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Modulation of synaptic plasticity by brain estrogen in the hippocampus

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ABSTRACT

The hippocampus is a center for learning and memory as well as a target of Alzheimer's disease in aged humans. Synaptic modulation by estrogen is essential to understand the molecular mechanisms of estrogen replacement therapy. Because the local synthesis of estrogen occurs in the hippocampus of both sexes, in addition to the estrogen supply from the gonads, its functions are attracting much attention.

Hippocampal estrogen modulates memory-related synaptic plasticity not only slowly but also rapidly. Slow actions of 17β-estradiol (17β-E2) occur via classical nuclear receptors (ERα or ERβ), while rapid E2 actions occur via synapse-localized ERα or ERβ. Elevation or decrease of the E2 concentration changes rapidly the density and morphology of spines in CA1–CA3 neurons. ERα, but not ERβ, drives this enhancement/suppression of spinogenesis. Kinase networks are involved downstream of ERα. The long-term depression but not the long-term potentiation is modulated rapidly by changes of E2 level.

Determination of the E2 concentration in the hippocampus is enabled by mass-spectrometry in combination with derivatization methods. The E2 level in the hippocampus is as high as approx. 8 nM for the male and 0.5–2 nM for the female, which is much higher than that in circulation. Therefore, hippocampus-derived E2 plays a major role in modulation of synaptic plasticity.

Many hippocampal slice experiments measure the restorative effects of E2 by supplementation of E2 to E2-depleted slices. Accordingly, isolated slice experiments can be used as *in vitro* models of *in vivo* estrogen replacement therapy for ovariectomized female animals with depleted circulating estrogen.

1. Introduction

Finding of local synthesis of estrogen and androgen in the adult brain opened a new field of estrogen function in relation to the regulation of daily memory formation [1–5]. For decades, neuromodulatory actions have been extensively investigated for circulating gonadal sex hormones in the hippocampus, a center of learning and memory, because the hippocampus is a target of sex hormones [6–11]. Steroid hormones have profound effects on synaptic plasticity in the brain other than the hippocampus, such as the hypothalamus.

Modulation of dendritic spines has been extensively studied in relation to memory processes and synaptic plasticity which are regulated by neurotransmitters, because synapse is a site of memory storage and spine is a postsynaptic structure. Slow modulation of synaptogenesis or electrophysiological properties is investigated by estrogen replacement for ovariectomized female rats [6–11]. An increase of synapses or an enhancement of synaptic transmission is observed upon s.c. injection of estrogen. Slow modulation of spines (postsynaptic structures) is also observed in slice cultures [6–11]. These slow genomic effects are mediated via nuclear estrogen receptors ERα/ERβ to initiate transcription processes. The rapid effect of estradiol (E2) (within 1–2 h) also occurs by modulating spine density or electrophysiological properties of the hippocampal slices [6,7,12,13]. These rapid modulations, relating to memory formation processes, favor locally synthesized steroids rather than circulating gonadal hormones which travel a long distance before reaching the brain. Rather than being a limiting factor, a weak activity of sex steroid production in the hippocampus is sufficient for the local usage within small neurons (i.e., an intracrine system). This intracrine system

Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DHEA, dehydroepiandrosterone; DPN, diarylpropionitrile; DHT, dihydrotestosterone; 17β-estradiol, E2; estrone, E1; GPR30, G protein coupled receptor 30; HSD, hydroxysteroid dehydrogenase; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LTD, long-term depression; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PSD, postsynaptic density; PREG, pregnenolone; PPT, propyl-pyrazole-triyl)tris-phenol; RIA, radioimmunoassay; StAR, steroidogenic acute regulatory protein; T, testosterone

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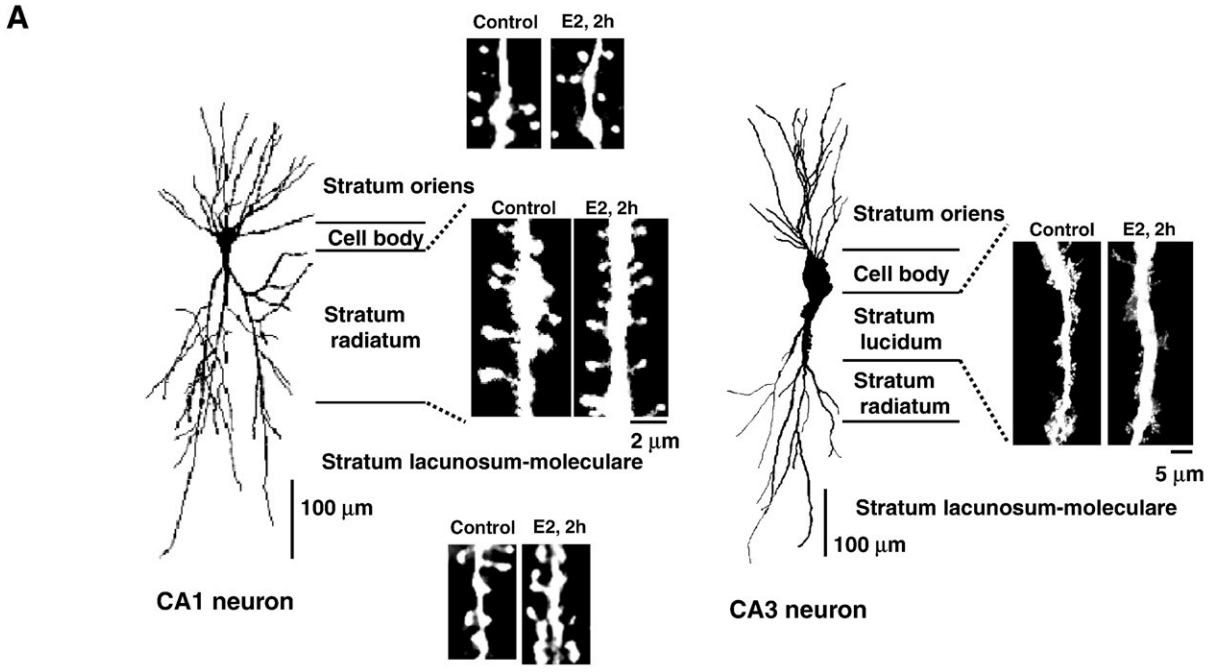
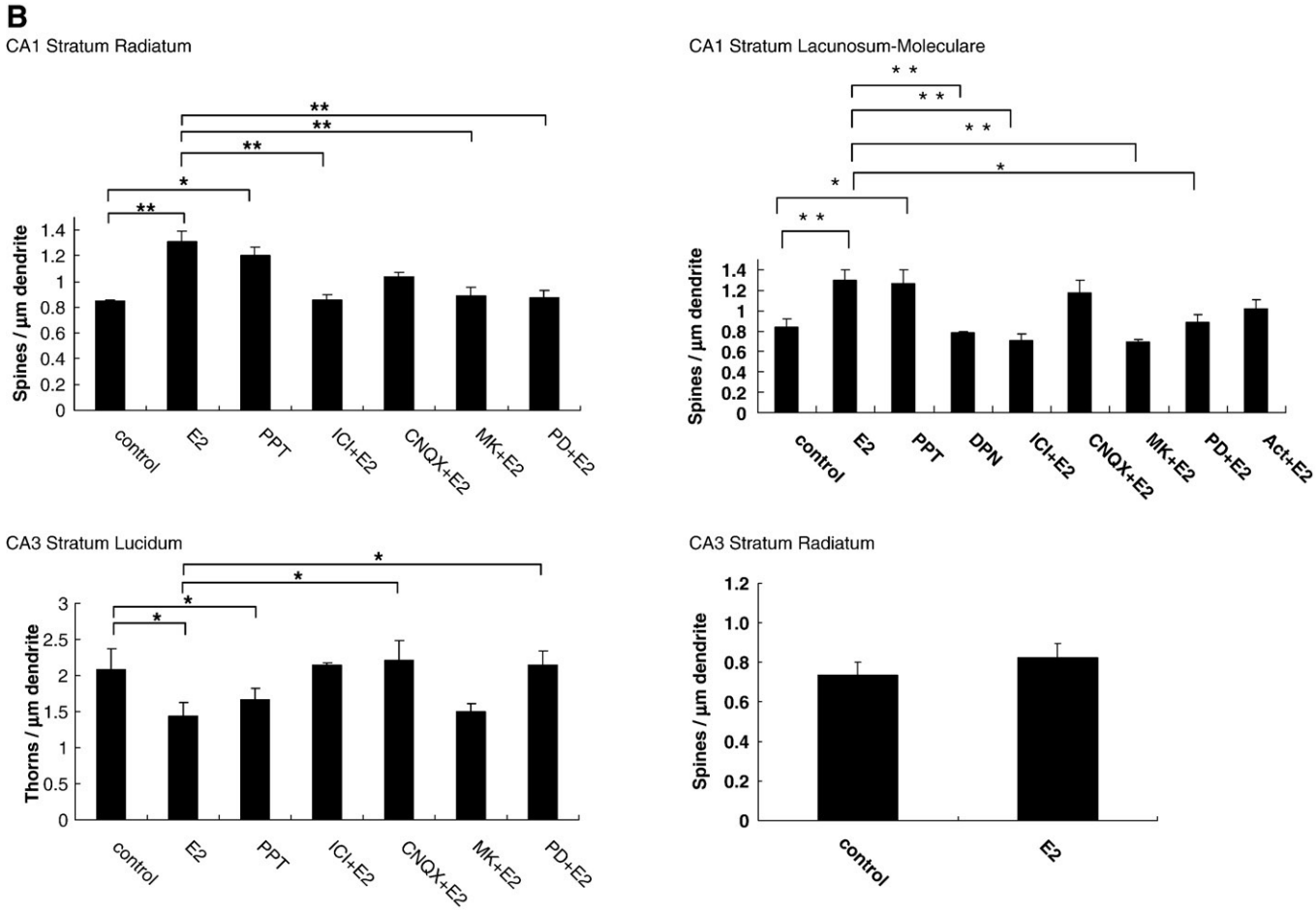
contrasts with the endocrine organs in which high expression levels of steroidogenic enzymes are necessary to supply steroids to many other organs via the blood circulation. For brain-derived sex hormones, the essential functions may be the rapid and continuous modulation of synaptic plasticity and cognitive functions.

The administration of a several-years therapy with estrogen for female patients with Alzheimer's disease following menopause is shown to be very effective in improving their capacity for learning and memory [14–17]. WHI (Women's Health Initiative) investigations with randomized methods, however, yielded contradictory results





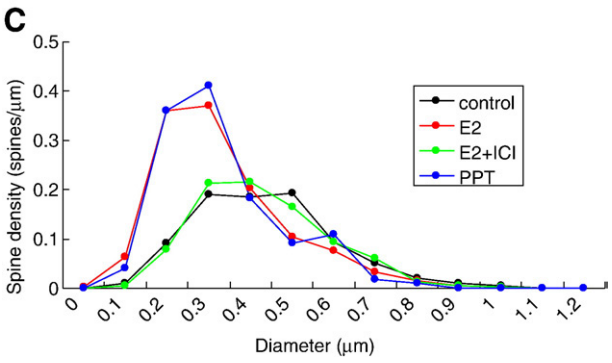


Fig. 1. Rapid estrogen modulation of spines (adult male rat, 12 weeks). Changes in the density and morphology of spines in CA1 or thorns (postsynapses) in CA3 upon 2 h-treatments of 17β-E2 (E2) and drugs in hippocampal slices. Confocal micrographs show spines/thorns along the dendrites of hippocampal pyramidal neurons in CA1 and CA3. (A, Left) A whole image of a Lucifer Yellow-injected CA1 neuron. Vertical bar 100 μm. Maximal intensity projections onto the *XY* plane from *z*-series confocal micrographs, showing spines along the dendrites. From top to bottom, spines along the basal dendrite in the stratum oriens (Control and E2), spines along the apical dendrite in stratum radiatum (Control and E2), and spines along the apical dendrite in stratum lacunosum-moleculare (Control and E2), horizontal bar 2 μm. (A, Right) A whole image of Lucifer Yellow-injected CA3 neuron. Vertical bar 100 μm. Maximal intensity projections onto the *XY* plane from *z*-series confocal micrographs, showing thorns on thorny excrescences along the primary/secondary dendrite in the stratum lucidum (Control and E2), bar 5 μm. Slices were treated in artificial cerebrospinal fluid (ACSF) for 2 h without drugs (Control) or with 1 nM E2 (E2). (B) ERα is responsible for changes by drug treatments of the total spine density (CA1) or thorn density (CA3) of pyramidal neurons in the CA1 stratum radiatum, the CA1 stratum lacunosum-moleculare, the CA3 stratum lucidum and the CA3 stratum radiatum. ICI suppresses the enhancing effect of spinogenesis by E2, suggesting that dimerization of ERα is involved in these events. Vertical axis is the number of spines or thorns per 1 μm of dendrite. A 2 h treatment in ACSF without drugs (Control), with 1 nM E2 (E2), with 100 nM PPT (PPT), with 100 nM DPN (DPN), with 1 nM E2 and 1 μM ICI 182,780 (ICI + E2), with 1 nM E2 and 20 μM CNQX (CNQX + E2), with 1 nM E2 and 50 μM MK-801 (MK + E2), with 1 nM E2 and PD98059 (50 μM for CA1 and 20 μM for CA3) (PD + E2), with 1 nM E2 and 20 μM CHX (CHX + E2), with 1 nM E2 and 4 μM Actinomycin D (Act + E2). Statistical significance (**P<0.01; *P<0.05). (C) Histogram of spine head diameters after a 2 h treatment in ACSF without drugs (control, black), with 1 nM E2 (red), with 1 nM E2 and ICI (green), and with 100 nM PPT (blue). E2 selectively increases small-head spines (<0.4 μm) (modified from Mukai et al. [23], Murakami et al. [21, 53] and Tsurugizawa et al. [22]).

showing less positive effects of estrogen replacement therapy [18]. In order to understand estrogen effect on learning and memory via estrogen replacement therapy, it is necessary to clarify the mechanisms of actions of E2, such as the signaling pathway, in the modulation/restoration of neuron–neuron communications in the hippocampus.

