Local Production of Sex Hormones and Their Modulation of Hippocampal Synaptic Plasticity

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It is believed that sex hormones are synthesized in the gonads and reach the brain via the blood circulation. In contrast with this view, the authors have demonstrated that sex hormones are also synthesized locally in the hippocampus and that these steroids act rapidly to modulate neuronal synaptic plasticity. The authors demonstrated that estrogens are locally synthesized from cholesterol through dehydroepiandrosterone and testosterone in adult hippocampal neurons. Significant expression of mRNA for P450(17α), P450arom, and other steroidogenic enzymes was demonstrated. Localization of P450(17α) and P450arom was observed in synapses of principal neurons. In contrast to the slow action of gonadal estradiol, hippocampal neuron-derived estradiol may act locally and rapidly within the neurons. For example, 1 to 10 nM estradiol rapidly enhances long-term depression (LTD). The density of thin spines is selectively increased within two hours upon application of estradiol in pyramidal neurons. Estrogen receptor ERα agonist has the same enhancing effect as estradiol on both LTD and spinogenesis. Localization of ERα in spines in addition to nuclei of principal neurons implies that synaptic ERα is responsible for rapid modulation of synaptic plasticity by endogenous estradiol. Activin A, a peptide sex hormone, may also play a role as a local endogenous modulator of synaptic plasticity. NEUROSCIENTIST 13(4):323–334, 2007. DOI: 10.1177/1073858407301396

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In recent years, increasing evidence has accumulated to support the local endogenous synthesis of estrogens, androgens, and nonsteroidal sex hormones in the mammalian brain, in areas such as the hippocampus (Kimoto and others 2001; Kawato and others 2002, 2003; Hojo and others 2004; Kretz and others 2004). In the 1980s, Baulieu and coworkers proposed a neurosteroid hypothesis, suggesting that pregnenolone (PREG), progesterone, and dehydroepiandrosterone (DHEA) may be endogenously synthesized in the brain due to the finding that PREG and DHEA have been present in the mammalian brain at concentrations greater than those in plasma (Corpechot and others 1981; Baulieu and Robel 1998). Because the concentration of PREG and DHEA does not decrease after adrenalectomy and castration, many experiments have been performed with the aim of demonstrating the de novo synthesis of DHEA within the brain (Corpechot and others 1981; Robel and others 1987).

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Direct demonstration of steroidogenesis in the mammalian brain had, however, long been not successful due to the extremely low levels of steroidogenic proteins in the brain (Warner and Gustafsson 1995). Therefore, sex steroids had not been considered to be brain-derived steroids and rather thought to reach the brain exclusively via blood circulation after crossing the blood-brain barrier (Baulieu and Robel 1998). This belief had been supported by many reports suggesting the absence of cytochrome P450(17α) (DHEA synthase) in the adult mammalian brain (Le Goascogne and others 1991; Mellon and Deschepper 1993) and also by the observation of the complete disappearance of testosterone in the brain within one day after castration (Baulieu and Robel 1998).

Extensive investigations have been performed to examine neuromodulatory actions of gonadal sex hormones in the brain, such as the hippocampus (Woolley and McEwen 1994; Woolley 1998), because the hippocampus is attractive as a center of learning and memory processes. Investigations often have focused on their role of slow modulation (occurring in a time scale of days) (Woolley and McEwen 1994; Woolley 1998; Pozzo-Miller and others 1999). The importance of the rapid effects of estrogens is also suggested from many observations of the modulation of synaptic plasticity of the hippocampus (Teyler and others 1980; Foy and others 1999; Bi and others 2000; Tsurugizawa and others 2005; Mukai and others 2006, 2007). Many scientists had, however, not seriously considered that these rapid modulations might favor the local hippocampal steroido-

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Table 1. Comparison of Relative mRNA Expression Level for Steroidogenic Enzymes in the Adult Rat (Three Months)

	Hippocampus	Hypothalamus	Adrenal/Testis/Ovary/Liver
P450scc	1	3	50000 (Ad)
P450(17α)	1	3	300 (Te)
P450arom	1	3	300 (Ov)
17β-HSD (type 1)	1	3	200 (Ov)
17β-HSD (type 3)	1	5	300 (Te)
3β-HSD (type 1)	1	3	5000 (Ov)
5α-reductase (type 1)	1	2	5 (Li)
ERα	1	5	20 (Ov)
ERβ	1	4	80 (Ov)

The level in the hippocampus is normalized to be 1. The hippocampus, hypothalamus, adrenal gland (Ad), testis (Te), ovary (Ov), and liver (Li) are compared. HSD, hydroxysteroid dehydrogenases. Values of mRNA expression level are approximate values obtained from semiquantitative reverse transcription PCR (RT-PCR) analyses (Hojo and others 2004; Ishii and Kawato, unpublished results).

genesis rather than gonadal hormones, which reach the brain, often traveling for long distances. A main reason for this is due to the extremely low level of expressions for P450s and hydroxyl steroid dehydrogenases (HSD) in the hippocampus, much lower than 1/100 of their levels in endocrine organs. To describe the biological significance of brain-derived steroids, it was essential to improve the sensitivity of measurements by nearly 1000-fold for immunostaining, Western blot, reverse transcription PCR (RT-PCR), and purified steroid detection.

