERβKO mice (Fig. 6A, C). Staining of ERα is localized in extranuclear, as well as nuclear regions of principal neurons in CA1, CA3 and the dentate gyrus (DG) regions. No positive immunoreactivity was obtained in ERαKO mice (Fig. 6B). Note that RC-19 immunoreactivity was weak in glial-like cells.

2.5. *Western blot analysis of ERα*

To further confirm the expression of ERα protein in the mouse hippocampus, we performed western blot analysis using RC-19 antibody. The hippocampus, hypothalamus, cerebral cortex and cerebellum were prepared from all types of mice (wild type, ERαKO and ERβKO mice) (Fig. 7A). In both wild type and ERβKO mice, only a single ERα band with a molecular mass of approx. 67 kDa was observed in the hippocampus as well as in the cerebral cortex and hypothalamus. The level of expression for ERα was almost same between hippocampi prepared from wild type and ERβKO mice. In ERαKO mice, no appearance of ERα was confirmed.

To assess the synaptic localization of ERα protein, we prepared several fractions such as post synaptic density (PSD), presynaptic membrane-rich fraction (PRE), high density membranes (HDM), cytoplasm (CYT) from whole brains except for cerebellum of wild type mice using a combination of density gradient centrifugations. Using RC-19, only a single protein band was observed in the PSD, PRE, HDM and CYT fractions (Fig. 7B).

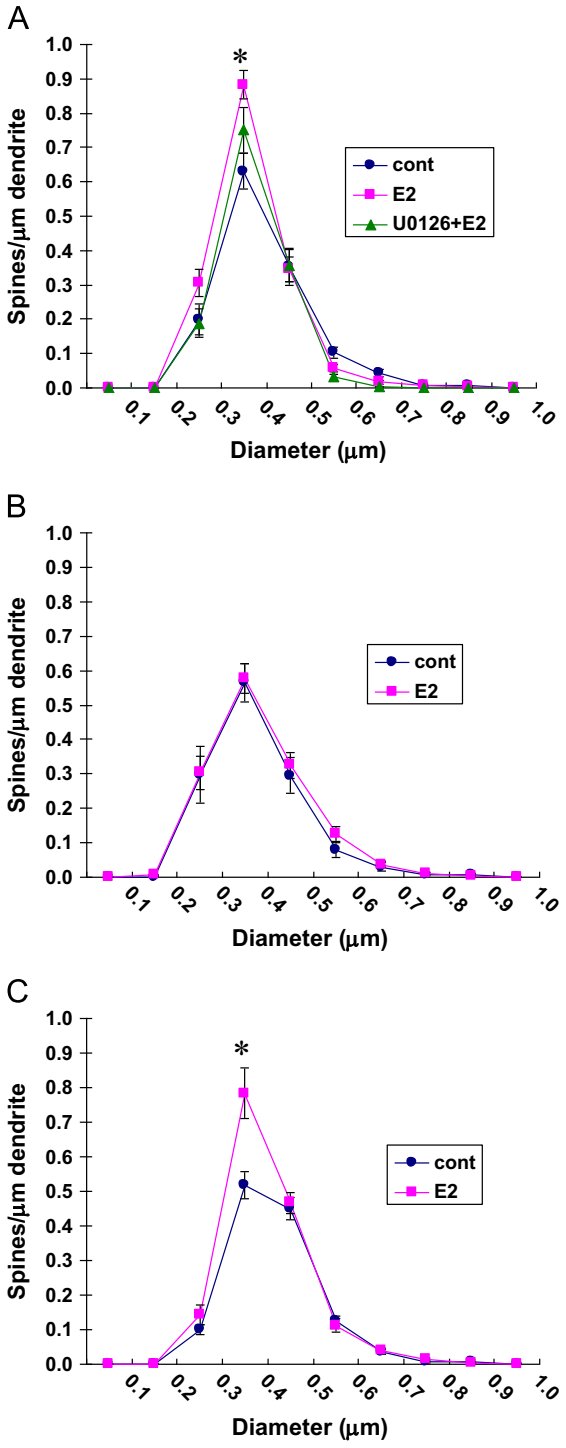

Fig. 3 – Histogram of spine head diameters after a 2 h treatment in ACSF without drugs (blue), with 10 nM E2 (red), or with 25 μM U0126 plus 10 nM E2 (green) in wild type mice (A), ERαKO mice (B) and ERβKO (C) mice. Horizontal axis is the spine head diameter and vertical axis is the average number of spines/μm $^{*}p<0.05$.

Extended analysis indicated that ERα was certainly localized in the PSD fraction (characterized by PSD-95) and PRE (characterized by synaptophysin). We did not detect ERα in other fractions such as myelin, mitochondria or dendritic raft.

3. Discussion

The current study clearly demonstrates different roles of ERα and ERβ in rapid modulation of spinogenesis and LTD by estradiol.

Estradiol-induced rapid spinogenesis: mice and rats study

In earlier study, we demonstrated that the application of the ERα selective agonist PPT, but not the ERβ selective agonist DPN, increased spine density in the CA1 region of the rat hippocampus. Furthermore, the ERα/ERβ antagonist ICI-182780 inhibited estradiol-induced spinogenesis (Mukai et al., 2007). These results indicated the involvement of ERα in the increase of spine density in rats. We here advanced the study from pharmacological investigations in rats to genetic investigations by using ERαKO and ERβKO mice, and we clearly demonstrated that the spinogenesis was modulated through ERα, but not ERβ.