2. Modulation of synaptic plasticity by brain sex steroids

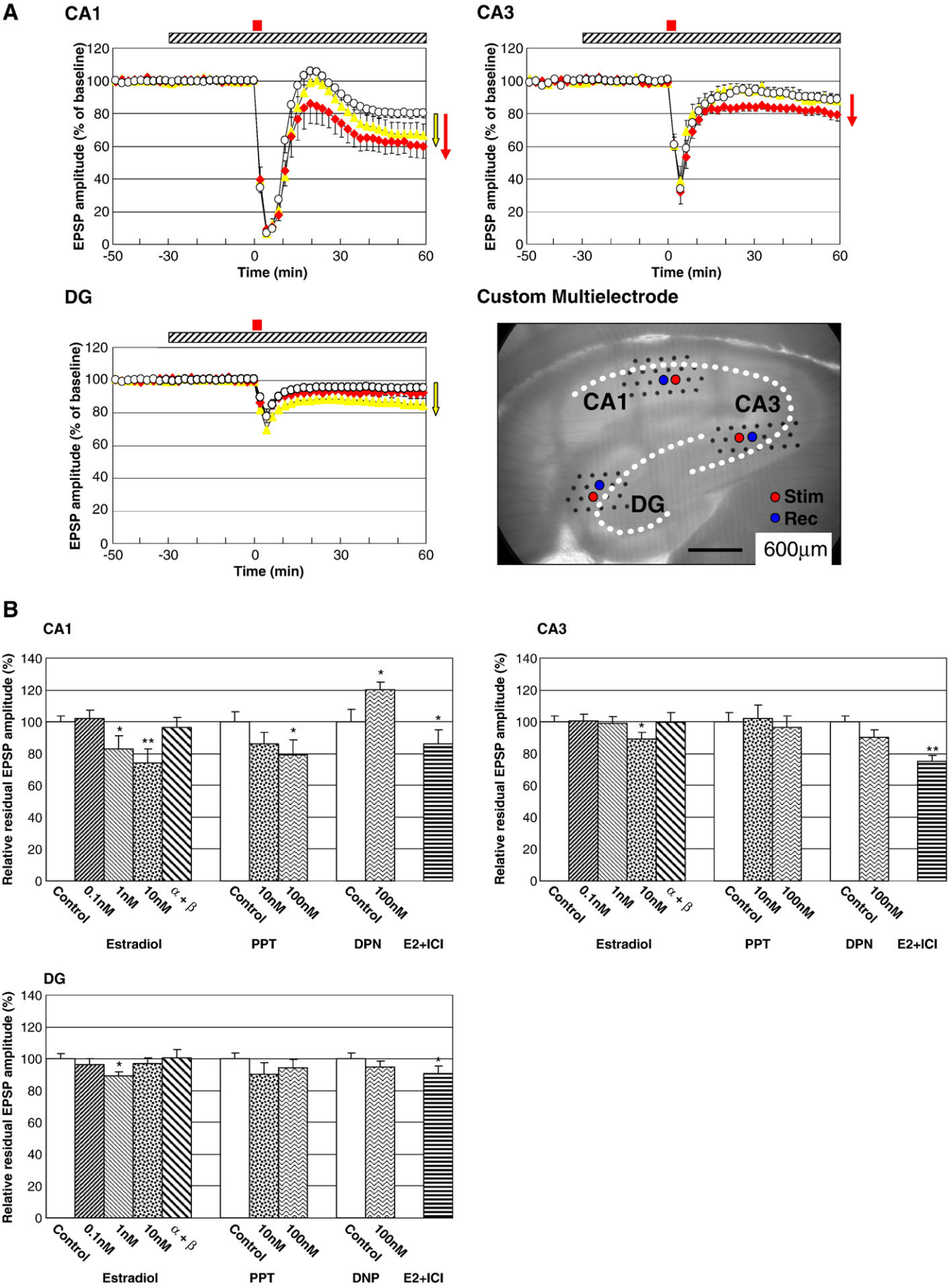
2.1. Spinogenesis

17β-Estradiol (E2) may rapidly modulate several different types of neuronal synaptic plasticity, including spinogenesis and synaptic transmission such as the long-term depression (LTD) or the long-term potentiation (LTP). Spinogenesis includes not only spine-synapses (spines forming synapses, roughly half of spines) but also free spines (spines without forming synapses, roughly half of spines), whereas LTD and LTP probe the characteristics of preformed synapses. Modulation of spinogenesis is an essential action of estrogen in memory processes, involving the production of new spines that create sites for new neuronal contacts. Dendritic spines are rapidly modulated upon E2 application, which is observed by single spine analysis of Lucifer-Yellow injected neurons in hippocampal slices from adult male rats (3 months) (Fig. 1A) [19–22]. Following a 2 h treatment with E2 in the stratum radiatum of the CA1 region, the treated dendrites have significantly more spines at 1 nM E2 (1.31 spines/μm) than dendrites at 0 nM E2 (0.85 spines/μm) (Fig. 1B) [23]. Propyl-pyrazole-trinyl-phenol (PPT, ERα agonist) [24] induces a significant enhancement of the spine density to 1.20 spines/μm. However, diarylpropionitrile (DPN, ERβ agonist) [24] increases the spine density only slightly (0.95 spines/μm). Blocking ERs by ICI 182,780 completely suppresses the enhancing effect of E2 on the spine density. Blocking the phosphorylation of ERK MAP kinase by PD98059 or U0126 completely prevents the E2-induced spinogenesis (Fig. 1B) [21]. Taken together, the enhancement of the CA1 spine density is probably induced by the activation of ERK MAP kinase via E2 and ERα within 2 h in resting neuronal synapses [25]. When the Ca^{2+} concentration in spines is further decreased by blocking NMDA receptors with MK-801, the enhancing effect by E2 is completely suppressed. The function of E2-bound ERα therefore requires the basal Ca^{2+} level of around 0.1–0.2 μM.

In adult hippocampal slices, the majority of spines (>95%) have distinct heads and necks, while the populations of stubby spines (roughly 5%, no neck) and filopodium (roughly 1%, no head) are very small. Therefore, in order to analyze the complex morphological changes in spines by kinase inhibitors upon E2-treatments, we have recently introduced a more precise description using the spine head diameter distribution, instead of the conventional classification, such as mushroom/thin/stubby/filopodium (Fig. 1C). Upon 1 nM E2 or PPT treatment, the density of small-head spine (0.2–0.4 μm) is selectively increased, while the density of large-head spine (>0.5 μm) is not significantly altered (Fig. 1C). ICI application completely reverses the spine head diameter change toward the control distribution.

The spine density is not always increased but is in some cases decreased by E2-treatments. The E2-induced spinogenesis is region specific and heterogeneous. In fact, in CA3 pyramidal neurons, the total density of thorns of thorny excrescences (spine-like postsynaptic structures in the stratum lucidum of CA3, having contacts with mossy fiber terminals originating from granule cells) decreased dramatically to approx. 70% upon a 2 h treatment of 1 nM E2 (Fig. 1B) [22]. PPT significantly decreases the density of thorns from 2.19 to 1.66 thorns/μm, but DPN does not significantly change the density of thorns. Blocking of ERK MAP kinase by PD98059 completely prevents the E2-induced decrease of thorns. Taken together, in the stratum lucidum of CA3, the decrease in the thorn density is probably induced by the activation of ERK MAP kinase by E2-bound ERα in resting neuronal synapses. When the Ca^{2+} concentration is decreased to below the basal level by blocking AMPA receptors with CNQX, or by changing the outer medium to Ca^{2+}-free ACSF, the suppression effect of E2 is completely inhibited (Fig. 1B). These results suggest that the decrease

Fig. 2. Rapid estrogen modulation of LTD (adult male rat). [panel A] Time dependence of maximal EPSP amplitude in CA1 (CA1), CA3 (CA3) and DG (DG) of hippocampal slices. E2 concentration was 0 nM (open circle), 1 nM (yellow closed triangle) and 10 nM (red closed diamond), respectively. (Custom Multielectrode) Custom-made 64 multielectrode probe (MED64, Panasonic, Japan) with the hippocampal slice. Stimulation (red circle) and recording (blue circle) electrodes are indicated. Here, 100% EPSP amplitude 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 to 3 min (closed bar above the graph). Hatched bar above the graph indicates the E2 administration period. The LTD enhancement was reproducibly observed in more than 90 slices out of 100 slices. [panel B] Comparison of modulatory effect on LTD by E2 and agonists in the CA1 (CA1), CA3 (CA3) and DG (DG) of hippocampal slices. Vertical axis represents relative EPSP amplitude at t = 60 min, where EPSP amplitude at t = 60 min of the control slice without drug application is taken as 100%. From left to right, 17β-E2 (E2), PPT (ERα agonist) and DPN (ERβ agonist) at indicated concentrations. Co-perfusion of 10 nM 17α-E2 with 1 nM 17β-E2 (α + β) blocks the 17β-E2 effect of LTD enhancement. Co-perfusion of 1 μM ICI with 10 nM 17β-E2 did not suppress the enhancing effect of LTD by estradiol. Importantly, ICI does not inhibit any type of E2 effects on electrophysiological signals, such as LTP, LTD or Kainate current presented in previous reports [23,52,54]. These results suggest that dimer formation of ERα/ERβ is not involved in the E2-modulation of synaptic transmission. An NMDA-induced chemical LTD is useful for adult hippocampal slices, because an electrical LTD is not inducible for adult hippocampal slices (in contrast, an electrical LTD is inducible for developmental hippocampus). The significance of the estradiol effect was confirmed at 60 min via statistical analysis (*p<0.05; **p<0.01) (modified from Mukai et al. [23]).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DG
Relative residual EPSP amplitude (%)
Control
0.1nM
1nM
10nM
α+β
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100nM
Estradiol
PPT
DPN
DNP
E2+ICI

of thorns requires the basal Ca^{2+} concentration which is maintained by spontaneous postsynaptic Ca^{2+} fluctuation via voltage activated calcium channels which are dependent upon AMPA receptor-mediated spontaneous voltage fluctuations, because the spontaneous Ca^{2+} influx within thorny excrescences occurs mainly via voltage activated calcium channels [26–30]. Note that blocking of NMDA receptors by MK-801 does not prevent the E2-induced decrease of thorns. This may be due to the much smaller contribution of NMDA receptors to the spontaneous Ca^{2+} influx within thorns than that of voltage activated calcium channels. The function of CA3-ERα also requires the basal Ca^{2+} concentration.

Usage of isolated hippocampal slices is suitable for an examination of E2 effect, particularly on glutamatergic neurons within slices. The rapid effect of estrogen is also observed *in vivo*. MacLusky et al. demonstrate that the E2 (60 μg/kg) increases the spine-synapse density due to synaptic rearrangements in ovariectomized adult rats as rapidly as 30 min after E2 injection, using electron micrographic analysis [31].

On the other hand, the slow genomic effects (1–4 days) of E2 on spine plasticity have been extensively investigated *in vivo* from the viewpoint of estrogen replacement therapy. For example, a supplement of estrogen to ovariectomized adult female rats increases the density of spines in the stratum radiatum of CA1 pyramidal neurons, resulting in a recovery of spines to the level of intact rat [31–34]. These enhancement effects on spinogenesis are also observed as rapidly as 4.5 h after s.c. injection of estrogen [31]. Results from *in vivo* investigations using whole rat may reflect not only the direct but also the indirect effects of E2 on glutamatergic neurons via cholinergic or serotonergic neurons, projecting to the hippocampus [31,35]. Estrogen mediates slow spine changes by means of NMDA receptors. Estradiol increases the binding of NMDA agonist, as well as the NR1 subunit levels in CA1 neurons [36,37]. Estrogen-induced increases in spine density are blocked by NMDA receptor antagonists [11,38].

In vitro investigations also show that the CA1 spine density increases following several days' treatment of cultured hippocampal slices with 0.5 mM (=0.2 mg/ml) exogenous E2 [8]. The contribution of hippocampus-derived E2 is reported by Kretz et al. who demonstrate that the suppression of endogenous E2 synthesis by letrozole treatments for 4 days significantly decreases the density of spines, spine-synapses, spinophilin (spine marker) and synaptophysin (presynaptic marker) in the stratum radiatum of the CA1 region in cultured slices [5]. No increase in the density of spines, spine-synapses or spinophilin expression is observed after exogenous application of 100 nM E2 to the medium of slice cultures that had not been treated with letrozole. Application of 100 nM E2, however, induces rescue effect which restores the synaptophysin expression that was once decreased by letrozole.

Compared with the case of estrogen, androgenic regulation on the formation and morphologic changes of dendritic spines is poorly understood in the hippocampus. Leranth et al. report that testosterone (T) is important for maintenance of normal spine density in male rat hippocampus, because the 2-day application of T propionate or dihydrotestosterone (DHT) retrieves the spine density in CA1 pyramidal neurons of gonadectomized rats in which the density is reduced without androgen supplement [39]. Part of the effects of T on dendritic spines in females, not in males, seems to be mediated by local conversion to estradiol [40]. Recently the rapid regulation (within 2 h) by androgen of spines is also shown in CA3 [41].