Endogenous synthesis and the action of peptide sex hormones such as activin and inhibin also have been described in the hippocampus. The up-regulation of inhibin β_A mRNA has been observed in the hippocampus injured by the kainic acid treatment (Tretter and others 2000). The induction of activin A (a homodimer of inhibin β_A peptides) has been demonstrated to play a role in the neuroprotective action of basic fibroblast growth factor on the acute excitotoxic injury of the hippocampus (Tretter and others 2000). Activin has been reported to have a neurotrophic effect in hippocampal neurons (Iwahori and others 1997). Little knowledge, however, is presently available about the role of activin in synaptic plasticity.

For brain-derived sex hormones, the rapid modulation (within one to two hours) on synaptic plasticity and cognitive functions are probably essential functions. Here we describe recent progress in investigations on the local synthesis of estrogens and androgens in the hippocampus. We also describe molecular mechanisms of the rapid action of estradiol on synaptic transmission and spinogenesis.

Steroid Synthesis Systems in the Adult Rat Hippocampus

Expression of Transcripts for Steroidogenic Enzymes

Highly sensitive molecular biology investigations are necessary for determining the presence of steroidogenic enzymes because of the very low level of expression of the mRNAs in the cerebrum and cerebellum (Warner and Gustafsson 1995).

Collectively from many studies, the relative level of mRNA expressed in the hippocampus has been suggested to be lowest for cytochrome P450scc and 3β-HSD and highest for steroidogenic acute regulatory protein (StAR) and 5α-reductase, with that of P450arom expressed at an intermediate level (see Table 1).

The concentration of cytochrome P450scc (CYP11A1) mRNA expressed in the brain is reported to be only 10^{-4} to 10^{-5} of that in the adrenal gland (Mellon and Deschepper 1993; Sanne and Krueger 1995). Our analysis showed approximately 1/50,000 for the P450scc level in the adult hippocampus as compared with that in the adrenal gland (Murakami and others 2006a; Table 1). As a result, the presence of P450scc mRNA could be demonstrated only by the RT-PCR method. The ribonuclease (RNase) protection assay for P450scc in the hippocampus, which was performed using a ^{32}P-labeled rat P450scc antisense riboprobe, however, yielded no detectable specific hybridization signals (Furukawa and others 1998). On the other hand, because StAR is most abundant, not only the PCR amplification but also the RNase protection assay demonstrated the presence of StAR transcripts, with an expression level of approximately 1/200 of the level in the adrenal gland (Furukawa and others 1998; King and others 2002).

The mRNAs for cytochrome P450(17α) (CYP17A) had not been detected in the adult rat brain by either RNase protection assays or RT-PCR (Mellon and Deschepper 1993). The expression of the mRNA for P450(17α) had been reported by many laboratories as only transient, occurring during rat embryonic and neonatal development (Compagnone and others 1995; Zwain and Yen 1999a, 1999b). We overcame this difficulty by carefully choosing the sequence of primer pairs that have high specificity by minimizing Gibbs free energy upon recombination of a 3′-primer with cDNA, using computer calculation (Hojo and others 2004). In the hippocampal tissues from adult male rats aged three months, we observed that P450(17α) transcripts expressed approximately 1/300 (Hojo and others 2004), when compared with those expressed in the testis.

The role of P450arom (CYP19) (estrogen synthase) in the hippocampus had also not been well elucidated, primarily because many studies had suggested the absence of P450arom in the adult rat and mouse hippocampus. The significant expression of mRNA for P450arom in the pyramidal and granule neurons of the adult rat hippocampus, however, has recently been demonstrated using in situ hybridization (Wehrenberg and others 2001). The level of the mRNA expression in the adult mouse hippocampus was approximately half of that in neonatal stages (Ivanova and Beyer 2000). We observed that the P450arom transcripts expressed approximately 1/300 (Hojo and others 2004), as compared with those expressed in the ovary, by using carefully designed primer pairs for RT-PCR.

The presence of mRNAs for 17β-HSD types 1 and 3 has been demonstrated in the human and rat hippocampus (Beyenburg and others 2000). We investigated the expression level of mRNA transcripts for 17β-HSD (types 1–4) using carefully designed primer pairs in the hippocampus from adult male rats. The mRNA level of 17β-HSD transcripts observed was approximately 1/200, relative to the level in the ovary for 17β-HSD (type 1), and 1/300, relative to the level in the testis for 17β-HSD (type 3), respectively (Hojo and others 2004).

The localization in neurons of several steroidogenic proteins has been demonstrated by means of in situ hybridization. For example, mRNAs for both StAR and 3β-HSD mRNA (10^{-2} for StAR and 10^{-3} for 3β-HSD of the levels in the adrenal gland) have been observed to be localized along the pyramidal cell layer in the CA1 to CA3 regions and the granule cell layer in the dentate gyrus of rats (Furukawa and others 1998) and mice (King and others 2002).

Glial cells have been considered to play an important role in steroidogenesis, as many reports have indicated the presence of mRNA for P450scc, P450(17α), 3β-HSD, and 17β-HSD in cultures of astrocytes and oligodendrocytes from embryonic and neonatal brains (Baulieu 1997; Jung-Testas and others 1989; Zwain and Yen 1999a, 1999b). Although similar levels of P450(17α) mRNA had been reported to be expressed in both astrocytes and neurons in primary cell cultures from the brains of neonatal rats, a much lower metabolic activity had been observed in neurons than astrocytes for the conversion of PREG to DHEA (Zwain and Yen 1999a, 1999b). These investigations are available on primary glial cell cultures, which are easily prepared from embryonic and neonatal brains. However, information regarding the synthesis system of neurosteroids in the "adult" rat brain is not directly available from these cell culture studies.