The estradiol-induced enhancement of spinogenesis was completely suppressed by MAPK inhibitor, indicating the involvement of MAPK in the signaling in mice as well as in rats. These results suggest that the estradiol-induced spinogenesis in mice is mediated by the same mechanism as that in rats. Accumulating evidence indicates that Erk MAPK is involved in a rapid, ER-dependent signaling pathway of estrogen (Lee et al., 2004). Erk MAPK is known to phosphorylate cortactin, a structural protein associated to actin (MacQueen et al., 2003). Cortactin interacts with both F-actin and actin-related protein (Arp) 2/3 complex as well as scaffold protein Shank in the PSD at the SH3 domain (Campbell et al., 1999; Weed et al., 1998), resulting in promotion of actin fiber remodeling within spines (Hering and Sheng, 2003; Racz and Weinberg, 2004). As a good example, upon BDNF stimulation, MAPK phosphorylates cortactin through interacting C-terminal of SH3 domain, resulting in a reorganization of spine morphology (Iki et al., 2005). Therefore it is probable that estradiol exerts its effect on spines through cortactin-actin pathway.

Besides cortactin, cofilin and LIM kinase (LIMK) are also good candidates for estrogen-induced actin reassembly, leading to spinogenesis (Aizawa et al., 2001; Clancy et al., 1992; Liston et al., 2013). It is known that corticosterone induces the phosphorylation of both LIMK and cofilin, leading to spinogenesis (Liston et al., 2013). Cofilin polymerizes actin filaments upon phosphorylation by LIMK (Clancy et al., 1992). RhoA may phosphorylate LIMK (Pilpel and Segal, 2004; Shi et al., 2009).

3.1. *Comparison of the estradiol-effect on density and diameter of spines between mice and rats*

In the mouse hippocampus, estradiol increased the total spine density by approx. 1.2-fold while it increased rat spine density by 1.4-fold. Spine head diameter was also demonstrated to be significantly affected by short (within 2 h) estradiol treatments in both mouse and rat hippocampus. In mice, the density of the middle-head spines (0.3 - 0.4 μm in head diameter) was significantly increased, while the density of large-head spines (> 0.4 μm) or small-head spines (< 0.3 μm) were not significantly altered. For the current estradiol effects, the classification with these three categories is much more useful than the classification with only two categories (e.g., thin and mushroom). Small-, middle-, and large-head spines may be different in the number of 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA)