2.2. Modulation of long-term depression (LTD) and long-term potentiation (LTP)

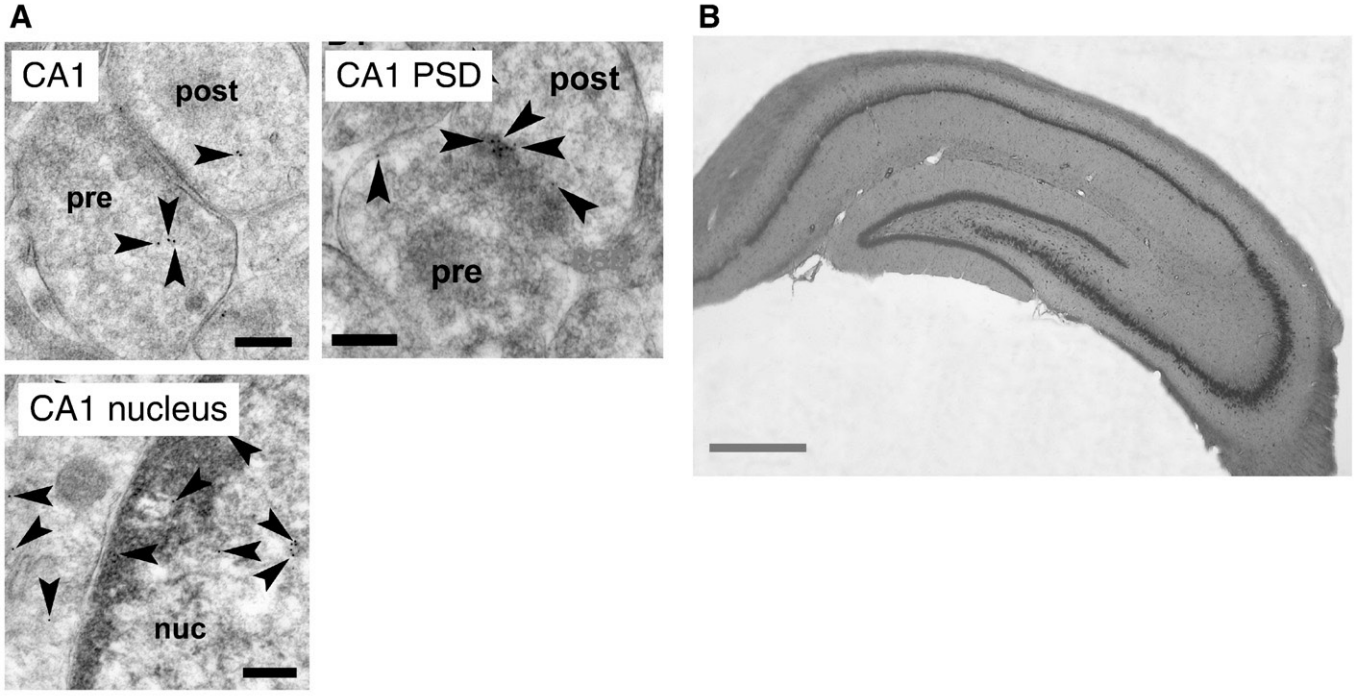
E2-induced rapid modulation of LTD or LTP occurs only in preexistent synapses, because newly generated spines induced by E2-treatments do not form new synapses within 2 h, as judged from no increase in the baseline magnitude of the excitatory postsynaptic potential (EPSP) signal during 2 h of E2 perfusion [23]. On the other hand, the slow effect of E2 (2–4 days) enhances LTP via formation of new synaptic contacts for estrogen supplemented ovariectomized 8-week female rats [42,43]. The electrophysiological properties of NMDA receptor-mediated transmission are altered by estrogens [44].

Evidence is emerging that E2 exerts a rapid influence (0.5–1 h) on the synaptic transmission of hippocampal slices from adult rats, as demonstrated by electrophysiology [7,9,13,45,46]. In the case of the occasionally observed enhancement of LTP by 1–10 nM E2 in CA1 pyramidal neurons, a baseline increase of 20–30% has always been observed upon the onset of 10 nM E2 perfusion in the initial slope of the EPSP, which has been attendant upon a further increase to approx. 160% upon high-frequency tetanic stimulation of the Schaffer collaterals of hippocampus from adult rat [6,7,47]. However, without this 20–30% baseline increase in EPSP slope (before the tetanic stimulation), the enhancement of LTP by E2 is not apparent, with regard to the pure tetanic stimulation-induced LTP. In other words, the magnitude of pure tetanic stimulation-induced LTP (approximately 130%) is nearly the same for the presence and the absence of 10 nM E2 [4,46]. It should be noted that in 3–4 week old puberty rats, 10 nM E2 even suppressed LTP-induction down to the same level as that for adult rats [9,46]. The effects of E2 on LTP are strongly dependent on the age of rats.

In memory processing, not only LTP (memory forming mechanism) but also LTD are essential. Mutant mice, which show enhanced LTP and suppressed LTD, have shown impaired learning of the Morris water maze [48]. This suggests that LTD may be required to "correct" wrong memories formed by initial LTP processes, which store not only correct information but also incorrect information.

We found that LTD is very sensitive to short 17β-E2 treatments (for 2 h) in hippocampal slices from adult male rats. We demonstrate, for the first time, a significant rapid enhancement of LTD by a 1–10 nM E2 perfusion in CA1, CA3 and DG (Fig. 2) [23]. Recordings are performed using 64 planar multielectrodes particularly arranged to stimulate the Schaffer collaterals in the stratum radiatum of CA1, the recurrent collateral fibers in the stratum radiatum of CA3, and the medial perforant pathways in the molecular layer of DG. LTD is induced pharmacologically by the transient application (3 min) of NMDA. This LTD is induced by the activation of phosphatase due to a moderate Ca^{2+} influx through NMDA receptors [49]. LTD is effectively induced by the transient application of NMDA (chemical LTD) for adult hippocampus, whereas low frequency electrical stimulation

Fig. 3. Estrogen receptor ERα, synaptic localization and neuronal expression (adult male rat). (A) Immunoelectron microscopic analysis of the distribution of ERα within axospinous synapses, in the stratum radiatum of hippocampal slices. (CA1) Gold particles (arrowheads) were localized in the pre- and postsynaptic regions in CA1. (CA1 PSD) In dendritic spines, gold particles were associated with PSD regions in CA1. (CA1 nucleus) Gold particles were also localized in the nuclei in CA1. Pre, presynaptic region; Post, postsynaptic region; Scale bar: 200 nm. (B) Immunohistochemistry of ERα (with RC-19) showing expression of ERα in pyramidal neurons (CA1–CA3) as well as granule cells (DG). (C) Western blot shows a single ERα band, stained with affinity column-purified RC-19 antibody used for immunoelectron microscopic analysis and immunohistochemistry. Reliable immunostaining requires the support of a single band in a Western blot check, otherwise immunostaining patterns show the expression of unknown proteins in addition to the target protein. Thus far, no ERβ antisera (nonpurified) show a single band in the Western blot of brain tissues. GPR30 also does not have a good antibody for Western blot identification. (C1) Western blot of PSD (postsynapic density), PRE (presynaptic membrane fraction), and CYT (cytosolic fraction) of the hippocampus. From left to middle, blot of PSD with RC-19 IgG (ERα), PSD-95 IgG (PSD-95) and MAP kinase IgG (MAPK). From middle to right, blot of PRE with RC-19 (ERα) and synaptophysin IgG (S-Physin). At rightmost lane, blot of CYT with RC-19 (ERα). The applied protein amount was 3-fold greater for PSD fraction than for other fractions. (C2) RC-19 does not bind to brain from ERαKO mice (left half) but binds to wild mice brain (right half). From left to right, hippocampus (HC) and hypothalamus (HT) from ERαKO mice, HC and HT from wild mice. (C3) MC-20 antiserum even binds to ERαKO mice brain, indicating that MC-20 binds to an unknown protein. From left to right, ovary (OV), cerebral cortex (CC), HC, and HT. (C4) MC-20 antiserum binds to a 62 kDa unknown protein in rat brain. (C5) AS409 antiserum binds to a 90 kDa unknown protein in rat brain (modified from Mukai et al. [23]).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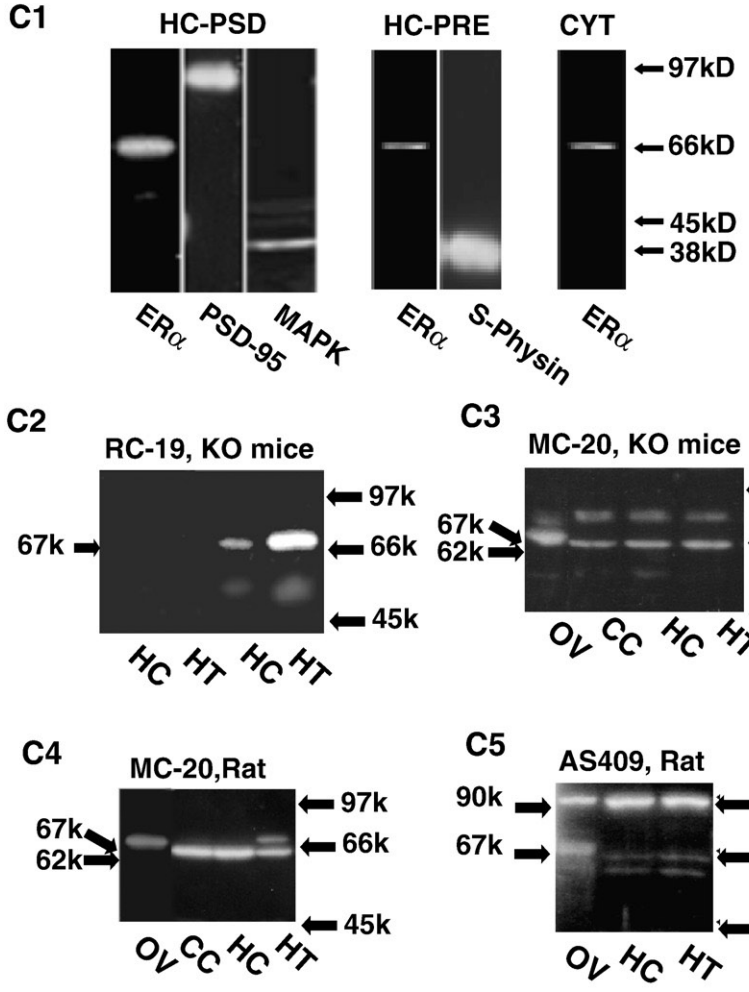
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cannot induce LTD in adult slices. Low frequency electrical stimulation can induce LTD in slices from rats younger than 2 weeks of age. A 30 min preperfusion of 10 nM E2 significantly enhances LTD resulting in a decrease in plateau EPSP amplitude (at 60 min after NMDA application), such as 80.4% → 59.7% (CA1), 88.8% → 79.1% (CA3) and 95.1% → 92.2% (DG) (Fig. 2) [23]. Investigations using specific estrogen agonists indicated that the contribution of ERα (but not ERβ) is essential for these E2 effects. PPT (ERα agonist) at 100 nM exhibits a significant LTD enhancement in CA1, while DPN induces a suppression of LTD in CA1, implying that the contribution of ERβ is opposite to that of ERα in the E2 effect on LTD. Taken collectively, E2-bound ERα may activate phosphatase at the moderate Ca^{2+} concentration of around 0.7–1 μM induced upon 30 μM NMDA application [50], and facilitated dephosphorylation of AMPA receptors may induce the enhancement of LTD. On the other hand, E2-bound ERα is not functional in LTP modulation at the transiently high Ca^{2+} concentration of roughly 5–12 μM under tetanic stimulation [19,50–52], because the phosphorylation of AMPA receptors by CaM kinase II is the dominant process at the high Ca^{2+} concentration.

2.3. Synaptic estrogen receptors

What is the receptor of 17β-E2 that mediates rapid actions (1–2 h) on synaptic plasticity in the hippocampus?

Classical nuclear type receptors ERα and ERβ are candidates for synaptic estrogen receptors. Because ICI does not suppress E2-induced rapid modulation of electrophysiological properties, such as LTD, LTP, and kainate-induced currents, many scientists consider classical estrogen receptors to not be involved in these modulations [45]. However, these results do not eliminate the possibility that ERα and ERβ could drive these synaptic transmissions. ICI has been indicated to display its effect by inhibiting the dimerization of ERα and ERβ. If dimerization processes are not involved in the rapid modulation of electrophysiological phenomena, then ICI cannot block these phenomena. On the other hand, the rapid enhancement of spinogenesis via ERα is significantly blocked by ICI (Fig. 1B) [23], therefore, dimerization processes occur for synaptic ERα in spinogenesis.

We identify the membrane estrogen receptor ERα localized in the spines of hippocampal pyramidal and granule neurons by means of immunoelectron microscopic analysis as well as Western blot analysis, using affinity-column purified anti-ERα antibody RC-19 (C-terminal antibody) [23]. Attention must be given to the fact that non-purified ERα antisera often react significantly with unknown proteins (62 kDa protein in the brain, Western blot), resulting in incorrect staining which differs from the real ERα distribution. In hippocampal slices the expression of 62 kDa protein is nearly the same as that of ERα, however, the cerebellum has a significant expression of 62 kDa protein, although real ERα expression is very poor [23].