Neuronal Localization of Proteins Investigated with Immunostaining

The role of neurons in steroid synthesis in mammalian brains had long been difficult to clearly determine, although some reports suggested the expression of several steroidogenic enzymes in the rat brain (Koenig and others 1995; Tsutsui and others 2000). The belief of the absence of P450(17α) had been partly due to the fact that many attempts to demonstrate the immunohistochemical reactivity in the rat brain had been unsuccessful for almost two decades (Le Goascogne and others 1991). We overcame many difficulties of nonspecific immunostaining by using affinity column-purified antibodies (instead of using nonpurified antisera), obtaining a good penetration of IgG by Triton X-100, as well as using fresh frozen slices of hippocampus (instead of using paraffin sections) from adult male rats. A significant localization of cytochromes P450scc, P450(17α), and P450arom was observed in pyramidal neurons in the CA1 to CA3 regions, as well as in granule cells in the dentate gyrus, by means of the immunohistochemical staining of hippocampal slices (Kimoto and others 2001; Kawato and others 2002, 2003; Hojo and others 2004). The colocalization of immunoreactivity against P450s and NeuN (marker of neuron) confirmed the presence of P450s in these neurons (Kimoto and others 2001; Kawato and others 2002; Hojo and others 2004). StAR was colocalized with P450s (Kimoto and others 2001; King and others 2002). These results imply that pyramidal neurons and granule cells are equipped with complete steroidogenic systems that catalyze the conversion of cholesterol to PREG, DHEA, testosterone, and estradiol.

An immunoelectron microscopic analysis using the postembedding immunogold method was very useful to determine the intraneuronal localization of P450(17α) in the hippocampal neurons of adult male rats. Surprisingly, we observed that both P450(17α) and P450arom were 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 to CA3 regions and of granule neurons in the dentate

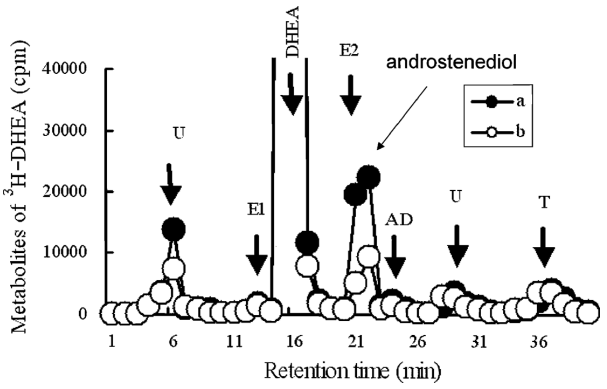

Fig. 1. Synthesis of estradiol and testosterone in adult hippocampal slices. High-performance liquid chromatography (HPLC) analysis shows the profile of ^{3}H-DHEA metabolites in the absence (line a) or in the presence (line b) of fadrozole (inhibitor of P450arom), after incubation of slices for five hours at 30 °C. In fadrozole-treated samples, E2 production was suppressed but androstenediol was still present at nearly the same position. E2, estradiol; T, testosterone; AD, androstenedione; E1, estrone; U, unknown metabolites; DHEA, dehydroepiandrosterone. The vertical axis indicates ^{3}H radioactivity (cpm).

gyrus. These results suggest a possibility of "synaptocrine" mechanisms of synthesis of estrogens and androgens, in addition to classic endocrine mechanisms in which sex steroids reach the brain via blood circulation.

The existence of these steroidogenic proteins was confirmed by Western immunoblot analyses. A single protein band was observed for each of these P450s (Kimoto and others 2001; Kawato and others 2002; Hojo and others 2004). The resulting molecular weights obtained for P450scc, P450(17α), and P450arom were nearly identical to those obtained from peripheral steroidogenic organs. The relative levels of these P450s in the hippocampus were approximately 1/500 (P450scc) and 1/300 (P450(17α) and P450arom) of that in the testis (P450scc and P450(17α)) and the ovary (P450arom), respectively.

From our observation in the adult hippocampus, the distributions of astroglial cells and oligodendroglial cells displayed very different patterns from those characteristics of the cells containing P450scc, P450(17α), and P450arom (Kimoto and others 2001; Kawato and others 2002; Hojo and others 2004). Because most P450-containing cells are neither astroglial cells nor oligodendroglial cells, the activity of neurosteroidogenesis in glial cells may be very low.

Synthesis of Estrogens and Androgens in the Hippocampus

A direct demonstration of the neuronal synthesis of DHEA in adult mammals was reported by our group for the first time (Kawato and others 2002; Hojo and others 2004). It had been assumed that DHEA and the sex steroids are supplied to the brain such as the hypothalamus, via the blood circulation, where they are converted to estradiol by P450arom (Baulieu 1997; Baulieu and Robel 1998). The absence of P450(17α) activity in the brain of adult mammals had been reported in a number of studies (Le Goascogne and others 1991; Baulieu and Robel 1998; Mensah-Nyagan and others 1999; Kibaly and others 2005). Incubations of [^{3}H]-PREG with brain slices, homogenates and microsomes, primary cultures of mixed glial cells, or astrocytes and neurons from rat and mouse embryos had failed to produce a radioactive metabolite 3H-DHEA (Baulieu and Robel 1998).