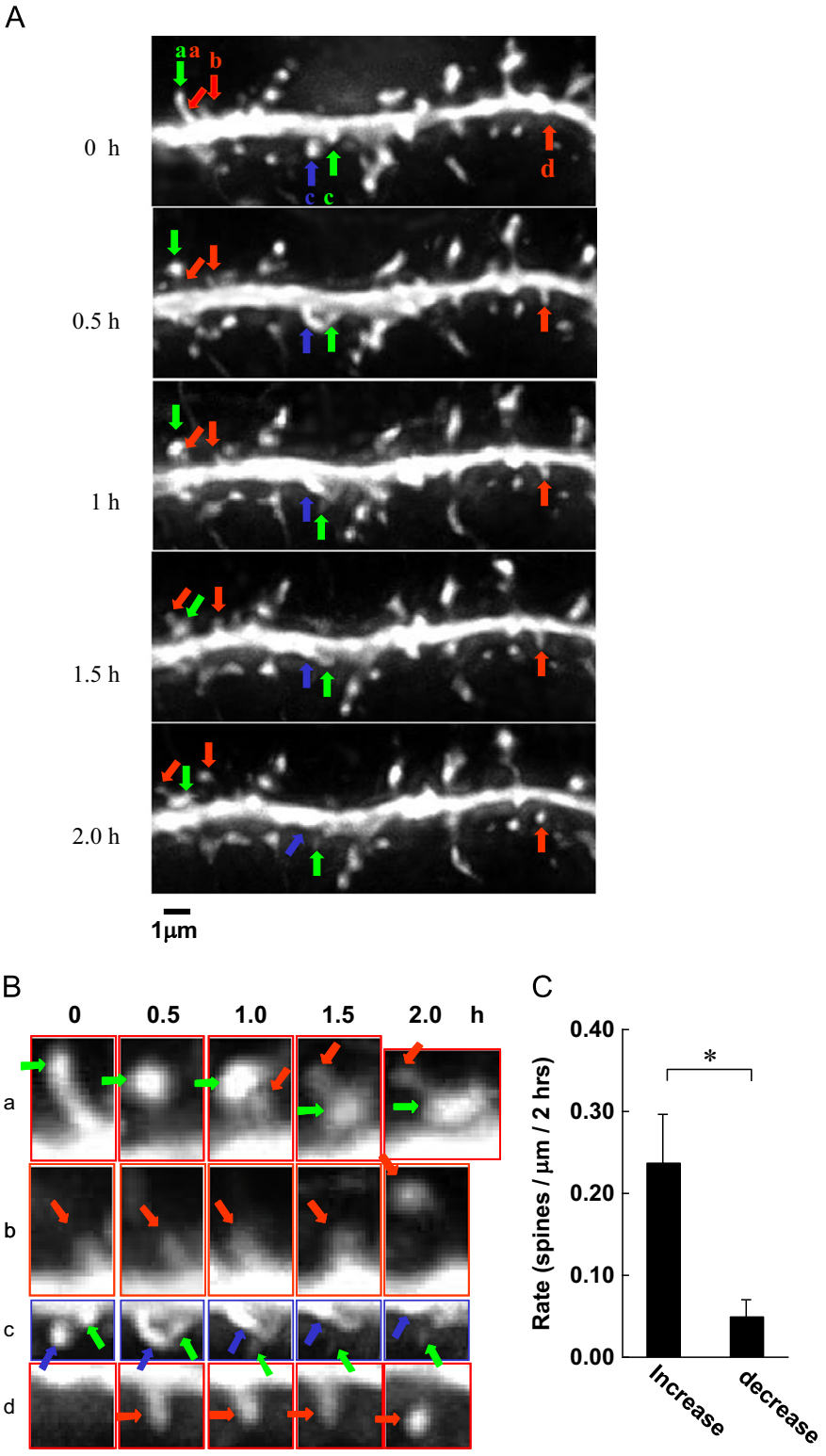

Fig. 4 – Typical direct tracing of estradiol-induced spinogenesis by time-lapse imaging of live hippocampal slices from YFP mice. Images were taken at t=0, 0.5, 1, 1.5 and 2 h from the beginning (t=0 h) of the 10 nM estradiol perfusion. (A) Spines along the same secondary dendrite of live hippocampal slice. (B) Magnified images of the spines. Newly appeared spines (a, b, d; red arrows) and disappeared spines (c; blue arrows) were indicated. Green arrows indicate spines, that were present over the 2 h time window, with dynamically changing shape. (C) The rate of increase and decrease of spines under the presence of estradiol ($n=4$). Vertical bar is the rate of increased spines (spines / μm / 2 h). Bar 1 μm $^{*}p<0.05$.

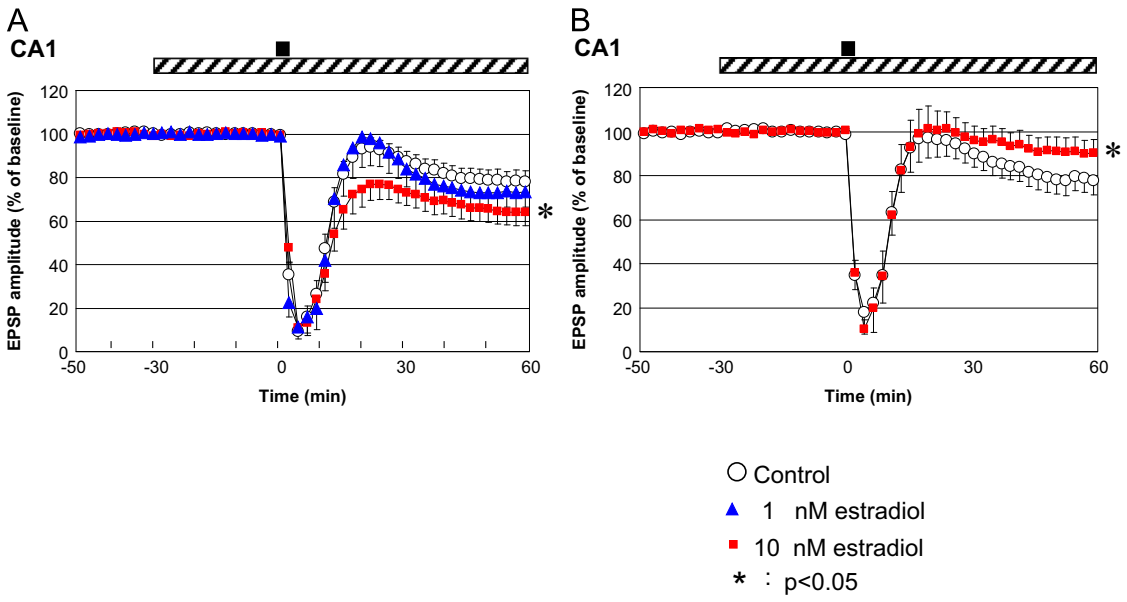

Fig. 5 – Rapid modulation of LTD by estradiol in the CA1 region for (A) wild type and (B) ERαKO mice. Estradiol concentration was 0 nM (open circle, $n=17$) and 1 nM (blue triangle, $n=8$) and 10 nM (red filled square, $n=10$), respectively; n is number of independent slices. Vertical axis indicates maximal amplitude of EPSP. Here, 100% refers to the EPSP value at $t=-40$ min prior to NMDA stimulation, irrespective of the test condition. LTD was induced by 30 μM NMDA perfusion at time $t=0$–3 min (black square above the graph). Hatched bar above the graph indicates period of time during which estradiol was administered. The significance of the estradiol effect was confirmed at 60 min with statistical analysis using ANOVA ($^{*}p<0.05$; $^{**}p<0.01$) as indicated in the figure. Illustrated data points and error bars represent the mean ± SEM from independent slices.

receptors, and therefore these three types of spines may have different efficiency in signal transduction. The number of AMPA receptors (including GluR1 subunit) in the spine increases as the size of postsynapse increases, whereas the number of NMDA receptors (including NR2B subunit) may be relatively constant (Shinohara et al., 2008).

In the rat hippocampus, estradiol increased small spines having the head diameter below the average diameter (Ishii et al., 2007; Mukai et al., 2007). The average head diameter of mouse spines are smaller than those of rat spines. Approximately about 22% of rat spines had the head diameter more than 0.5 μm, but in mice only 12% of spines had such a large head diameter.