A post-embedding immunogold electron microscopic analysis demonstrates the synaptic localization of ERα in the glutamatergic neurons in CA1, CA3 and DG (Fig. 3). ERα is also localized in the nuclei. Western blot analysis demonstrates that ERα (67 kDa) and ERK MAP kinase are tightly associated with postsynaptic density fractions (PSD). Because the E2-induced modulation of LTD and spine density appears so rapidly in the time range of 1–2 h, the synaptic ERα observed at PSD or postsynaptic compartments probably plays an essential role in driving rapid signaling. The specific binding of purified RC-19 antibody to real ERα (67 kDa) in the hippocampus is verified as the absence of reactivity of RC-19 with ERα knock-out mice hippocampus (Fig. 3) [23]. These analyses are essential in the hippocampus and other brain regions, as we found that non-purified MC-20 antisera, frequently used in previous investigations, often react with unknown 62 kDa proteins in several brain regions, and do

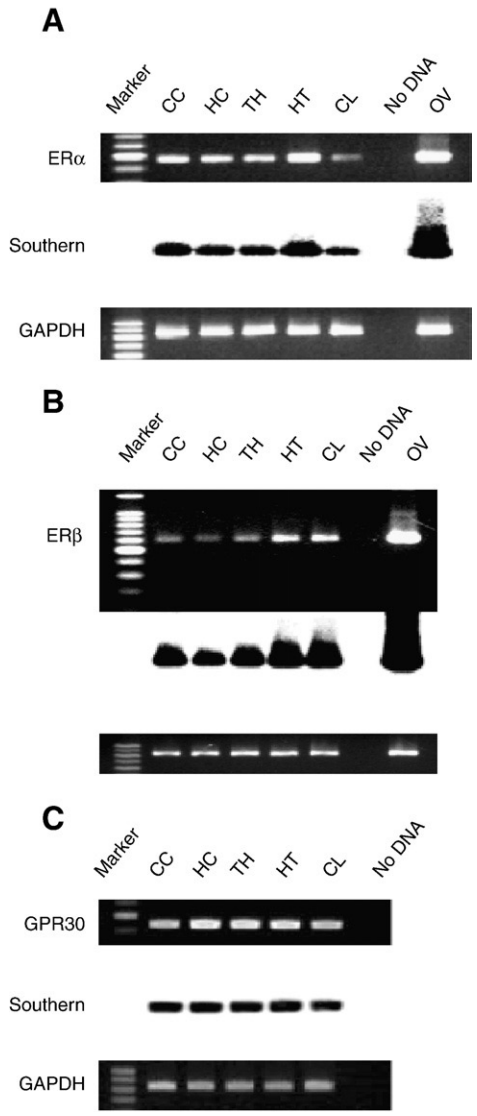
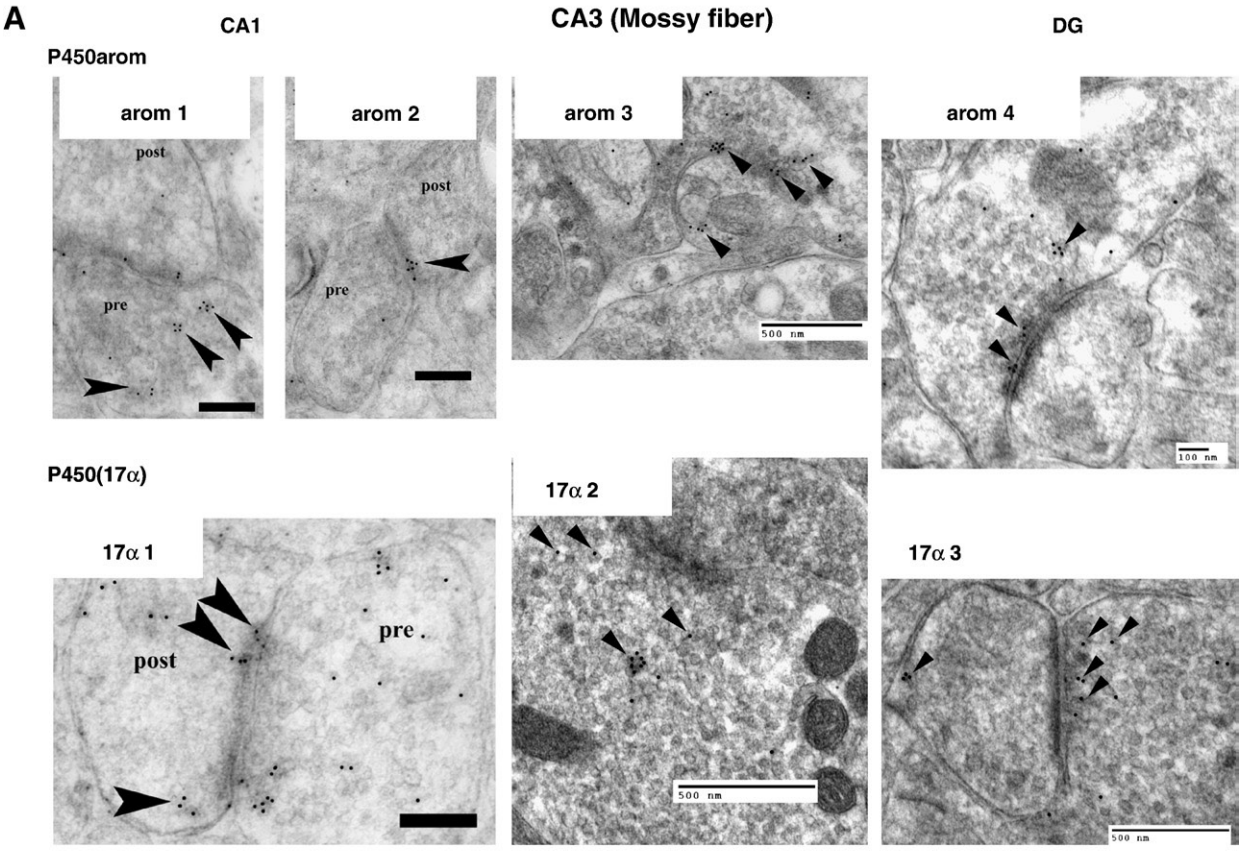


Fig. 4. mRNA expression of estrogen receptors in the brain. Estrogen receptor expression of (A) ERα, (B) ERβ and (C) GPR30 by RT-PCR analysis (adult male rat). In each panel from left to right, Cerebral Cortex (CC), Hippocampus (HC), Thalamus (TH), Hypothalamus (HT), Cerebellum (CL), the sample without template DNA (no cDNA), and positive control ovary (OV). Total RNAs used were 100 ng for each estrogen receptor. As judged from the PCR cycles used, the relative expression level is roughly ERα (34 cycle)/ERβ (36 cycle)/GPR30 (30 cycle) = 8:1:64 in the hippocampus. In cerebellum, the ERβ level is much higher than that of ERα. ERβ is the sum of types 1 and 2. Interestingly, no difference in the expression level of ERα is observed between male and female hippocampus in the adult stage (data not shown) (modified from Mukai et al. [23]).

Fig. 5. Sex-hormone synthase cytochrome P450; synaptic localization and neuronal expression (adult male rat). (A) Immunoelectron microscopic analysis of the distribution of P450arom and P450(17a) within synapses, in the hippocampal CA1 (arom 1, 2 and 17α 1), CA3 mossy fiber (arom 3 and 17α 2), and DG (arom 4 and 17α 3) regions. Gold particles (indicated by arrow heads) are observed to be localized in the presynaptic region (Pre), and the postsynaptic region (Post) of pyramidal neurons in CA1 and CA3, and granule neurons in DG. In the presynaptic region (Pre), gold particles are associated with small synaptic vesicles. In spines (Post), gold particles are found within the spine heads. Scale bar: 200 nm for (arom 1, 2 and 17α 1), 500 nm for (arom 3, 17α 2 and 3), and 100 nm for (arom 4). (B) Immunohistochemical staining of P450arom and P450(17α) in the coronal section of adult male rat hippocampus. Scale bar: 800 μm. (C) Western blot analysis of P450arom and P450(17α) in microsomes of hippocampus (HC), lung (Lu), ovary (OV) and testis (Te). Lung (Lu) is the negative control. Ovary (OV) for P450arom and testis (Te) for P450(17α) are used as the positive control. (D) Western immunoblot analysis of P450arom and P450 (17α) in subcellular fractions of adult male rat hippocampus. From left to right, postsynaptic membrane-rich fraction (Post), presynaptic membrane-rich fraction (Pre), postsynaptic density fraction (PSD), microsomes (MS) and ovary (OV) for P450arom, and Post, Pre, PSD, MS and testis (Te) for P450(17α). The amount of protein applied to the gels was 20 μg for each hippocampal fraction, and 1 μg for ovary or testis (modified from Hojo et al. [4]).


A
CA1
CA3 (Mossy fiber)
DG
P450arom
arom 1
arom 2
arom 3
arom 4
post
pre
500 nm
100 nm
P450(17α)
17α 1
17α 2
17α 3
post
pre
500 nm
B
P450arom
P450(17α)
C
P450arom
Lu OV HC
66 kDa
45 kDa
P450(17α)
Lu Te HC
66 kDa
45 kDa
D
P450arom
Post Pre PSD MS OV
P450(17α)
Post Pre PSD MS Te

not significantly react with real ERα (67 kDa) (Fig. 3) [23]. ERα antisera are often verified for their reactivity only in endocrine organs such as the ovary, in which ERα is highly expressed. Therefore, the staining of interneurons and absence of staining of primary neurons with non-purified antisera (such as MC-20 or AS409) probably do not show the real ERα distribution in the hippocampus [53] (Fig 3). Antisera should be purified before application to the hippocampus. ERα knock-out mice may be useful in investigating the participation of ERα in modulation of synaptic plasticity. However, thus far, no data is available for real ERα knock-out mice. Electrophysiological investigations are performed by using knock-down mice (not knock-out mice) by Moss et al. [45,54]. They report no essential contribution of ERα to the E2-induced rapid enhancement of the kainate currents of CA1 neurons. They reach this conclusion due to the observation of a very small difference in the E2 effect on the kainate currents between wild-type and ERα-Neo knock-down mice which have been constructed by the method of Neomycin insertion into exon 1 (the previously named exon 2) [55]. It should be noted that in Neomycin-insertion ERα-Neo knock-down mice, N-terminal-modified ERα (61 kDa) is expressed [55–57]. Because the N-terminal-modified ERα is demonstrated to still be active on E2 binding and to drive genomic processes [55–57], the participation of ERα in the electrophysiological properties of the CA1 cannot be excluded from their investigations. Therefore, it is necessary to investigate real ERα knock-out mice in which, for example the whole exon 2 of the mouse ERα gene is deleted [58].

ERβ has, however, not yet been identified as a synaptic membrane receptor. ERβ is reported to associate with membranes in genetically expressed CHO cells and MCF-7 cells [59,60]. Several investigations of immunostaining of ERβ suggest the extranuclear expression of ERβ including dendritic appearance in the hippocampal principal neurons [61]. The subcellular immunostaining patterns of these reports might reflect the relatively minor expression of ERβ and the major expression of unknown proteins, due to multiple reactivity of non-purified ERβ antisera to several unknown proteins in the Western blot analysis of hippocampal tissues. The purity of commercially available ERβ antisera is worse than that of ERα antisera as judged from our Western blot analysis.

Recently transmembrane G-protein coupled estrogen receptor GPR30 is identified in the plasma membrane of SKBR3 breast cancer cells that lack ERα and ERβ [62], as well as in the endoplasmic reticulum membranes of COS7 after genetic expression of GPR30 fused with green fluorescent protein [63]. The expression of GPR30 is also observed in the hippocampal neurons (Fig. 3C) [64]. Synaptic expression of GPR30, however, has yet to be demonstrated, and further investigations are necessary to reveal its contribution to the rapid E2 modulation of synaptic plasticity (Fig. 4) [65].

3. Synthesis of sex steroids in the hippocampus

3.1. Pathway of synthesis (Fig. 7)

Sex steroids had been thought to reach the brain exclusively via blood circulation after crossing the blood–brain barrier [66]. However, recent studies using immunohistochemical staining and Western immunoblot analysis reveal a significant localization of steroidogenic proteins such as cytochromes P450scc, P450(17α), P450arom and StAR in pyramidal neurons in CA1–CA3, as well as in granule cells in DG, of adult hippocampus (12 week) [1–4,53], and also developmental hippocampus [5,67–69]. The hippocampal expression of mRNAs for steroidogenic enzymes is also demonstrated using RT-PCR and/or *in situ* hybridization for P450scc [70,71], StAR [72,73], 3β-hydroxysteroid dehydrogenase (3β-HSD) [72,74], P450(17α) [4], P450arom (CYP19) [4,75,76], 17β-hydroxysteroid dehydrogenase (17β-HSD) type 1-4 [4,77], and 5α-reductase (Fig. 5) [78,79]. These results imply that hippocampal pyramidal neurons and granule neurons are equipped with complete steroidogenic systems which catalyze the conversion of cholesterol to pregnenolone (PREG), dehydroepiandrosterone (DHEA), T and E2. In fact, a direct demonstration of the neuronal synthesis of DHEA and T in adult mammals is reported for the first time by Kawato et al. [1,2,4]. We succeeded in demonstrating the synthesis of DHEA, T and E2 in the adult (12 week) hippocampal slices by means of careful HPLC analysis [2,4]. The significant conversion from [^{3}H]-PREG to ^{3}H-DHEA, from ^{3}H-DHEA to ^{3}H-androstenediol, to ^{3}H-T and to ^{3}H-E2 is observed after incubation with the slices for 5 h [4]. The rate of production for ^{3}H-E2 from ^{3}H-T is very slow, and the production rate of ^{3}H-dihydrotestosterone from ^{3}H-T is much more rapid than that of E2. These activities are abolished by the application of specific inhibitors of cytochrome P450s. Surprisingly, ^{3}H-E2 is extremely stable and is not significantly converted to other steroid metabolites such as estrone. On the other hand, DHT is rapidly converted to 3α, 5α-androstanediol. To demonstrate the rapid net production of neurosteroids upon synaptic stimulation, the NMDA-induced production of PREG and E2 is investigated in hippocampal slices [1,2,4]. Upon stimulation with NMDA for 30 min, the hippocampal levels of PREG and E2 increase to approx. 2-fold that of the basal levels. This implies that the NMDA-induced Ca^{2+} influx drives the net production of PREG and E2. E2 synthesis is also demonstrated in cultured hippocampal slices from neonatal rats in the absence and presence of

Table 1
Mass spectrometric analysis of the concentration of steroids in the hippocampus and plasma of adult rats.