We recently succeeded in demonstrating the synthesis of DHEA, testosterone, and estradiol in the adult hippocampal slices by means of careful high-performance liquid chromatography (HPLC) analysis (Kawato and others 2002; Hojo and others 2004). The purification of neurosteroids from very fatty brain tissues required the application of a set of sophisticated methods, which included purification with organic solvent, column chromatography, and HPLC (Kimoto and others 2001; Hojo and others 2004; Wang and others 1997). The significant conversion from [^{3}H]-PREG to [^{3}H]-DHEA, as well as from [^{3}H]-DHEA to [^{3}H]-androstenediol, [^{3}H]-testosterone, and [^{3}H]-estradiol, was observed after incubation with the slices for five hours (Hojo and others 2004; Fig. 1). The conversion from [^{3}H]-testosterone to [^{3}H]-estradiol and [^{3}H]-dihydrotestosterone was also demonstrated. These activities were abolished by the application of specific inhibitors of cytochrome P450s. Interestingly, [^{3}H]-estradiol was rather stably present and not significantly converted to other steroid metabolites. On the other hand, dihydrotestosterone was rapidly converted to 3α, 5α-androstanediol.

We determined the concentration of DHEA and estradiol as well as PREG in acute hippocampal slices from adult male rats by means of radioimmunoassay (RIA) or liquid chromatography/tandem mass spectroscopy (LC/MS/MS) after careful purification of steroids with HPLC. The basal concentrations of PREG, DHEA, and estradiol in the male rat hippocampus were approximately 18, 0.3, and 0.6 nM, which were 6 to 10 times greater than those typical of plasma (Kimoto and others 2001; Kawato and others 2002; Hojo and others 2004). To demonstrate the rapid net production of neurosteroids upon synaptic stimulation, the *N*-methyl-D-aspartate (NMDA)–induced production of PREG and estradiol was investigated in hippocampal slices (Kimoto and others 2001; Kawato and others 2002; Hojo and others 2004). Upon stimulation with NMDA for 30 minutes, the hippocampal level of PREG and estradiol increased to approximately twofold of the basal levels. This implies that the NMDA-induced Ca^{2+} influx drives net production of PREG and estradiol. Estradiol synthesis has also been demonstrated in cultured hippocampal slices in the absence and presence of letrozole, an inhibitor of P450arom. After 4 days of treatment with letrozole, the amount of estradiol released into the medium was significantly decreased (Kretz and others 2004).

Interestingly, PREG sulfate and DHEA sulfate have been reported to be absent in the rat brain, as measured by direct mass spectroscopic analysis, although cholesterol sulfate is present (Higashi and others 2003; Liu and others 2003; Liere and others 2004). In many previous publications, PREG sulfate or DHEA sulfate had been determined indirectly—that is, by measuring PREG or DHEA after solvolysis of water-soluble fractions, which may contain some PREG derivatives different from sulfated steroids (Corpechot and others 1981; Baulieu 1997; Liere and others 2000; Kimoto and others 2001; Liu and others 2003). Because numerous publications have reported that sulfated steroids are important participants in neuromodulation, these results merit careful consideration (Wu and others 1991; Vallee and others 1997; Baulieu and Robel 1998).

Is the local concentration of brain neurosteroids sufficiently high to allow action as local mediators? The concentration of estradiol detected in the hippocampus was about 0.6 nM (basal) and 1.3 nM after the NMDA stimulation, respectively. The local concentration of estradiol immediately after synthesis in the pyramidal neurons is likely to be approximately 10-fold higher than the bulk concentration of 1.3 nM, due to the relatively small volume of the P450-immunoreactive cells in the total hippocampus. These considerations suggest that the local concentration of estradiol could be as high as 1 to 10 nM.