3.2. *Individual spines dynamically fluctuate*

Time lapse imaging of spines from YFP mice showed the dynamic modulation of spine density and morphology by estradiol in the adult hippocampus by tracing the same spines at every 30 min. In time lapse imaging of control slices, although both appearances and disappearances of spines were observed, the rate of appearance and disappearance of spines is identical, resulting in the equilibrium state without any stimulation. In the presence of estradiol, the equilibrium state is shifted toward the increased level of spine density, however, the rate of appearance and disappearance of spines is again balanced, resulting in statistical increase in the spine density. Interestingly, not only small-head spines but also middle- and large-head spines changed their head structures in adult slices, suggesting that the stability may not be always proportional to sizes.

The current results are consistent with the estradiol-induced increase of the spine density using fixed slices (fixed after 2 h estradiol treatments), but the fixed slice analysis has weak points, due to its statistical comparison between different slices. Time lapse imaging of the same spines is a direct analysis. Although density and shapes of spines dynamically fluctuate naturally in live slices (Murphy and Segal, 1996; Yuste and Bonhoeffer, 2004), the average spine density does not significantly change during slice incubation over 1–5 h without drugs (Mukai et al., 2007).

3.3. *Difficulty in dye-injection staining of mouse spines prevents investigations in mice*

Fluorescent dye-injection staining in hippocampal slices is a good method to visualize spine heads using z-series confocal images. To our surprise, fluorescence staining of mouse hippocampal neurons by dye-injection was much more difficult than that in rats, because of the faster leakage of dyes from dendrites of neurons in mice (leaking within 10 h) in comparison with rats (retaining dyes stably for 2–3 weeks). To overcome these problems, we performed confocal imaging within 2 h after the injection of Alexa Fluor 488, before significant dye leakage occurred. Alexa Fluor 488 was used because it has stronger fluorescence intensity than Lucifer Yellow often used in previous studies of rats (Mukai et al., 2007).

Partly due to the staining difficulty, only a few studies demonstrate estradiol effects in mice dendritic spines in brain slices. Using golgi staining, instead of fluorescent staining, slow

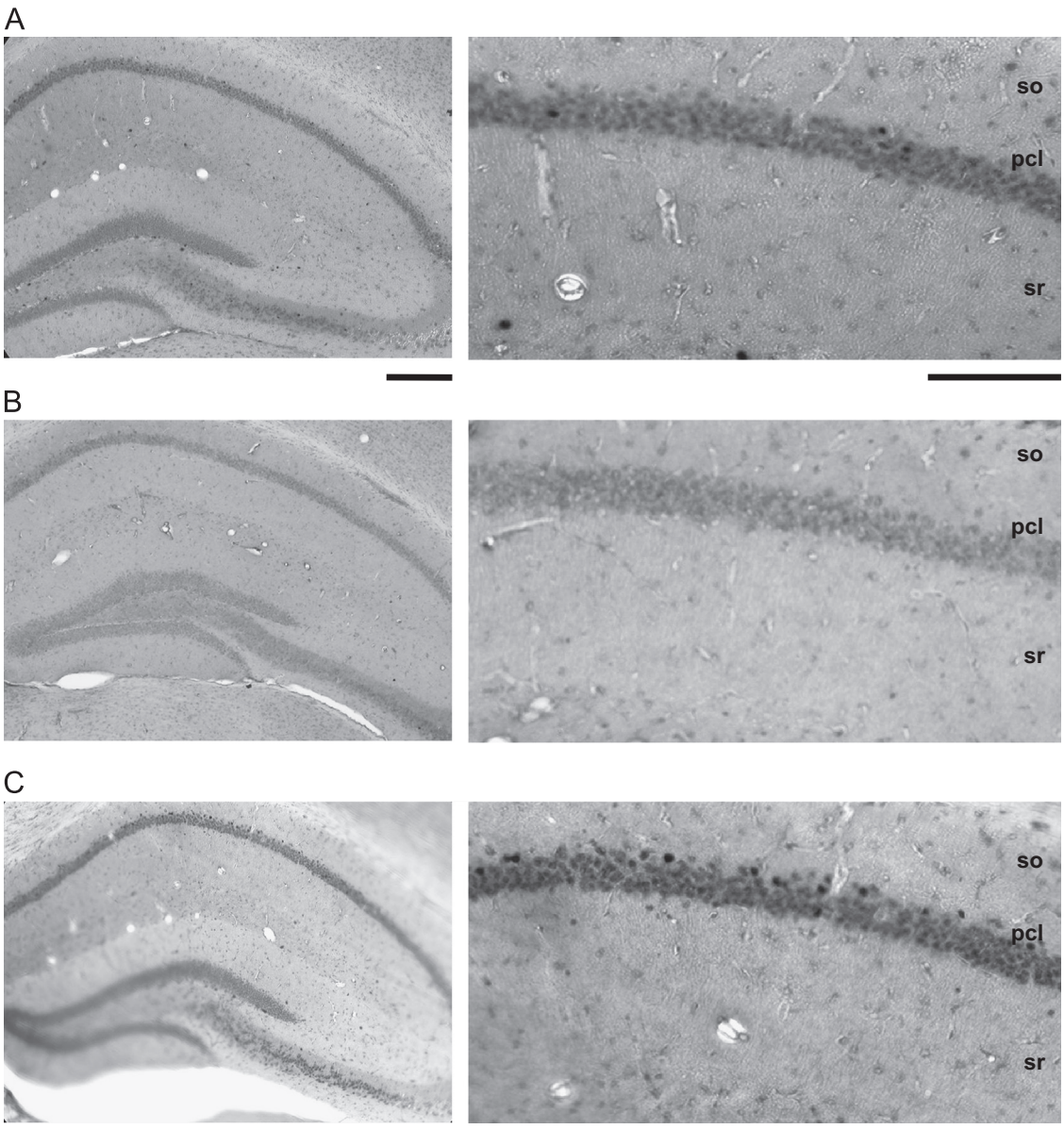

Fig. 6 – Immunohistochemical staining of ERα with RC-19 antibody in hippocampal slices of (A) wild type, (B) ERαKO and (C) ERβKO mice. Left; Coronal section of the whole hippocampal formation. Right; Magnified images of the CA1 region of the hippocampus. Abbreviations: so, stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. Scale bar. 200 μm in left and 100 μm in right. Several independent experiments were performed.