(A) Male	Hippocampus		Plasma	
	Intact[a]	Castrated	Intact	Castrated
17β-E2 (ng/g wet weight or ml)	2.3 ± 0.4[b] (n[c] = 6)	1.9 ± 0.2 (n = 16)	0.004 ± 0.001 (n = 5)	0.002 ± 0.000 (n = 14)
17β-E2 (nM)[d]	8.4 ± 1.5	6.9 ± 0.8	0.014 ± 0.003	0.006 ± 0.001
T (ng/g wet weight or ml)	4.9 ± 0.7 (n = 8)	0.9 ± 0.2 (n = 16)	4.2 ± 0.5 (n = 8)	0.06 ± 0.02 (n = 16)
T (nM)	16.9 ± 2.3	3.1 ± 0.8	14.6 ± 1.7	0.20 ± 0.08
DHT (ng/g wet weight or ml)	1.9 ± 0.5 (n = 8)	0.06 ± 0.01 (n = 16)	0.18 ± 0.03 (n = 8)	0.012 ± 0.003 (n = 16)
DHT (nM)	6.6 ± 1.7	0.22 ± 0.04	0.63 ± 0.10	0.04 ± 0.01
(B) Female 17β-E2	Proestrus	Estrus	Diestrus-1	Diestrus-2
Hippocampus (nM)	1.7 ± 0.4 (n = 3)	1.0 ± 0.3 (n = 3)	0.5 ± 0.1 (n = 3)	0.7 ± 0.2 (n = 3)
Plasma (nM)	0.120 ± 0.015 (n = 3)	0.020 ± 0.015 (n = 3)	0.008 ± 0.005 (n = 3)	0.026 ± 0.005 (n = 3)

The levels of hippocampal sex-steroids are significantly higher than those of circulating sex-steroids. Castration depletes circulating T completely but does not considerably change the hippocampal E2 level (modified from Hojo et al. [79]).

[a] Intact shows the averaged values from intact and sham-operated rats, because there were no significant differences between these two groups of rats.

[b] Data are expressed as mean ± SEM.

[c] Number of animals (i.e. the number of hippocampi).

[d] Concentration in nM is calculated using the average volume of 0.14 ml for one whole hippocampus that has 0.14 ± 0.02 g wet weight (n = 86). We assume that tissue having 1 g of wet weight has an approximate volume of 1 ml, since the major part of tissue consists of water whose 1 ml weight is 1 g.

letrozole, an inhibitor of P450arom. After a 4 day treatment with letrozole, the amount of E2 released into the medium is significantly decreased [5].

3.2. Concentration of estrogen and androgen

Why do we need E2 concentrations higher than 1 nM in order to obtain significant effects of E2 in slices or neurons, even though the maximal circulating E2 level achieved in cycling female rats is 0.1 nM at proestrus (Table 1) [80]? Does this suggest that the endogenous concentration of E2 in the brain is higher than 1 nM? To answer these questions, an accurate determination of the physiological concentration of E2 and other steroids is necessary for an understanding/explanation of its modulatory action on synaptic plasticity described in Section 2.

In most experiments, scientists did not know (could not measure) the endogenous E2 level in brain slices or cultured neurons. In fact, the brain E2 concentration is much higher than that in circulation (Table 1). In addition, the concentration of E2 in ACSF-treated slices is not invariant and is often very different from that in freshly isolated hippocampus. Because of technical problems, the accurate determination of the E2 concentration in whole hippocampus, slices or cultures neurons had been impossible. Based on RIA determination, the concentration of E2 detected in the male rat hippocampus is roughly 0.6 nM (basal) and 1.3 nM after the NMDA-stimulation, respectively, although the values are 6–10 times greater than those typical of plasma [4]. RIA is a very sensitive method for steroid detection, but has uncertainty regarding specificity and accuracy. Even with mass-spectrometric assay such as gas chromatography with mass-spectrometry (GC-MS/MS), liquid chromatography with mass-spectrometry (LC-MS) and liquid chromatography with tandem-mass-spectrometry (LC-MS/MS), the presence of 17β-E2, DHT and estrone (E1) had not yet been observed, though DHEA and T have been observed in the whole brain extracts [81–84].

We therefore substantially improved the determination methodology using liquid chromatography-tandem-mass spectrometry (LC-MS/MS) in combination with picolinoyl-derivatization of pre-purified E2/T/DHT/E1 fractions obtained via normal phase HPLC (Fig. 6) [79,85]. Pre-purification of E2/T/DHT/E1 fractions via normal phase HPLC is necessary to remove contaminating fats and lipids, and reverse phase LC of LC-MS/MS is not sufficient for this kind of pre-purification. By LC-MS/MS analysis, basal level of E2 is determined to be approx. 8 nM [86], and this E2 level is not decreased by castration to deplete circulating T (Table 1) [79]. This level is sufficient to allow E2 to act as a local mediator that modulates synaptic plasticity [6,7,9,45,46]. On the other hand, the concentration of T in the hippocampus is approx. 17 nM, in which 3 nM T is synthesized in the hippocampus of male rats. The level of DHT is 7 nM in the hippocampus, and circulating DHT is 0.6 nM. Interestingly, castration depletes circulating T completely but does not considerably change the hippocampal E2 level (Table 1) [79]. Hippocampal E2 may be preferentially synthesized from hippocampal T, rather than from circulating T which is preferentially converted to DHT. If this is also the case in the hypothalamus, the so-called 'Testosterone Hypothesis' in the male animal (that male brain E2 is synthesized from circulating T, resulting in sexual dimorphism) will require re-examination, because brain estradiol may be preferentially synthesized from brain-derived T, rather than from circulating T. Of course for this re-examination, the hypothalamus rather than the hippocampus should be examined.

One of the functional differences between E2 produced from circulating T and E2 produced from hippocampus-derived T may be the time-dependencies of their levels (Fig. 7B). The brain is permeated with circulating T (male), or E2 (female), the levels of which change slowly depending on the circadian rhythm. On the other hand, the endogenous synthesis of E2 (for both male and female) is a transient event depending on neural excitation such as an LTP or LTD event, because the E2 level is significantly elevated upon Ca^{2+} influx by NMDA stimulation [4].

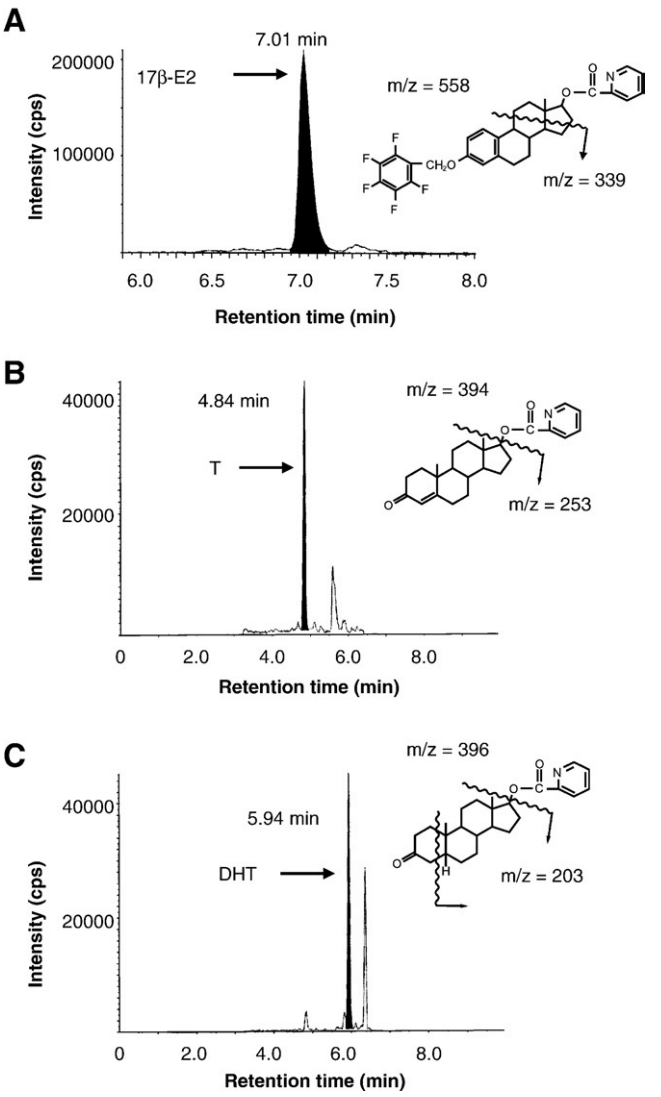


Fig. 6. Mass-spectrometric determination of sex-steroids (adult hippocampus). LC-MS/MS chromatograms and steroid derivatives of (A) 17β-E2, (B) T, and (C) DHT. Shaded portions indicate the intensity of the fragmented ions of 17β-E2-pentafluorobenzoxy-picolinoyl ($m/z = 339$, A), T-picolinoyl ($m/z = 253$, B) and DHT-picolinoyl ($m/z = 203$, C), respectively. The vertical axis indicates the intensity of the fragmented ions. The horizontal axis indicates the retention time of the fragmented ions, $t = 7.01$ min for E2 (A), $t = 4.84$ min for T (B) and $t = 5.94$ min for DHT (C). The time of injection to the LC system was defined as $t = 0$ min (modified from Hojo et al. [79]).

In many previous works, the concentration of endogenous E2 within neurons or glia has not been determined accurately (due to technical difficulties), therefore results are explained only with the concentration of exogenously added E2. This situation leads to many misunderstandings and conflicts. For *in vitro* experiments using slices or primary cultures of neuron/glia, the concentration of exogenously added E2 must be higher than that of endogenous E2 in order to cause a significant effect. Importantly, the endogenous E2 concentration is dependent on the outer medium (i.e., whether the outer medium contains steroids or not). Many experiments using acute hippocampal slices show restorative effects of E2 upon supplementation of E2 (1–10 nM) to E2-depleted slices whose E2 level is less than 0.5 nM. The depletion of E2 in slices occurs during recovery treatments, because a significant amount of E2 is released