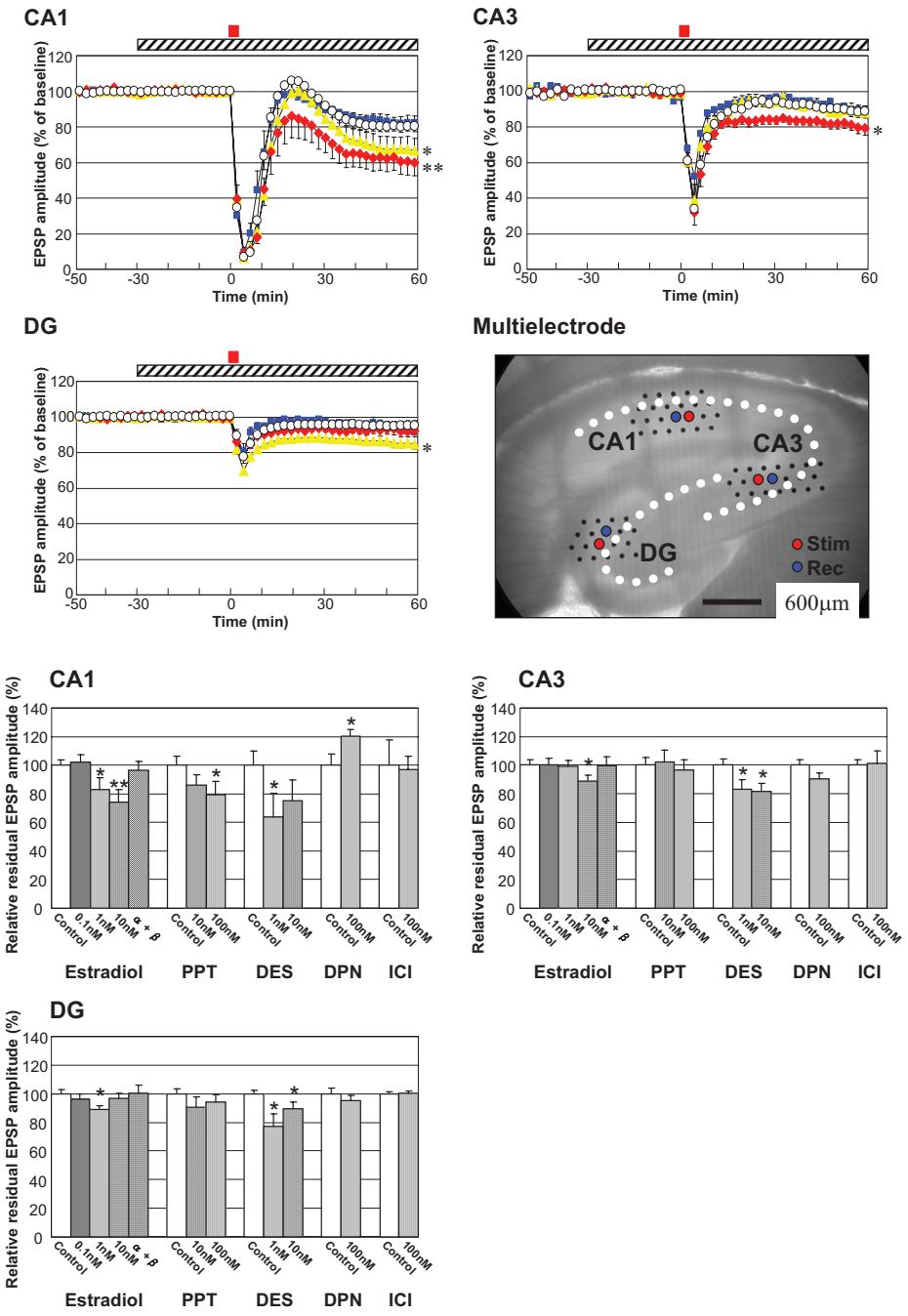

Fig. 2. Rapid modulation of long-term depression (LTD) by 17β-estradiol in the hippocampal acute slice. (*Upper CA1, CA3, DG*) Time dependence of maximal excitatory postsynaptic potential (EPSP) amplitude in the CA1 (*CA1*), CA3 (*CA3*), and dentate gyrus (*DG*). Estradiol concentration was 0 nM (open circle) and 0.1 nM (blue closed square), 1 nM (yellow closed triangle) and 10 nM (red closed diamond), respectively. (*Multielectrode*) Custom-made 64-multielectrode probe 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 minutes prior to *N*-methyl-D-aspartate (NMDA) stimulation, irrespective of the test condition. LTD was induced by 30 μM NMDA perfusion at time, t = 0 to 3 minutes (closed bar above the graph). Hatched bar above the graph indicates period of time during which estradiol was administered. (*Lower CA1, CA3, DG*) Comparison of modulation effect of LTD by 17β-estradiol and agonists in the CA1, CA3, and DG of hippocampal slices. Vertical axis is relative EPSP amplitude at t = 60 minutes, where EPSP amplitude at t = 60 minutes of the control slice without drug application is taken as 100%. From *left* to *right*, 17β-estradiol (Estradiol), propyl-pyrazole-trinyl-phenol (PPT), diethylstilbestrol (DES), diarylpropionitrile (DPN), and ICI 182, 780 at indicated concentrations. In Estradiol, α + β represents that 10 nM 17α-estradiol was perfused with 1 nM 17β-estradiol. Note that coperfusion of 1 μM ICI with 10 nM 17β-estradiol did not suppress the enhancing effect of LTD by estradiol. The significance of estradiol effect is confirmed at 60 min via statistical analysis, $^{*}P < .05$, $^{**}P < .01$.

These levels are sufficient to allow estradiol to act as local mediators that modulate synaptic transmission (Gu and Moss 1996; Foy and others 1999; Ito and others 1999; Bi and others 2000; Shibuya and others 2003; Mukai and others 2006). Functional differences between blood-derived estradiol (reproductive modulator) and brain-synthesized estradiol (neuronal modulator) may be due to the time dependence of their levels. The brain is filled with a low concentration of blood-derived estradiol, which has level changes that are dependent on the circadian rhythm, whereas the endogenous synthesis of estradiol is a transient event occurring mainly during synaptic transmission, which drives Ca^{2+} influx (Hojo and others 2004).

Rapid Modulation of Synaptic Plasticity by Estrogens

17β-Estradiol may rapidly modulate two different types of synaptic plasticity of neurons. One is synaptic transmission, such as LTP or long-term depression (LTD), and the other is spinogenesis. LTD and LTP probe the characteristics of preformed synapses, whereas spinogenesis analyzes not only spine synapses (spines forming synapses) but also free spines (spines without forming synapses). Estradiol-induced modulation of LTD or LTP occurs only in preexistent synapses because newly generated spines by estradiol treatments do not form new synapses within two hours, as judged from no increase in the baseline magnitude of the excitatory postsynaptic potential (EPSP) signal during two hours of estradiol perfusion.

Evidence is emerging that estradiol exerts a rapid influence (0.5–1 hour) on the synaptic transmission of adult rat hippocampal neurons, as demonstrated by electrophysiology (Gu and Moss 1996; Foy and others 1999; Ito and others 1999; Bi and others 2000; Shibuya and others 2003; Teyler and others 1980). In the case of the enhancement of LTP by 1 to 10 nM estradiol in CA1 pyramidal neurons, an immediate increase by approximately 20% has been observed upon the onset of estradiol perfusion in the initial slope of the EPSP, which has been attendant on a further approximate 130% increase on high–frequency tetanic stimulation of Schaffer collaterals (Bi and others 2000; Foy and others 1999; Kawato 2004; Mukai and others 2006).