effects of estradiol on spines are shown *in vivo* in the mouse hippocampus (Li et al., 2004). Estrogen supplement for five days in ovariectomized (OVX) female mice increase the density of CA1 spines with the head diameter larger than 0.3 μm. These results suggest the slow rescue effect by estradiol from ovariectomy-induced decreased level of the spine density. Electron micrographic investigations of spine-synapse are performed for female mouse neonatal slice cultures. By using ERαKO and ERβKO mice hippocampal cultures, ERα but not ERβ was shown to contribute spine-synapse increase upon estradiol supplementation (Zhou et al., 2014). Indirect effects of estradiol may also occur *in vivo* through cholinergic or serotonergic neurons, which project to the hippocampus, in addition to the direct influence of estradiol on glutamatergic neurons (Leranth et al., 2000; Leranth et al., 2003).

3.4. *Rapid estradiol effect on LTD: mice and rats analysis*

In the current study, involvement of ERα in the modulation of LTD was clearly demonstrated using ERαKO mice which are genetically devoid of the receptor. These results add further confidence to previous studies in rats showing the involvement of ERα in enhancement of LTD by using selective estrogen receptor agonists (Mukai et al., 2007). An ERα agonist, PPT, induced the enhancement of LTD, however, the ERβ agonist, DPN, suppressed LTD in CA1. In the current study, the enhancement effect of LTD by estradiol was abolished in ERα (+/-) where the expression of ERα was reduced to approx. 50% of that in wild type mice as revealed by Western blot analysis (Fig. S2). In ERα (-/-) mice, estradiol effect through ERβ suppressed LTD, suggesting that only ERβ activation could abolish LTD itself. This effect is

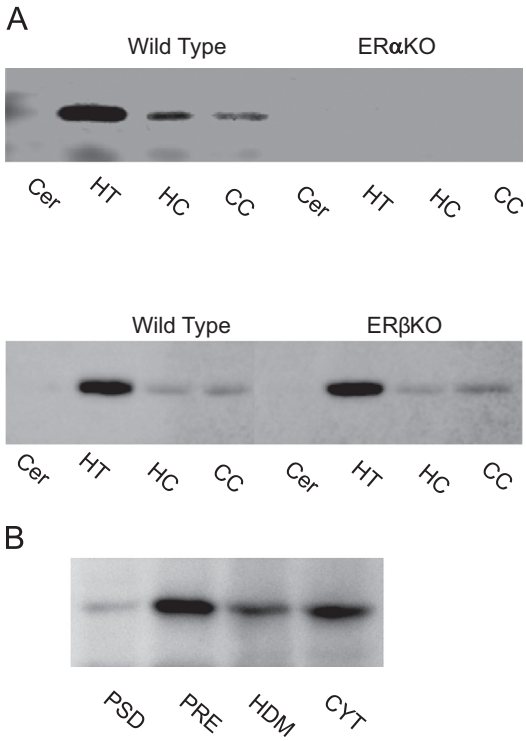

Fig. 7 – (A) Upper panel; Staining of ERα with RC-19 in tissue homogenates from wild type and ERαKO mice. From left to right, cerebellum (Cer), hypothalamus (HT), hippocampus (HC) and cerebral cortex (CC) from wild type mice, Cer, HT, HC and CC from ERαKO mice. Lower panel; Staining of ERα with RC-19 in tissue homogenates from wild type and ERβKO mice. From left to right, Cer, HT, HC and CC from wild type mice, Cer, HT, HC and CC from ERβKO mice. The amount of protein applied to each lane was 20 μg. (B) Staining of ERα with RC-19 in the subcellular fractions from the wild type mouse brain. From left to right, postsynaptic density fraction (PSD), presynaptic membrane-rich fraction (PRE), high density membrane fraction (HDM) and cytoplasmic fraction (CYT). The amount of protein applied to each lane was 20 μg.

consistent with the previous result showing suppression of LTD by DPN, ERβ-specific agonist. On the other hand, estradiol still enhanced LTD in ERβ (+/-) mice, where the expression of ERβ is supposed to be reduced but no change in ERα expression. Taken together, ERα showed a predominant role in the estradiol-induced enhancement of LTD.

Concerning the involvement of ERα in rapid estradiol effects, important investigations were performed using knock-down mice by Moss and coworkers (Gu and Moss, 1996; Gu et al., 1999), showing that no essential contribution of ERα to estradiol-induced rapid enhancement of the kainate currents in CA1 neurons. They reached this conclusion due to the very small difference in estradiol effects on the kainate currents between wild type and ERα-Neo KO mice which have been constructed by the method of Neomycin insertion into exon 1 (the previously named exon 2) (Couse et al., 1995). However, in Neomycin-inserted ERα-Neo KO mice express N-terminal-modified ERα (61 kDa) (Couse et al., 1995; Kos et al., 2002; Pendaries et al., 2002). Since the N-terminal-modified ERα has been demonstrated to be active on estradiol binding and show genomic responses (Chen et al., 2009; Kos et al., 2002; Pendaries et al., 2002), the participation of ERα to electrophysiological properties in CA1 cannot be excluded from their investigations (Chen et al., 2008; Couse et al., 1995; Kos et al., 2002; Pendaries et al., 2002).