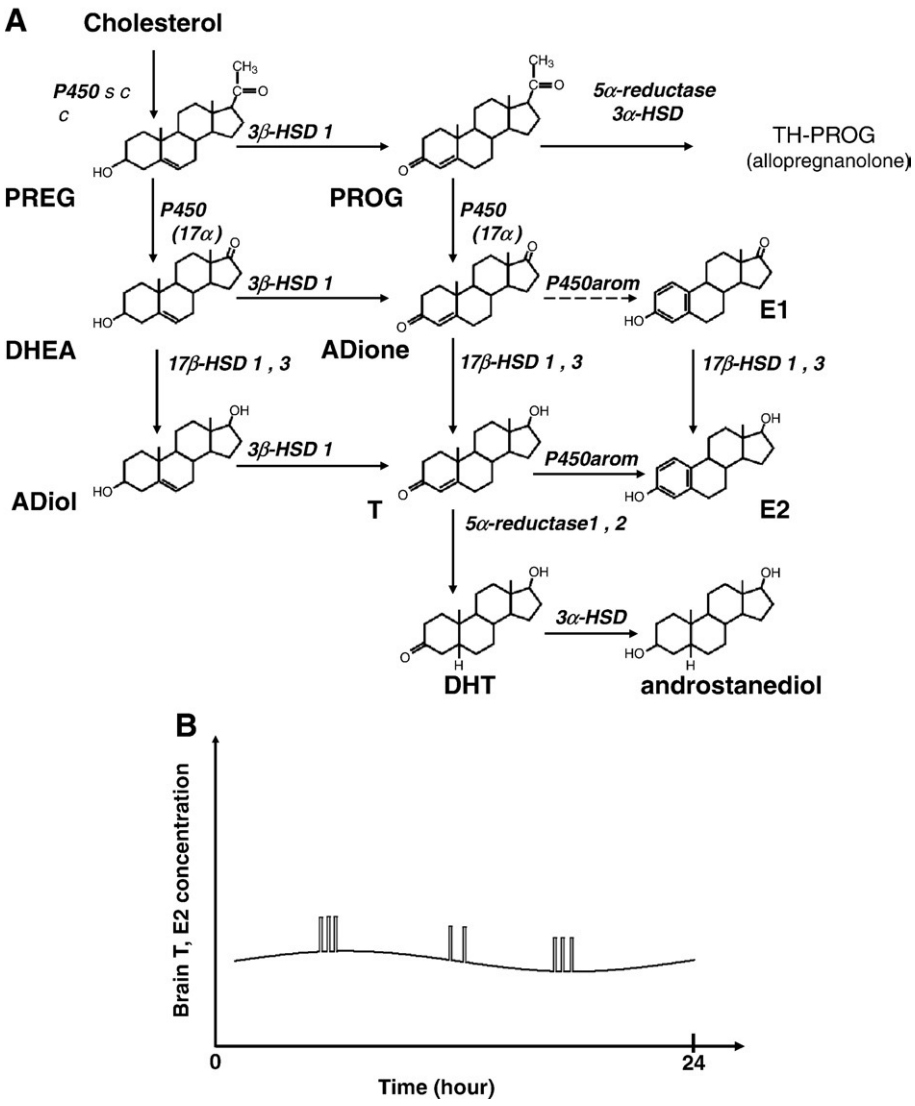


Fig. 7. Schematic illustrations. (A) Pathway of sex-steroid synthesis in the rat hippocampus, thus far identified. A real arrow (→) indicates an active pathway. A chain arrow from androstenedione (ADione) to estrone (E1) indicates an extremely weak conversion in male rat. The abbreviated names of steroids and enzymes involved in each reaction are indicated. Only 3β-HSD type 1 is present but other types of 3β-HSD (types 2–4) are absent. Androstenediol (ADiol) is a major product of DHEA. Once produced, E2 is very stable and is not inactivated to species such as E1 within 5 h. Roughly half of PREG and PROG is incorporated into hippocampus from circulation. Tetrahydro-PROG (TH-PROG) is also referred to as allopregnanolone. Because cytochrome P450scc is expressed extremely weakly in the adult hippocampus (10^{-5} of that in the adrenal cortex), the production of PREG from cholesterol may be performed very slowly. In the female rat hippocampus, ADione → E1 pathway may act as strongly as ADione → T. Identification of sexual difference in hippocampal sex-steroid synthesis requires further investigations. Incorporation of T, E2, PREG and PROG from the blood circulation may occur, as judged from the 50–80% decrease of their concentrations in the hippocampus by castration or ovariectomy. Estrogen replacement therapy should take into account these mechanisms of endogenous estrogen synthesis in the brain. Precursor replacement therapy, such as estriol (E3) supplementation for E3 → E1 → E2 conversion within the brain may be useful for the female, while DHEA supplementation for DHEA → T → E2 may be useful for the male. (B) Hippocampus-derived E2 or T may exhibit a great difference in function from circulating E2 or T. The level of circulating E2 or T changes depending on circadian rhythm (slow and homeostatic), but the level of hippocampus-derived E2 or T may change depending on neuronal activity in order to regulate synaptic plasticity or memory processes. Endogenous synthesis is dependent on synaptic activity, such as Ca^{2+}-influx dependent activation of StAR [1], and dephosphorylation-dependent (phosphatase-dependent) activation of P450arom [107]. Endogenous synthesis may also be dependent on GnRH in the female hippocampus [88].

to the steroid-free outer medium during incubation of isolated slices for 1–2 h with ACSF. In this sense, these isolated slice experiments may represent an *in vitro* model of *in vivo* estrogen replacement therapy (injecting E2 at 10–50 μg/kg rat weight) for circulating estrogen-depleted ovariectomized female animals (circulating E2 less than 0.01 nM). In the case of cultured slices or cultured neuron/glia, the endogenous E2 level may be 6–8 nM (65–83 fmol/mg protein) in slices determined via RIA or mass-spectrometric assay [87] or roughly 0.1 nM (120 pg/5 ml) in the outer medium (released E2) [5,68,88]. Therefore, the expected effect of exogenously added E2 may strongly depend on the concentration difference between the exogenously added E2 level and the endogenous E2 level.

4. Difference between classical slow genomic pathway and rapid synaptic pathway of estrogen

Not only slow (gene transcriptional) but also rapid (kinase driving) estrogen signaling pathways function independently in the brain (Fig 8).

4.1. Slow action via genomic pathway

Classical genomic effects have been studied extensively in the past few decades, focusing mainly on restorative effects on brain function by supplemented estrogen in ovariectomized female rats. In classical slow genomic pathway, gonadal E2 → reaches neurons via the

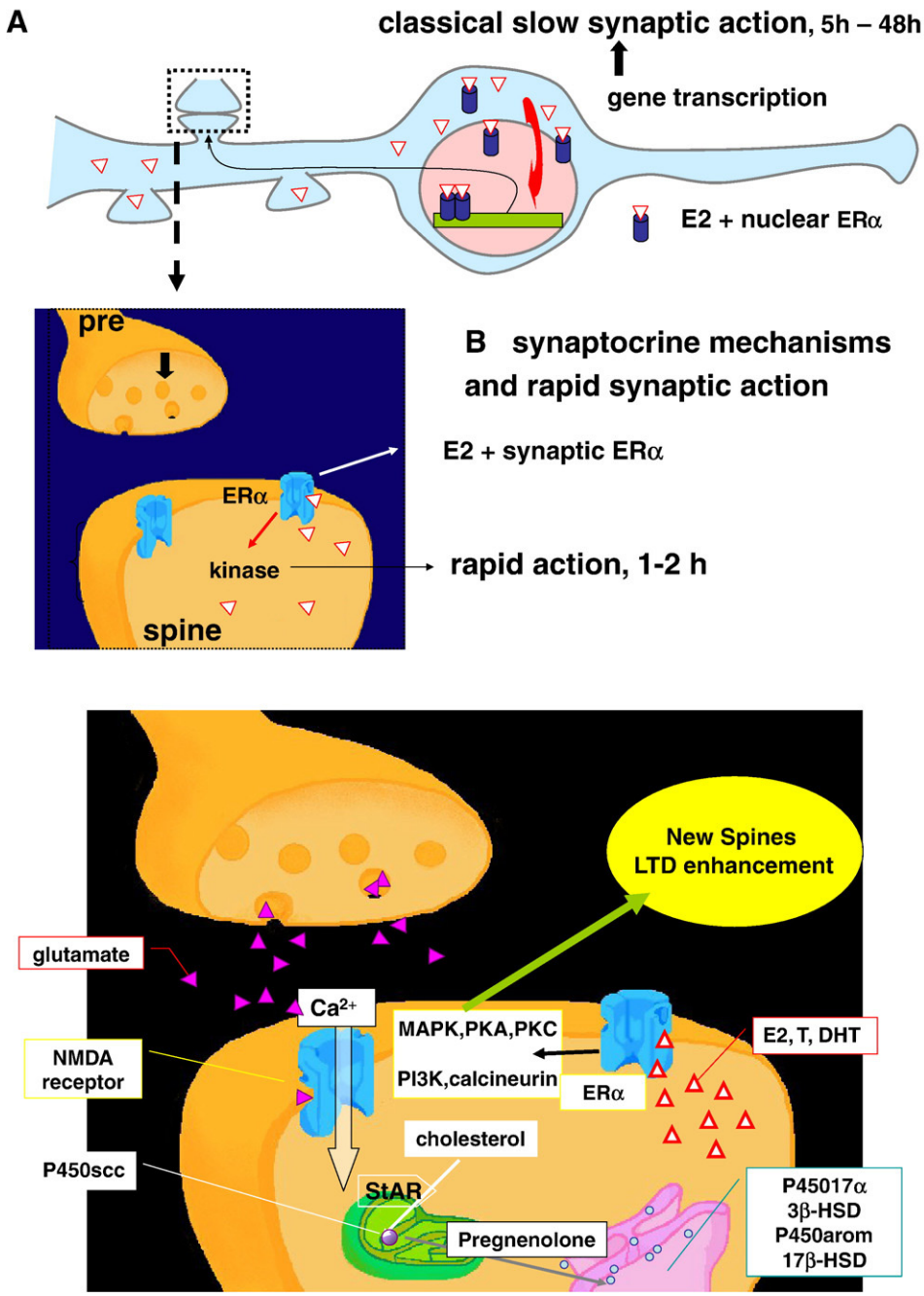


Fig. 8. (A) Schematic illustration for the slow modulation of synaptic plasticity via gene transcription. The site of the delayed action of E2 is ERα present in cytoplasm and nuclei of neurons. The neuronal synthesis (intracrine mechanism) of sex-steroids produces more E2 than E2 from circulation. StAR and P450scc are present in the mitochondria, and P450 (17α), 3β-HSD, 17β-HSD and P450arom are localized in the endoplasmic reticulum. (B) Schematic illustration for the synaptic synthesis (synaptocrinology mechanism) of sex hormones, and the rapid modulation of the synaptic plasticity via synaptic ER. The site of rapid action of E2 is synaptic ERα. Synaptic ERβ might also function. StAR and P450scc are present in the mitochondria. P450(17α), 3β-HSD, 17β-HSD and P450arom are localized in the membranes in the synaptic compartment. Only NMDA type glutamate receptor is illustrated, while AMPA type glutamate receptor is omitted for clarity. Glial steroidogenesis also occurs, but may be much weaker than neuronal steroidogenesis, as judged from the much weaker expression of steroidogenic enzymes in glial cells in the hippocampus.

circulation → cytoplasmic ERα or ERβ → nucleus → gene transcription → new proteins (NMDA-R NR1 subunit, etc.) → neuroprotection, synaptogenesis, and enhancement of LTP. Because the activation of both the transcriptional and translational machinery of the cell is necessary to invoke classical steroid actions, a time-lag of several hours to days must be present between the beginning of the steroid action and its physiological consequences.

The chronic genomic effects of E2 on synaptic plasticity have been extensively investigated. For example, the dendritic spine density in CA1 pyramidal neurons is sensitive to both naturally occurring estrogen fluctuations in rats [10], and experimentally induced estrogen depletion and replacement [32]. Recent evidence suggests that estrogens mediate these morphological changes by means of NMDA receptors. E2 increases the binding of NMDA or glutamate to NMDA receptors, as well as the NR1 level in CA1 dendrites [36,37,89]. Moreover, the estrogen-induced increase in dendritic spine density is blocked by NMDA receptor antagonists [11,38,90], and the electrophysiological properties of NMDA receptor-mediated transmission are altered by estrogen [7,8,44]. The investigation of the signaling pathway of endogenous E2, which results in the modulation of synaptic plasticity of neural networks in the hippocampus, may contribute to an understanding of therapeutic effects because several-

years of therapy with estrogen for female patients of Alzheimer's disease following menopause is shown to be very effective in improving their capacity for learning and memory [14–17].

4.2. Gene transcription as a downstream event of rapid synaptic action

Rapid synaptic actions of E2 are not limited to those within synapses but also may include the triggering of gene transcription in nuclei. Many kinases (MAP kinase, A-kinase, PI3 kinase, C-kinase, etc.) are activated in the rapid synaptic actions of E2 [91–93]. These kinases (or target proteins) probably travel to nuclei, resulting in gene transcription. For example, A-kinase → phosphorylation of CREB → gene transcription → new protein → new synapses [94]; MAP kinase → gene transcription → new protein→ new synapses → protection of neuron from damage [94]; PI3 kinase → Akt kinase → suppression of GSK-3β→ suppression of hyperphosphorylation of tau → inhibition of disassembling of microtubles → protection from Alzheimer's disease [95,96]; PI3 kinase → Akt kinase → suppression of apoptosis signal from mitochondria →protection of neuron from damage; C-kinase → NFκB → gene transcription → new protein → new synapses [97].

5. Estrogen action and synthesis in non-mammalian brain

Studies of estrogen effects on non-mammalian brains including the hippocampus are useful for mammalian study [98]. Estrogen actions are extensively investigated in the songbird brain (e.g. zebra finch) [99] [100]. Volumes of song control nuclei (HVC (high vocal center), RA (nucleus robustus arcopallialis), and Area X) are affected by estrogen [101] which action is mediated by P450arom activity at synapses [102]. Local and rapid increase of E2 in auditory cortex is revealed during social interaction [103]. Rapid production and action of estrogen might be regulated by the phosphorylation of P450arom [98]. In the songbird hippocampus E2, but not DHT, facilitates the acquisition of spatial memory [104]. A significant level of P450arom expression is observed in the hippocampus [105]. Taken together, it is suggested that locally synthesized E2 from circulating T probably acts in paracrine- or synaptocrine-fashion in the brain.

6. Model explanation of synaptocrine and intracrine mechanisms

Fig. 8 shows a hypothetical model for the synaptic synthesis of brain steroids (synaptocrine mechanism) and the modulation of the synaptic plasticity of neurons by brain steroids. Brain steroid synthesis proceeds in the following manner. First, glutamate release from the presynapse induces a Ca^{2+} influx through the NMDA receptors. The Ca^{2+} influx drives StAR [106] to transport cholesterol into the mitochondria, where P450scc converts cholesterol to PREG. The conversion of 'PREG → DHEA → androstenediol → T → E2, or T → DHT → androstanediol, or progesterone (PROG) → androstenedione → T' is performed at spines, in addition to the endoplasmic reticulum in the cell body by P450(17α), 3β-HSD, 17β-HSD, P450arom, 5α-reductase and 3α-HSD. The produced E2 binds to synaptic ERα and drives a signaling pathway including many kinases (MAP kinase, A-kinase, C-kinase, PI3 kinase or even phosphatases), finally resulting in the modulation of AMPA receptors (AMPA type glutamatergic receptors) or NMDA receptors (NMDA type glutamatergic receptors). Modulation indicates, for example, phosphorylation of these receptors or AMPA receptor insertion/endocytosis. Note, of course, that brain steroids are synthesized also in the endoplasmic reticulum and mitochondria in the cell bodies of neurons (intracrine mechanisms). The genomic pathway via nuclear ERα receptors also functions in delayed E2 effects, such as neuroprotection, spinogenesis, maintaining homeostasis, etc (intracrine mechanisms). Because the levels of E2, T and DHT are much lower in the circulation [79,86], hippocampus-synthesized sex-steroids may play a central role in the modulation of synaptic plasticity or memory process.