However, without this 20% baseline increase in EPSP slope (before the tetanic stimulation), the enhancement of LTP by estradiol is not apparent, with regard to the pure titanic stimulation-induced LTP. In other words, the magnitude of pure tetanic stimulation-induced LTP is nearly the same between the presence and absence of estradiol.

When considering the role of estrogen in memory processing, not only LTP (memory-forming mechanism) but also LTD is essential. LTD is not simply a "forgetting" mechanism; it may be a positive mechanism used to "correct" wrong memories formed by initial LTP processes that store not only correct information but also wrong information.

We found that LTD was very sensitive to 17β-estradiol treatments. We demonstrated, for the first time, a significant rapid enhancement of LTD by 1 to 10 nM estradiol perfusion in the adult male rat hippocampal CA1, CA3, and dentate gyrus (DG) (Mukai and others 2007; Fig. 2). Recordings were performed using novel 64 planar multielectrodes (MED64, Panasonic, Japan), 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 was induced pharmacologically by the transient application (3 minutes) of NMDA. This LTD was induced by the activation of phosphatase due to a moderate Ca^{2+} influx through NMDA receptors. The plateau EPSP amplitude at 60 minutes after the NMDA application was 80.4% (CA1), 88.8% (CA3), and 95.1% (DG), respectively. A 30-minute preperfusion of 10 nM estradiol significantly enhanced LTD, resulting in the residual EPSP amplitude at 60 minutes of 59.7% (CA1), 79.1% (CA3), and 92.2% (DG) (Mukai and others 2007; Fig. 2). Investigations using specific estrogen agonists indicated that the contribution of ERα (but not ERβ) was essential to these estradiol effects. Propyl-pyrazole-trinyl-phenol (PPT, ERα agonist) (Harrington and others 2003) at 100 nM exhibited a significant LTD enhancement in CA1, whereas diarylpropionitrile (DPN, ERβ agonist) (Harrington and others 2003) did induce a suppression of LTD in CA1, implying that the contribution of ERβ was opposite to that of ERα in the estradiol effect on LTD.

Modulation of spinogenesis is another important role of estrogen in memory processes, involving production of new spines that create sites for new neuronal contacts. We demonstrated that dendritic spines were rapidly modulated by estradiol application, using single spine analysis of Lucifer Yellow–injected neurons in adult male hippocampal slices (Tsurugizawa and others 2005; Mukai and others 2007; Murakami and others 2006b). Following a two-hour treatment with estradiol in the stratum radiatum of the CA1 region, the treated dendrites have significantly more spines at 1 nM estradiol (1.31 spines/μm) than dendrites at 0 nM estradiol (0.85 spines/μm) (Mukai and others 2007; Murakami and others 2006b; Fig. 3*A*). PPT at 100 nM induced a significant enhancement of the spine density to 1.20 spines/μm. However, DPN at 100 nM increased the spine density only slightly (0.95 spines/μm). Blocking of ERα by ICI 182,780 and of NMDA receptors by MK-801 completely suppressed the enhancing effect of estradiol on the spine density. Blocking of phosphorylation of Erk mitogen activated protein (MAP) kinase by PD98059 completely prevented the estradiol-induced spinogenesis. The morphological changes in CA1 spines induced by two-hour estradiol treatments were also assessed. In control slices (0 nM estradiol), the relative population of spines was approximately 24% for the mushroom spine, 62% for the thin spine, 1% for filopodium, and 13% for the stubby spine. Upon 1 nM estradiol treatment, the density of the thin spine was selectively increased, from 0.57 spines/μm to 0.97 spines/μm, whereas the density of the mushroom and stubby spines was not significantly altered. (Fig. 3*A*). Filopodium was increased from almost null (0.01 spines/μm) to 0.11 spines/μm.

Interestingly, in CA3 pyramidal neurons, the total density of thorns or thorny excrescences (spine-like