3.5. *Different effects of estradiol on spinogenesis and LTD*

Estradiol increased spines within 2 h, and LTD are enhanced within 1.5 h. The newly appeared spines might not affect LTD within this time range, since new spine-synapse formation may require 12–24 h (Pozzo-Miller et al., 1999). Newly appeared spines may be unpaired free spines (spines without forming synapses) within 1–2 h. Therefore, LTD modulation should be performed in pre-formed synapses. Modulation pathway is not known at the moment, but one hypothesis might be "synaptic ERα→MAPK activation →phosphorylation of NMDA receptor (NR2B)→moderate increase in Ca influx under NMDA stimulation→activation of calcineurin→LTD enhancement". On the other hand, synaptic ERβ might deactivate MAPK under moderate Ca influx (~0.8 μM) (Lee et al., 1998), resulting in LTD suppression.

3.6. *Estradiol levels in the hippocampus*

The concentration of endogenous estradiol is determined to be approx. 8 nM in a freshly isolated adult male hippocampus by mass-spectrometric analysis (Hojo et al., 2008; Hojo et al., 2009). However, it should be noted that in order to obtain 'acute' slices (often used for analysis of synaptic plasticity) by incubation of hippocampal slices with artificial cerebrospinal fluid (ACSF), the levels of estradiol decreased to below 0.5 nM (Fig.S3) (Hojo et al., 2011). This situation is the same for all control slices of wild-type and ERKO mice. Therefore, the total spine density was nearly the same between control slices of wild-type, ERαKO and ERβKO mice, within experimental error (Fig. 2). In this study, the exogenous application of 10 nM estradiol was used to elevate the hippocampal estradiol level from the steroid-depleted level in 'acute' slice (< 0.5 nM), in order to rapidly revert to the endogenous level. Exogenous application of estradiol was employed to modulate hippocampal estrogen levels, because until now the method of rapid elevation of endogenous estradiol concentration through activation of its synthesis enzymes is not yet established. In such acute slices without estradiol, ER and kinases are probably not endogenously activated. In physiological conditions *in vivo*, hippocampal estradiol (8 nM) may significantly regulate ER, kinases and spines.

3.7. *Difference in estradiol level between female and male hippocampus*

In female, the level of endogenous estradiol is approx. 1 nM (much lower than 8 nM) in a freshly isolated hippocampus, determined by mass-spectrometric analysis (Kato et al., 2013). Concerning slow genomic processes (~ one day treatments), it was shown that 10 mg letrozole/kg body weight suppressed hippocampal LTP in adult female (12 week-old) but not in male (Vierk et al., 2012). These results could be interpreted in the following way: Supplementation of 10 mg letrozole/kg completely suppressed the function of a low level estradiol

(~1 nM) in female, but only partially suppressed the function of a high level estradiol (~8 nM) in male. A higher dose of letrozole (> 10 mg letrozole / kg b.w.) may be necessary to completely suppress male LTP.

3.8. *Function of ERβ*

Agreement is still not obtained for ERβ functions. ERβ was shown not to contribute to increase in spine-synapses (Zhou et al., 2014) and in dendritic spines (Phan et al., 2011; the current study). On the other hand, in female OVX adult rats, several earlier studies showed that ERβ played an important role in morphology change of spines, which was found by treatments of ERβ agonist, WAY200070 (Kramar et al., 2009; Liu et al., 2008). We observed that ERß agonist DPN rescued CORT-induced suppression of LTP in male hippocampus (Ooishi et al., 2012). Thus, clarification of ERβ functions in synaptic plasticity awaits further studies.

3.9. *Age dependent difference in LTD modulation by estradiol*

Grassi, Petorrossi and co-workers (Pettorossi et al., 2013) show that blocking androgen receptor AR by flutamide converts LTD (upon low frequency electric stimulation) to LTP in very young hippocampal slices (two to three week old) and in adolescent hippocampal slices (seven week-old). They show that blocking estrogen receptor ER by ICI suppresses LTP, but not affects LTD. They suggest that estradiol is necessary for LTP and testosterone is necessary for LTD in rats that are younger than adolescents. We cannot directly compare their no LTD modulation by estradiol (in very young brain) and the current chemical-LTD modulation by estradiol (in adult brain). LTD cannot be induced by low frequency stimulation in nine week-old adult mouse hippocampus (corresponding to approx. 12 week-old adult rat hippocampus). Therefore we used chemical-LTD by 3 min NMDA perfusion. Concerning chemical-LTD, we observed that the perfusion of testosterone (10 nM) and dihydro-testosterone (DHT) (10 nM) prior to 3 min NMDA perfusion did not affect LTD in CA1 (data not shown). The discrepancy between the current results and results of Grassi and Petorrossi may be due to the age of hippocampus, since estradiol effects are very different between different ages. Their experimental conditions (using two to three week-old and seven week-old rats, corresponding to one to two week-old and five week-old mice, approximately) are very different from the conditions of current experiments (nine week-old adult mice). Response to estradiol is very different between two week-old rat hippocampus and 12 week-old rat hippocampus. In two week-old rat hippocampus, estradiol supply immediately elevates EPSP baseline, and enhances LTP upon high-frequency stimulation, while in 12 week-old rat hippocampus (corresponding to approx. nine week-old mouse) estradiol has no effect on LTP-induction, nor on EPSP baseline (Ooishi et al., 2012). Importantly in adult stage, LTD is used to delete wrong synaptic memory for rewriting new memory (Migaud et al., 1998). The current results suggest that estradiol could facilitate this deletion of wrong memory. In addition, we recently found that testosterone and DHT suppressed estradiol-supported LTP-induction upon sub-threshold stimulation in adult rat hippocampal slices (Hasegawa et al., 2014 in this Issue of Brain Research). Therefore in such a condition testosterone and DHT work as suppressors.