An immunoelectron microscopic analysis using a postembedding immunogold method is very useful in determining the intraneuronal localization of P450 or ERα in the hippocampal neurons of adult male rats. Surprisingly, we observed that both P450(17α) and P450arom are localized not only in the endoplasmic reticulum but also in the presynaptic region as well as the postsynaptic region of pyramidal neurons in the CA1 and CA3 regions and of granule neurons in DG (Fig. 4). These results suggest the 'synaptic' synthesis of estrogen and androgen, in addition to classical microsomal synthesis of sex steroids.

References

[1] T. Kimoto, T. Tsurugizawa, Y. Ohta, J. Makino, H. Tamura, Y. Hojo, N. Takata, S. Kawato, Neurosteroid synthesis by cytochrome p450-containing systems localized in the rat brain hippocampal neurons: N-methyl-D-aspartate and calcium-dependent synthesis, Endocrinology 142 (2001) 3578–3589.

[2] S. Kawato, Y. Hojo, T. Kimoto, Histological and metabolism analysis of P450 expression in the brain, Methods Enzymol. 357 (2002) 241–249.

[3] S. Kawato, M. Yamada, T. Kimoto, Brain neurosteroids are 4th generation neuromessengers in the brain: cell biophysical analysis of steroid signal transduction, Adv. Biophys. 37 (2003) 1–48.

[4] Y. Hojo, T.A. Hattori, T. Enami, A. Furukawa, K. Suzuki, H.T. Ishii, H. Mukai, J.H. Morrison, W.G. Janssen, S. Kominami, N. Harada, T. Kimoto, S. Kawato, Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes P45017alpha and P450 aromatase localized in neurons, Proc. Natl. Acad. Sci. U S A 101 (2004) 865–870.

[5] O. Kretz, L. Fester, U. Wehrenberg, L. Zhou, S. Brauckmann, S. Zhao, J. Prange-Kiel, T. Naumann, H. Jarry, M. Frotscher, G.M. Rune, Hippocampal synapses depend on hippocampal estrogen synthesis, J. Neurosci. 24 (2004) 5913–5921.

[6] R. Bi, G. Broutman, M.R. Foy, R.F. Thompson, M. Baudry, The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus, Proc. Natl. Acad. Sci. U S A 97 (2000) 3602–3607.

[7] M.R. Foy, J. Xu, X. Xie, R.D. Brinton, R.F. Thompson, T.W. Berger, 17beta-estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation, J. Neurophysiol. 81 (1999) 925–929.

[8] L.D. Pozzo-Miller, T. Inoue, D.D. Murphy, Estradiol increases spine density and NMDA-dependent Ca2+ transients in spines of CA1 pyramidal neurons from hippocampal slices, J. Neurophysiol. 81 (1999) 1404–1411.

[9] K. Shibuya, N. Takata, Y. Hojo, A. Furukawa, N. Yasumatsu, T. Kimoto, T. Enami, K. Suzuki, N. Tanabe, H. Ishii, H. Mukai, T. Takahashi, T.A. Hattori, S. Kawato, Hippocampal cytochrome P450s synthesize brain neurosteroids which are paracrine neuromodulators of synaptic signal transduction, Biochim. Biophys. Acta 1619 (2003) 301–316.

[10] C.S. Woolley, Estrogen-mediated structural and functional synaptic plasticity in the female rat hippocampus, Horm. Behav. 34 (1998) 140–148.

[11] C.S. Woolley, B.S. McEwen, Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism, J. Neurosci. 14 (1994) 7680–7687.

[12] H. Mukai, N. Takata, H.T. Ishii, N. Tanabe, Y. Hojo, A. Furukawa, T. Kimoto, S. Kawato, Hippocampal synthesis of estrogens and androgens which are paracrine modulators of synaptic plasticity: synaptocrinology, Neuroscience 138 (2006) 757–764.

[13] T.J. Teyler, R.M. Vardaris, D. Lewis, A.B. Rawitch, Gonadal steroids: effects on excitability of hippocampal pyramidal cells, Science 209 (1980) 1017–1018.

[14] M. Baldereschi, A. Di Carlo, V. Lepore, L. Bracco, S. Maggi, F. Grigoletto, G. Scarlato, L. Amaducci, Estrogen-replacement therapy and Alzheimer's disease in the Italian Longitudinal Study on Aging, Neurology 50 (1998) 996–1002.

[15] S.C. Waring, W.A. Rocca, R.C. Petersen, P.C. O'Brien, E.G. Tangalos, E. Kokmen, Postmenopausal estrogen replacement therapy and risk of AD: a population-based study, Neurology 52 (1999) 965–970.

[16] C. Kawas, S. Resnick, A. Morrison, R. Brookmeyer, M. Corrada, A. Zonderman, C. Bacal, D.D. Lingle, E. Metter, A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: the Baltimore Longitudinal Study of Aging, Neurology 48 (1997) 1517–1521.

[17] P. Collins, C. Webb, Estrogen hits the surface, Nat. Med. 5 (1999) 1130–1131.

[18] S.A. Shumaker, C. Legault, S.R. Rapp, L. Thal, R.B. Wallace, J.K. Ockene, S.L. Hendrix, B.N. Jones 3rd, A.R. Assaf, R.D. Jackson, J.M. Kotchen, S. Wassertheil-Smoller, J. Wactawski-Wende, Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial, Jama 289 (2003) 2651–2662.

[19] H. Mukai, T. Tsurugizawa, M. Ogiue-Ikeda, G. Murakami, Y. Hojo, H. Ishii, T. Kimoto, S. Kawato, Local neurosteroid production in the hippocampus: influence on synaptic plasticity of memory, Neuroendocrinology 84 (2006) 255–263.

[20] Y. Komatsuzaki, G. Murakami, T. Tsurugizawa, H. Mukai, N. Tanabe, K. Mitsuhashi, M. Kawata, T. Kimoto, Y. Ooishi, S. Kawato, Rapid spinogenesis of pyramidal neurons induced by activation of glucocorticoid receptors in adult male rat hippocampus, Biochem. Biophys. Res. Commun. 335 (2005) 1002–1007.

[21] G. Murakami, T. Tsurugizawa, Y. Hatanaka, Y. Komatsuzaki, N. Tanabe, H. Mukai, Y. Hojo, S. Kominami, T. Yamazaki, T. Kimoto, S. Kawato, Comparison between

basal and apical dendritic spines in estrogen-induced rapid spinogenesis of CA1 principal neurons in the adult hippocampus, Biochem. Biophys. Res. Commun. 351 (2006) 553–558.
[22] T. Tsurugizawa, H. Mukai, N. Tanabe, G. Murakami, Y. Hojo, S. Kominami, K. Mitsuhashi, Y. Komatsuzaki, J.H. Morrison, W.G. Janssen, T. Kimoto, S. Kawato, Estrogen induces rapid decrease in dendritic thorns of CA3 pyramidal neurons in adult male rat hippocampus, Biochem. Biophys. Res. Commun. 337 (2005) 1345–1352.
[23] H. Mukai, T. Tsurugizawa, G. Murakami, S. Kominami, H. Ishii, M. Ogiue-Ikeda, N. Takata, N. Tanabe, A. Furukawa, Y. Hojo, Y. Ooishi, J.H. Morrison, W.G. Janssen, J.A. Rose, P. Chambon, S. Kato, S. Izumi, T. Yamazaki, T. Kimoto, S. Kawato, Rapid modulation of long-term depression and spinogenesis via synaptic estrogen receptors in hippocampal principal neurons, J. Neurochem. 100 (2007) 950–967.
[24] W.R. Harrington, S. Sheng, D.H. Barnett, L.N. Petz, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression, Mol. Cell. Endocrinol. 206 (2003) 13–22.
[25] H. Ishii, T. Tsurugizawa, M. Ogiue-Ikeda, M. Asashima, H. Mukai, G. Murakami, Y. Hojo, T. Kimoto, S. Kawato, Local production of sex hormones and their modulation of hippocampal synaptic plasticity, Neuroscientist 13 (2007) 323–334.
[26] D.T. Monaghan, V.R. Holets, D.W. Toy, C.W. Cotman, Anatomical distributions of four pharmacologically distinct 3H-L-glutamate binding sites, Nature 306 (1983) 176–179.
[27] A. Baude, Z. Nusser, E. Molnar, R.A. McIlhinney, P. Somogyi, High-resolution immunogold localization of AMPA type glutamate receptor subunits at synaptic and non-synaptic sites in rat hippocampus, Neuroscience 69 (1995) 1031–1055.
[28] J.M. Fritschy, O. Weinmann, A. Wenzel, D. Benke, Synapse-specific localization of NMDA and GABA(A) receptor subunits revealed by antigen-retrieval immunohistochemistry, J. Comp. Neurol. 390 (1998) 194–210.
[29] C.A. Reid, R. Fabian-Fine, A. Fine, Postsynaptic calcium transients evoked by activation of individual hippocampal mossy fiber synapses, J. Neurosci. 21 (2001) 2206–2214.
[30] C.A. Reid, The role of dendritic spines: comparing the complex with the simple, Eur. J. Pharmacol. 447 (2002) 173–176.
[31] N.J. MacLusky, V.N. Luine, T. Hajszan, C. Leranth, The 17alpha and 17beta isomers of estradiol both induce rapid spine synapse formation in the CA1 hippocampal subfield of ovariectomized female rats, Endocrinology 146 (2005) 287–293.
[32] E. Gould, C.S. Woolley, M. Frankfurt, B.S. McEwen, Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood, J. Neurosci. 10 (1990) 1286–1291.
[33] C.S. Woolley, E. Gould, B.S. McEwen, Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons, Brain. Res. 531 (1990) 225–231.
[34] C.S. Woolley, B.S. McEwen, Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat, J. Neurosci. 12 (1992) 2549–2554.
[35] C. Leranth, M. Shanabrough, T.L. Horvath, Hormonal regulation of hippocampal spine synapse density involves subcortical mediation, Neuroscience 101 (2000) 349–356.
[36] N.G. Weiland, Estradiol selectively regulates agonist binding sites on the N-methyl-D-aspartate receptor complex in the CA1 region of the hippocampus, Endocrinology 131 (1992) 662–668.
[37] A.H. Gazzaley, N.G. Weiland, B.S. McEwen, J.H. Morrison, Differential regulation of NMDAR1 mRNA and protein by estradiol in the rat hippocampus, J. Neurosci. 16 (1996) 6830–6838.
[38] D.D. Murphy, M. Segal, Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones, J. Neurosci. 16 (1996) 4059–4068.
[39] C. Leranth, O. Petnehazy, N.J. MacLusky, Gonadal hormones affect spine synaptic density in the CA1 hippocampal subfield of male rats, J. Neurosci. 23 (2003) 1588–1592.
[40] N.J. MacLusky, T. Hajszan, C. Leranth, Effects of dehydroepiandrosterone and flutamide on hippocampal CA1 spine synapse density in male and female rats: implications for the role of androgens in maintenance of hippocampal structure, Endocrinology 145 (2004) 4154–4161.
[41] Y. Hatanaka, H. Mukai, K. Mitsuhashi, Y. Hojo, G. Murakami, Y. Komatsuzaki, R. Sato, S. Kawato, Androgen rapidly increases dendritic thorns of CA3 neurons in male rat hippocampus, Biochem. Biophys. Res. Commun. 381 (2009) 728–732.
[42] C.C. Smith, L.L. McMahon, Estrogen-induced increase in the magnitude of long-term potentiation occurs only when the ratio of NMDA transmission to AMPA transmission is increased, J. Neurosci. 25 (2005) 7780–7791.
[43] C.C. Smith, L.L. McMahon, Estradiol-induced increase in the magnitude of long-term potentiation is prevented by blocking NR2B-containing receptors, J. Neurosci. 26 (2006) 8517–8522.
[44] D.A. Cordoba Montoya, H.F. Carrer, Estrogen facilitates induction of long term potentiation in the hippocampus of awake rats, Brain. Res. 778 (1997) 430–438.
[45] Q. Gu, R.L. Moss, 17 beta-Estradiol potentiates kainate-induced currents via activation of the cAMP cascade, J. Neurosci. 16 (1996) 3620–3629.
[46] K. Ito, K.L. Skinkle, T.P. Hicks, Age-dependent, steroid-specific effects of oestrogen on long-term potentiation in rat hippocampal slices, J. Physiol. 515 (Pt 1) (1999) 209–220.
[47] S. Kawato, Endocrine disrupters as disrupters of brain function: a neurosteroid viewpoint, Environ. sci. 11 (2004) 1–14.
[48] M. Migaud, P. Charlesworth, M. Dempster, L.C. Webster, A.M. Watabe, M. Makhinson, Y. He, M.F. Ramsay, R.G. Morris, J.H. Morrison, T.J. O'Dell, S.G. Grant, Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein, Nature 396 (1998) 433–439.
[49] H.K. Lee, K. Kameyama, R.L. Huganir, M.F. Bear, NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus, Neuron 21 (1998) 1151–1162.
[50] J. Lisman, A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory, Proc. Natl. Acad. Sci. U S A 86 (1989) 9574–9578.
[51] S.N. Yang, Y.G. Tang, R.S. Zucker, Selective induction of LTP and LTD by postsynaptic [Ca2+]i elevation, J. Neurophysiol. 81 (1999) 781–787.
[52] M. Ogiue-Ikeda, N. Tanabe, H. Mukai, Y. Hojo, G. Murakami, T. Tsurugizawa, N. Takata, T. Kimoto, S. Kawato, Rapid modulation of synaptic plasticity by estrogens as well as endocrine disrupters in hippocampal neurons, Brain. Res. Rev. 57 (2008) 363–375.
[53] G. Murakami, N. Tanabe, H.T. Ishii, M. Ogiue-Ikeda, T. Tsurugizawa, H. Mukai, Y. Hojo, N. Takata, A. Furukawa, T. Kimoto, S. Kawato, Role of cytochrome p450 in synaptocrinology: endogenous estrogen synthesis in the brain hippocampus, Drug. Metab. Rev. 38 (2006) 353–369.
[54] Q. Gu, K.S. Korach, R.L. Moss, Rapid action of 17beta-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors, Endocrinology 140 (1999) 660–666.
[55] J.F. Couse, S.W. Curtis, T.F. Washburn, J. Lindzey, T.S. Golding, D.B. Lubahn, O. Smithies, K.S. Korach, Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene, Mol. Endocrinol. 9 (1995) 1441–1454.
[56] M. Kos, S. Denger, G. Reid, K.S. Korach, F. Gannon, Down but not out? A novel protein isoform of the estrogen receptor alpha is expressed in the estrogen receptor alpha knockout mouse, J. Mol. Endocrinol. 29 (2002) 281–286.
[57] C. Pendaries, B. Darblade, P. Rochaix, A. Krust, P. Chambon, K.S. Korach, F. Bayard, J.F. Arnal, The AF-1 activation-function of ERalpha may be dispensable to mediate the effect of estradiol on endothelial NO production in mice, Proc. Natl. Acad. Sci. U S A 99 (2002) 2205–2210.
[58] S. Dupont, A. Krust, A. Gansmuller, A. Dierich, P. Chambon, M. Mark, Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes, Development 127 (2000) 4277–4291.
[59] A. Pedram, M. Razandi, E.R. Levin, Nature of functional estrogen receptors at the plasma membrane, Mol. Endocrinol. 20 (2006) 1996–2009.
[60] M. Razandi, A. Pedram, G.L. Greene, E.R. Levin, Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells, Mol. Endocrinol. 13 (1999) 307–319.
[61] T.A. Milner, K. Ayoola, C.T. Drake, S.P. Herrick, N.E. Tabori, B.S. McEwen, S. Warrier, S. E. Alves, Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation, J. Comp. Neurol. 491 (2005) 81–95.
[62] P. Thomas, Y. Pang, E.J. Filardo, J. Dong, Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells, Endocrinology 146 (2005) 624–632.
[63] C.M. Revankar, D.F. Cimino, L.A. Sklar, J.B. Arterburn, E.R. Prossnitz, A transmembrane intracellular estrogen receptor mediates rapid cell signaling, Science 307 (2005) 1625–1630.
[64] E. Brailoiu, S.L. Dun, G.C. Brailoiu, K. Mizuo, L.A. Sklar, T.I. Oprea, E.R. Prossnitz, N.J. Dun, Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system, J. Endocrinol. 193 (2007) 311–321.
[65] H. Sakamoto, K. Matsuda, K. Hosokawa, M. Nishi, J.F. Morris, E.R. Prossnitz, M. Kawata, Expression of G protein-coupled receptor-30, a G protein-coupled membrane estrogen receptor, in oxytocin neurons of the rat paraventricular and supraoptic nuclei, Endocrinology 148 (2007) 5842–5850.
[66] E.E. Baulieu, P. Robel, Dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) as neuroactive neurosteroids, Proc. Natl. Acad. Sci. U S A 95 (1998) 4089–4091.
[67] L. Fester, V. Ribeiro-Gouveia, J. Prange-Kiel, C. von Schassen, M. Bottner, H. Jarry, G.M. Rune, Proliferation and apoptosis of hippocampal granule cells require local oestrogen synthesis, J. Neurochem. 97 (2006) 1136–1144.
[68] J. Prange-Kiel, L. Fester, L. Zhou, H. Lauke, J. Carretero, G.M. Rune, Inhibition of hippocampal estrogen synthesis causes region-specific downregulation of synaptic protein expression in hippocampal neurons, Hippocampus 16 (2006) 464–471.
[69] S. Higo, Y. Hojo, H. Ishii, T. Kominami, K. Nakajima, D. Poirier, T. Kimoto, S. Kawato, Comparison of sex-steroid synthesis between neonatal and adult rat hippocampus, Biochem. Biophys. Res. Commun. 385 (2009) 62–66.
[70] S.H. Mellon, C.F. Deschepper, Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain, Brain Res 629 (1993) 283–292.
[71] J.L. Sanne, K.E. Krueger, Expression of cytochrome P450 side-chain cleavage enzyme and 3 beta-hydroxysteroid dehydrogenase in the rat central nervous system: a study by polymerase chain reaction and in situ hybridization, J. Neurochem. 65 (1995) 528–536.
[72] A. Furukawa, A. Miyatake, T. Ohnishi, Y. Ichikawa, Steroidogenic acute regulatory protein (StAR) transcripts constitutively expressed in the adult rat central nervous system: colocalization of StAR, cytochrome P-450SCC (CYP XIA1), and 3beta-hydroxysteroid dehydrogenase in the rat brain, J. Neurochem. 71 (1998) 2231–2238.
[73] S.L. King, M.J. Marks, S.R. Grady, B.J. Caldarone, A.O. Koren, A.G. Mukhin, A.C. Collins, M.R. Picciotto, Conditional expression in corticothalamic efferents reveals a developmental role for nicotinic acetylcholine receptors in modulation of passive avoidance behavior, J. Neurosci. 23 (2003) 3837–3843.
[74] C. Ibanez, R. Guennoun, P. Liere, B. Eychenne, A. Pianos, M. El-Etr, E.E. Baulieu, M. Schumacher, Developmental expression of genes involved in neurosteroidogenesis:

3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase in the rat brain, Endocrinology 144 (2003) 2902–2911.
[75] T. Ivanova, C. Beyer, Ontogenetic expression and sex differences of aromatase and estrogen receptor-alpha/beta mRNA in the mouse hippocampus, Cell Tissue Res. 300 (2000) 231–237.
[76] U. Wehrenberg, J. Prange-Kiel, G.M. Rune, Steroidogenic factor-1 expression in marmoset and rat hippocampus: co-localization with StAR and aromatase, J. Neurochem. 76 (2001) 1879–1886.
[77] S. Beyenburg, M. Watzka, I. Blumcke, J. Schramm, F. Bidlingmaier, C.E. Elger, B. Stoffel-Wagner, Expression of mRNAs encoding for 17beta-hydroxisteroid dehydrogenase isozymes 1, 2, 3 and 4 in epileptic human hippocampus, Epilepsy Res. 41 (2000) 83–91.
[78] R.C. Agis-Balboa, G. Pinna, A. Zhubi, E. Maloku, M. Veldic, E. Costa, A. Guidotti, Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis, Proc. Natl. Acad. Sci. U S A 103 (2006) 14602–14607.
[79] Y. Hojo, S. Higo, H. Ishii, Y. Ooishi, H. Mukai, G. Murakami, T. Kominami, T. Kimoto, S. Honma, D. Poirier, S. Kawato, Comparison between hippocampus-synthesized and circulation-derived sex steroids in the hippocampus, Endocrinology 150 (2009) 5106–5112.
[80] M. Warner, J.A. Gustafsson, Nongenomic effects of estrogen: why all the uncertainty? Steroids 71 (2006) 91–95.
[81] M.J. Ebner, D.I. Corol, H. Havlikova, J.W. Honour, J.P. Fry, Identification of neuroactive steroids and their precursors and metabolites in adult male rat brain, Endocrinology 147 (2006) 179–190.
[82] T. Higashi, H. Sugitani, T. Yagi, K. Shimada, Studies on neurosteroids XVI. Levels of pregnenolone sulfate in rat brains determined by enzyme-linked immunosorbent assay not requiring solvolysis, Biol. Pharm. Bull. 26 (2003) 709–711.
[83] P. Liere, Y. Akwa, S. Weill-Engerer, B. Eychenne, A. Pianos, P. Robel, J. Sjovall, M. Schumacher, E.E. Baulieu, Validation of an analytical procedure to measure trace amounts of neurosteroids in brain tissue by gas chromatography-mass spectrometry, J. Chromatogr. B Biomed. Sci. Appl. 739 (2000) 301–312.
[84] S. Liu, J. Sjovall, W.J. Griffiths, Neurosteroids in rat brain: extraction, isolation, and analysis by nanoscale liquid chromatography-electrospray mass spectrometry, Anal. Chem. 75 (2003) 5835–5846.
[85] Y. Hojo, G. Murakami, H. Mukai, S. Higo, Y. Hatanaka, M. Ogiue-Ikeda, H. Ishii, T. Kimoto, S. Kawato, Estrogen synthesis in the brain–role in synaptic plasticity and memory, Mol. Cell. Endocrinol. 290 (2008) 31–43.
[86] K. Yamashita, M. Okuyama, Y. Watanabe, S. Honma, S. Kobayashi, M. Numazawa, Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography-electrospray ionization tandem mass spectrometry, Steroids 72 (2007) 819–827.
[87] E. Munetsuna, Y. Hojo, M. Hattori, H. Ishii, S. Kawato, A. Ishida, S.A. Kominami, T. Yamazaki, Retinoic acid stimulates 17beta-estradiol and testosterone synthesis in rat hippocampal slice cultures, Endocrinology 150 (2009) 4260–4269.
[88] J. Prange-Kiel, H. Jarry, M. Schoen, P. Kohlmann, C. Lohse, L. Zhou, G.M. Rune, Gonadotropin-releasing hormone regulates spine density via its regulatory role in hippocampal estrogen synthesis, J. Cell. Biol. 180 (2008) 417–426.
[89] M. Cyr, O. Ghribi, T. Di Paolo, Regional and selective effects of oestradiol and progesterone on NMDA and AMPA receptors in the rat brain, J. Neuroendocrinol. 12 (2000) 445–452.
[90] M. Cyr, O. Ghribi, C. Thibault, M. Morissette, M. Landry, T. Di Paolo, Ovarian steroids and selective estrogen receptor modulators activity on rat brain NMDA and AMPA receptors, Brain Res. Brain. Res. Rev. 37 (2001) 153–161.
[91] C.D. Toran-Allerand, M. Singh, G. Setalo Jr., Novel mechanisms of estrogen action in the brain: new players in an old story, Front Neuroendocrinol. 20 (1999) 97–121.
[92] P. Mendez, F. Wandosell, L.M. Garcia-Segura, Cross-talk between estrogen receptors and insulin-like growth factor-I receptor in the brain: cellular and molecular mechanisms, Front Neuroendocrinol. 27 (2006) 391–403.
[93] S.M. Belcher, A. Zsarnovszky, Estrogenic actions in the brain: estrogen, phytoestrogens, and rapid intracellular signaling mechanisms, J. Pharmacol. Exp. Ther. 299 (2001) 408–414.
[94] E.R. Kandel, J.H. Schwartz, T.M. Jessel, Principles of Neural Science, 4th Edition, McGraw-Hill, 2000.
[95] P. Cardona-Gomez, M. Perez, J. Avila, L.M. Garcia-Segura, F. Wandosell, Estradiol inhibits GSK3 and regulates interaction of estrogen receptors, GSK3, and beta-catenin in the hippocampus, Mol. Cell. Neurosci. 25 (2004) 363–373.
[96] A. Takashima, GSK-3 is essential in the pathogenesis of Alzheimer's disease, J. Alzheimers Dis. 9 (2006) 309–317.
[97] P.J. Meberg, W.R. Kinney, E.G. Valcourt, A. Routtenberg, Gene expression of the transcription factor NF-kappa B in hippocampus: regulation by synaptic activity, Brain Res. Mol. Brain Res. 38 (1996) 179–190.
[98] J. Balthazart, G.F. Ball, Is brain estradiol a hormone or a neurotransmitter? Trends Neurosci 29 (2006) 241–249.
[99] M.S. Brainard, A.J. Doupe, What songbirds teach us about learning, Nature 417 (2002) 351–358.
[100] G.F. Ball, J. Balthazart, Seasonal and hormonal modulation of neurotransmitter systems in the song control circuit, J. Chem. Neuroanat. (2009).
[101] L. Fusani, M. Gahr, Hormonal influence on song structure and organization: the role of estrogen, Neuroscience 138 (2006) 939–946.
[102] B.A. Schlinger, A.P. Arnold, Circulating estrogens in a male songbird originate in the brain, Proc. Natl. Acad Sci. U S A 89 (1992) 7650–7653.
[103] L. Remage-Healey, N.T. Maidment, B.A. Schlinger, Forebrain steroid levels fluctuate rapidly during social interactions, Nat. Neurosci. 11 (2008) 1327–1334.
[104] J.G. Oberlander, B.A. Schlinger, N.S. Clayton, C.J. Saldanha, Neural aromatization accelerates the acquisition of spatial memory via an influence on the songbird hippocampus, Horm. Behav. 45 (2004) 250–258.
[105] C.J. Saldanha, B.A. Schlinger, P.E. Micevych, T.L. Horvath, Presynaptic N-methyl-D-aspartate receptor expression is increased by estrogen in an aromatase-rich area of the songbird hippocampus, J. Comp. Neurol. 469 (2004) 522–534.
[106] V. Papadopoulos, Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: biological role in steroidogenic cell function, Endocr. Rev. 14 (1993) 222–240.
[107] J. Balthazart, G.F. Ball, Fast regulation of steroid biosynthesis: a further piece in the neurosteroid puzzle, Trends Neurosci. 23 (2000) 57–58.