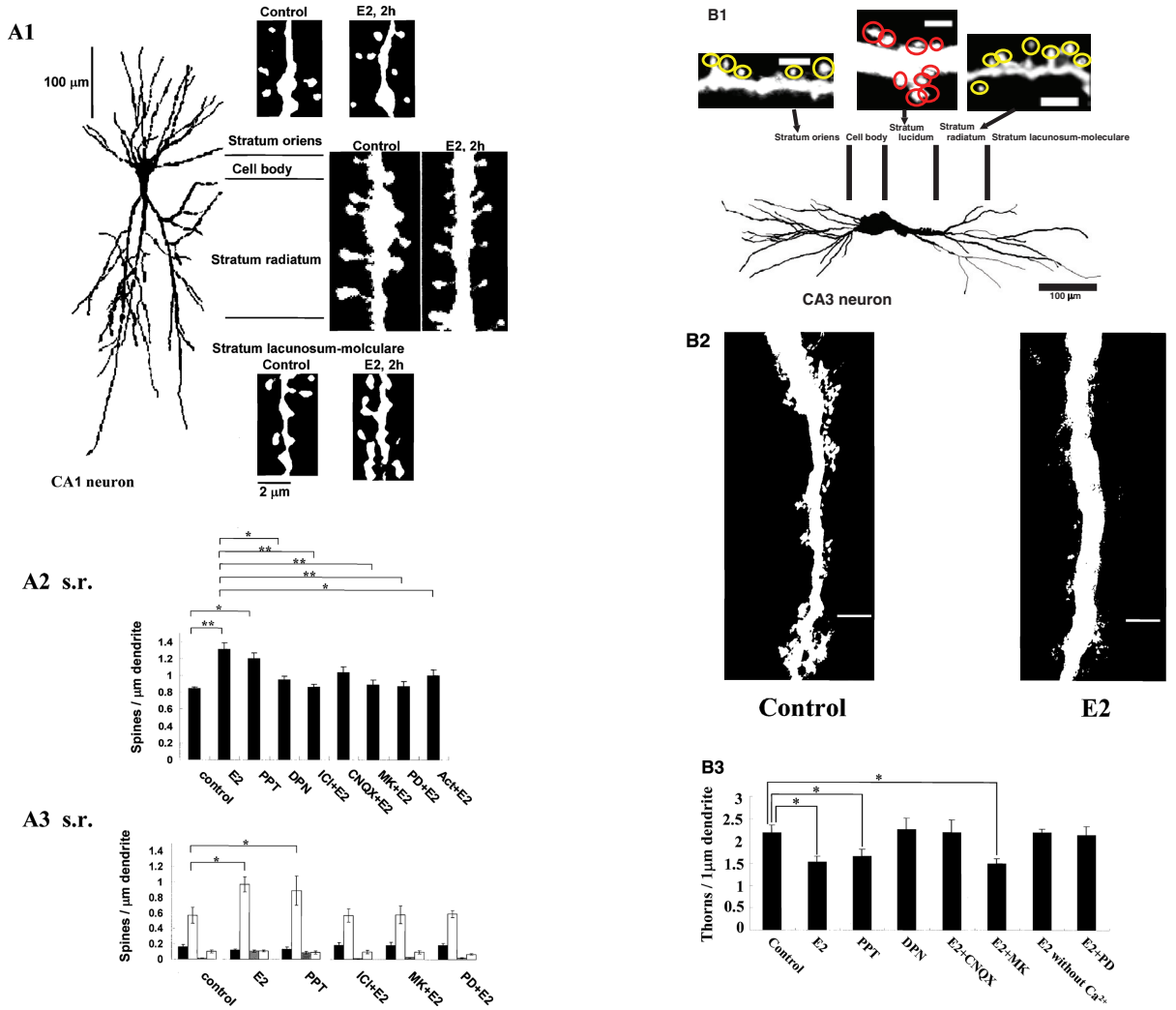

Fig. 3. Changes in the density and morphology of spines (CA1) or thorns (CA3) upon treatments of 17β-estradiol (E2) and drugs in hippocampal acute slices. Spines/thorns were analyzed along the dendrites of pyramidal neurons. (*A1*) Confocal micrographs showing spines along the secondary dendrites of hippocampal CA1 pyramidal neurons. (*Left*) A whole image of Lucifer Yellow–injected CA1 neuron. Vertical bar 100 μm. (*Right*) Maximal intensity projections onto the *XY* plane from z-series confocal micrographs, showing spines along the dendrites. From top to bottom, the distribution of spines along the basal dendrite in the stratum oriens (control and E2), distribution of spines along the apical dendrite in the stratum radiatum (control and E2), and the distribution of spines along the apical dendrite in the stratum lacunosum-moleculare (control and E2); horizontal bar 2 μm. Slices were treated in artificial cerebrospinal fluid (ACSF) for two hours without drugs (control) or with 1 nM E2 (E2). (*A2*) Effect of drug treatments on the total spine density of CA1 neurons in the stratum radiatum (s.r.). Vertical axis is the average number of spines per 1 μm of dendrite. A two-hour treatment in ACSF without drugs (control), with 1 nM E2 (E2), with 100 nM propyl-pyrazole-trinyl-phenol (PPT), with 100 nM diarylpropionitrile (DPN), with 1 nM E2 and 1 μM ICI 182,780 (ICI + E2), with 1 nM E2 and 50 μM MK-801 (MK + E2), with 1 nM E2 and 50 μM PD98059 (PD + E2), and with 1 nM E2 and 4 μM actinomycin D (Act + E2). (*A3*) Density of four subtypes of spines in the stratum radiatum. A two-hour treatment in the ACSF without drugs (control group), with 1 nM E2 (E2 group), with 1 nM E2 and 1 μM ICI (ICI + E2 group), with 1 nM E2 and 50 μM MK-801 (MK + E2 group), and with 1 nM E2 and 50 μM PD98059 (PD + E2 group). In each group, from left to right, (a) mushroom (black column), (b) thin (dotted column), (c) filopodium (hatched column), and (d) stubby (open column). (*B1*) Maximal intensity projections onto the *XY* plane from z-series confocal micrographs, showing thorns and spines along the primary and secondary dendrites of hippocampal CA3 pyramidal neurons. (*Upper left*) Spines along the basal dendrite in stratum oriens; bar 2 μm. (*Upper middle*) Thorny excrescences along the apical dendrite in the stratum lucidum; bar 2 μm. (*Upper right*) Spines along the apical dendrite in the stratum radiatum; bar 2 μm. Thorny excrescences have bulbous-shaped huge heads named "thorns" (red circles), which are different from spines with separated distribution (yellow circles). (*Lower image*) A whole image of Lucifer Yellow–injected CA3 neuron. Horizontal bar 100 μm. (*B2*) Maximal intensity projections onto the *XY* plane from z-series confocal micrographs, showing thorns in the stratum lucidum without drug treatments (control) and thorns after estradiol treatments (E2). Bar 5 μm. (*B3*) Effect of drug treatments on the average number of thorns per 1-μm dendritic segment. A two-hour treatment in the ACSF (containing Ca^{2+}) without estradiol (control), with 1 nM estradiol (E2), with 100 nM PPT (PPT), with 100 nM DPN (DPN), with 1 nM estradiol and 20 μM cyano-nitroquinoxaline-dione (E2 + CNQX), with 1 nM estradiol and 50 μM MK-801 (E2 + MK), with 1 nM estradiol in the ACSF not containing Ca^{2+} (E2 without Ca^{2+}), and with 1 nM estradiol and 20 μM PD98059 in ACSF containing Ca^{2+} (E2 + PD). Statistical significance, $^{*}P < .05$, $^{**}P < .01$.