In summary, the current study using ERα and ERβ knockout mice confirmed the involvement of synaptic ERα but not ERβ in the rapid spinogenesis and enhancement of LTD induced by estradiol in glutamatergic neurons of hippocampus, in which ERα is expressed in post synapses. These results advance the previous rat study using selective agonists of ERα and ERβ.

4. Materials and methods

4.1. *Animals*

ERα knockout mice [ERα (–/–)] were obtained by inbreeding ERα (–/+) mice. The development of ERαKO mice was accomplished by deleting the whole exon 2 of the mouse ERα gene (Dupont et al., 2000). Note that nomenclature of ERα exon changed recently, and the current exon 1 and exon 2 (Kos et al., 2002; Pendaries et al., 2002) correspond to the previous exon 2 and exon 3, respectively. ERβ knockout mice [ERβ (–/–)] were obtained by inbreeding ERβ (–/+) with ERβ (–/–) mice. The development of ERβKO mice was accomplished by disruption within exon 3 of the mouse ERβ gene (Dupont et al., 2000). Cg-TgN transgenic mice expressing YFP under the *Thy1* promoter were purchased from The Jackson Laboratory (ME, USA) and were mated with C57BL/6 J mice.

The experimental procedure of this research was approved by the Committee for Animal Research of Univ of Tokyo.

4.2. *Chemicals*

N-methyl-D-aspartate (NMDA), cyano-nitroquinoxaline-dione (CNQX), 17β-estradiol, ICI 182,780, (Propyl-pyrazole-trinyl)tris-phenol (PPT), (4-hydroxyphenyl)-propionitrile (DPN) and U0126 were purchased from Sigma (MO, USA). Alexa fluoro 488 was obtained from Molecular Probes (OR, USA).

4.3. *Imaging and analysis of dendritic spines*

Experimental set up 1, for spine analysis in fixed hippocampal slices. Adult male mice aged nine weeks were deeply anesthetized and decapitated. The brains were removed and placed in cutting solution. Cutting solution consisted of (mM): 120 Choline chloride, 3.0 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 8.0 $MgCl_2$ and 25 glucose, and was equilibrated with 95% O_2 / 5% CO_2. The hippocampus was then dissected and 300 μm thick transverse ventral slices to the long axis, from the middle third of the hippocampus, were prepared with a vibratome (Dosaka, Japan). Hippocampal slices were transferred into an incubating chamber containing ACSF and held at 25 °C for 2 h for slice recovery. 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. These widely used 'acute' slices were then incubated with or without 10 nM estradiol. Next, the slices were pre-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C

overnight. Neurons within slices were visualized by an injection of Alexa fluoro 488 (Molecular Probes, Eugene, OR, USA) under a Nikon E600FN microscope (Japan) equipped with a C2400–79 H infrared camera (Hamamatsu Photonics, Japan) and with a 40× water immersion lens (Nikon). Dye was injected with a glass electrode whose tip was filled with 5% Alexa fluoro 488 for less than 10 min, using Axopatch 200B (Axon Instruments, CA, USA). Approximately five neurons under the surface of a slice were injected.

Experimental set up 2, for time-lapse imaging of spines in live hippocampal slices. Hippocampal 'acute' slices were prepared from YFP mice for estradiol treated groups with the same procedures written in *Experimental set-up 1*, and transferred to a measurement glass chamber (under the confocal microscope). After 2 h recovery, confocal imaging was performed for these live slices under perfusion with ACSF containing 10 nM estradiol. To obtain dynamic change of spines, images were taken every 30 min for 2 h.

4.4. Confocal laser microscopy and morphological analysis

The imaging was performed from sequential z-series scans with a LSM5 PASCAL confocal microscope (Carl Zeiss, Germany) at high zoom (3.0) with a 63x water immersion lens, NA 1.2 (Carl Zeiss) yielding 0.0435 μm per pixels. For Alexa fluoro 488, the excitation and emission wavelengths were 488 nm and 515 nm, respectively. For YFP, the excitation and emission wavelengths were 515 nm and 535 nm respectively. For the analysis of spines, a three-dimensional image was reconstructed from approximately 30 sequential z-series sections of every 0.45 μm. Confocal images were then deconvoluted using AUTODEBLUR software (AutoQuant, USA). In each slice, two to three neurons with three of averaged 50 μm length dendrite were analyzed. Density, head diameter and neck length were analyzed with Spiso-3D (automated mathematical software calculating geometrical parameters of spines) that was developed by Bioinformatics Project of Kawato's group (Mukai et al., 2011). Spiso-3D has an equivalent capacity with Neurolucida (MicroBrightField, USA) which needs time-consuming manual operation. We analyzed the secondary dendrites in the stratum radiatum, lying between 100 and 200 μm from the soma. Spine shapes were classified into three categories as follows. (1) A small-head spine, which has a head diameter between 0.2-0.3 μm. (2) A middle-head spine, which has a head diameter between 0.3-0.4 μm. (3) A large-head spine, which has a head diameter between 0.4-1.0 μm. These three categories enabled to distinguish different responses of spine subpopulations to the current estradiol treatments.

Because the majority of spines (>97%) had a distinct head and neck, and stubby spines and filopodium did not contribute much to overall changes, we analyzed spines having a distinct head. The neck length was calculated as the distance between the center of the spine and the central line of dendrite. The significance of the drug effect was examined using the two-tailed paired student t-test. We also used the Tukey-Kramer posthoc multiple comparisons test when one way ANOVA tests yielded $p<0.05$ in the analysis of dendritic spine morphology and density.

4.5. Measurements of LTD with custom multi-electrode probes

Hippocampal acute slices from adult male mice aged nine weeks were prepared with a vibratome in a manner identical to that described in *experimental set up 1*. MED64 multi-electrode apparatus (Panasonic, Japan) was used for the electrophysiological measurements (Mukai et al., 2007; Oka et al., 1999; Shimono et al., 2000). After incubation for at least 1 h with ACSF at 25 °C for recovery, slices were positioned on a novel custom multi-electrode probe in which 64 planar microelectrodes were particularly arranged to cover densely the important regions containing essential synaptic contacts of pyramidal neurons in CA1. By using the current custom microelectrode arrangement, the correct stimulation of schaffer collaterals in the stratum radiatum of CA1 can be performed.

Bipolar constant current pulses (approximately 60 μA, 0.1 ms) served as a test stimulus. The responses of excitatory postsynaptic potential (EPSP) were measured with selected electrodes at which EPSP were filtered through a 1 Hz-10 kHz bandpass filter and recorded at a 20 kHz sampling rate. Estradiol (1–10 nM) was perfused (at 30 °C with perfusion rate of 2 mL/min) for 30 min prior to the induction of LTD, which was induced by a transient perfusion of 30 μM NMDA for 3 min (Lee et al., 1998; Mukai et al., 2007). The significance of the estradiol or drug effect was analyzed at 60 min through statistical analysis using ANOVAs ($^{*}p<0.05$; $^{**}p<0.01$).

4.6. Immunohistochemical staining of hippocampal slices

Immunohistochemical staining was performed essentially as described elsewhere (Kawato et al., 2002; Kimoto et al., 2001; Mukai et al., 2007). Briefly, hippocampal slices were prepared from nine weeks old male mice deeply anesthetized and perfused transcardially with PBS, followed by fixative solution of 4% paraformaldehyde in PBS. The hippocampi were post-fixed, cryoprotected and frozen-sliced coronally at 20 μm thickness with a cryostat (CM1510, Leica, Germany). Brains from several animals were used, and a single representative coronal section including the hippocampus was selected from each brain. Staining for ERα was performed using the avidin-biotin peroxidase complex technique. Column purified C-terminal peptide antibody, RC-19 was pre-treated with 5% BSA for 5 h to pre-absorb non-specific contaminated IgGs against BSA. After application of RC-19 IgG (1/1000), the slices were incubated for 24 h at 4 °C, in the presence of 0.5% Triton X-100 and 3% skim milk, with gentle shaking. Triton X-100 treatment was necessary to facilitate penetration of IgG into cells in slices. Biotinylated anti-rabbit IgG (1/200) in PBS was then applied, followed by a 30 min incubation with streptavidin-horseradish peroxidase complex (Vector Laboratories, CA, USA). Immunoreactive products were detected by immersing the slices in a detection solution (0.1 M Tris–HCl, pH 7.2, containing 0.05% diaminobenzidine, 0.01% H_2O_2 and 0.3% ammonium nickel sulfate). After dehydration and embedding in Entellan Neu (Merck, Germany), the immunoreactive cells in the slices were examined under a microscope, and digital images with a 2272 × 1704 pixel resolution were taken by a digital camera (COOLPIX4500, Nikon, Japan).

4.7. *Western immunoblot analysis*

For Western blot analysis, homogenates of whole tissues were prepared from the hippocampus, hypothalamus, cerebral cortex and cerebellum of nine weeks old male mice. Fraction samples were prepared by a combination of centrifugations for the whole brains except for cerebellum prepared from seven mice (Cohen et al., 1977). Detailed descriptions of centrifugation procedures to obtain fractions including the PSD fraction are described previously (Mukai et al., 2007). All samples were suspended in 62.5 mM Tris–HCl buffer (pH 6.8) containing 5 mM dithiothreitol, 5% sucrose, 3% sodium dodecylsulfate and 0.01% bromophenol blue. Twenty microgram of each fraction was subjected to electrophoresis using a 7.5% polyacrylamide gel. After transfer to polyvinylidene fluoride membranes (Immobilon-P, MA, USA), the blots were probed with purified antibody RC-19 (diluted to 1/3000) for 12–18 h at 4 °C, and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cell Signaling, MA, USA). The protein bands were detected with enhanced chemiluminescence (ECL) plus western blotting detection reagents (Amersham, NJ, USA). To obtain high quality images of chemiluminescence from protein bands using ECL plus, we used LAS3000 Image Analyzer (Fuji Film, Japan) with a 16- bit wide dynamic range.

4.8. *Statistical analysis*

Data are expressed as mean ± SEM. The significance of estradiol or drug effect was examined through statistical analysis using Tukey-Kramer post-hoc multiple comparisons test when one-way ANOVA tests yielded $^{*}P<0.05$. A difference was considered significant at a value of $^{*}p<0.05$ or $^{**}p<0.01$.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2014.12.002.

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