postsynaptic structures in CA3), having contact with mossy fiber terminals that originated from granule cells, decreased dramatically to approximately 70% upon two-hour treatments of 1 nM estradiol (Tsurugizawa and others 2005; Fig. 3*B*). These results imply that the spine density is not always increased by the estradiol treatments and that the estradiol-induced spinogenesis is highly region specific and heterogeneous.

The rapid effect of estrogens has also been observed in vivo. Leranth, MacLusky, and coworkers have demonstrated that estradiol (60 μg/kg) increases the spine-synapse density due to synaptic rearrangements in ovariectomized rats after 30 minutes using electronmicrographic analysis (MacLusky and others 2005a).

Over decades, the slow genomic effects (one to four days) of estradiol on spine plasticity have been extensively investigated in vivo. For example, supplement of estrogens in ovariectomized female rats (Gould and others 1990; Woolley and McEwen 1992; Leranth and others 2000; Leranth and others 2002) increases the density of spines in the stratum radiatum of CA1 pyramidal neurons, resulting in recovery of spines to the level of wild rat. These effects of enhancement in spinogenesis have also been observed as rapid as 4.5 hours after the estrogen injection (MacLusky and others 2005b). In vitro investigations have also shown that spine density in CA1 increases following several days' treatment of cultured hippocampal slices with exogenous estradiol (Pozzo-Miller and others 1999). The contribution of endogenous estradiol has been reported by Rune and coworkers, who demonstrated that the suppression of endogenous estradiol synthesis by letrozole treatments for four days significantly decreased the spine density in the stratum radiatum of the CA1 region in cultured slices (Kretz and others 2004).

What is a receptor of 17β-estradiol in terms of its rapid action (0.5–2 hours) on synaptic plasticity in the hippocampus? Putative synaptic membrane estrogen receptors for rapid estradiol action have been poorly understood. There have been many attempts to identify membrane estrogen receptors. At the present stage, the most probable candidates for membrane estrogen receptors may be ERα, ERβ, and GPR30. GPR30 is a transmembrane G-protein-coupled protein and therefore a candidate for membrane estrogen receptors (Thomas and others 2005; Revankar and others 2005).

Why are classic nuclear-type receptors ERα and ERβ candidates? Because the ICI do not suppress estradiol–induced rapid modulation of electrophysiological properties such as LTD, LTP, and kinate-induced currents, classic estrogen receptors are suggested to be not involved in these modulations (Gu and Moss 1996). However, these results do not indicate that estrogen receptors driving these synaptic transmissions must be the non-classic type, different from ERα and ERβ. ICI has been indicated to display its effect by inhibiting dimerization of ERα and ERβ; therefore, if dimerization processes are not necessary for ERα and ERβ in the rapid modulation of electrophysiological phenomena, then ICI cannot block these phenomena. Rapid enhancement of spinogenesis via ERα, on the other hand, was significantly blocked by ICI (Mukai and others 2007; Murakami and others 2006b); therefore, dimerization processes should occur for synaptic ERα in spinogegesis.

After several years of careful investigations, we successfully identified 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 novel purified anti-ERα antibody RC-19 (Mukai and others 2007). A postembedding immunogold electron microscopic analysis demonstrated the synaptic localization of ERα in the glutamatergic neurons in CA1, CA3, and DG. ERα was localized not only in the nuclei but also within both the dendritic spines and axon terminals of principal neurons. Western blot analysis demonstrated that ERα (67 kDa) and MAP kinase were tightly associated with postsynaptic density fractions (PSD). Because the estradiol-induced modulation of LTD and spine morphology appeared so rapidly in the time range of one to two hours, the synaptic ERα observed at PSD and the postsynaptic compartment probably plays an essential role in driving rapid processes. It should be noted that specific binding of purified RC-19 antibody to real ERα (67 kDa) in the hippocampus was qualified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass-spectrometric analysis of RC-19 reacted proteins as well as the absence of reactivity of RC-19 with ERα knockout (KO) mice hippocampus (Mukai and others 2007). These analyses are essential in the hippocampus, because we found that non-purified MC-20 antisera, frequently used in previous investigations, often reacted with 62-kDa unknown proteins in the brain and did not significantly react with real ERα (67 kDa) (Mukai and others 2007). AS409, another frequently used antiserum, mainly reacted with unknown proteins different from real ERα (Mukai and others 2007). Nonpurified antisera may largely react with proteins having amino acid sequences similar to the real antigen in the hippocampus, in which an extremely low level of ERα is expressed as compared with that in the ovary. ERα antisera are normally examined for their reactivity only in endocrine organs such as the ovary, in which ERα is highly expressed. Therefore, staining patterns with nonpurified antisera probably do not show real ERα distribution in the hippocampus.

So far, ERβ has not been identified as a synaptic membrane receptor. ERβ has been reported to associate with membranes in genetically expressed Chinese hamster ovary (CHO) cells and MCF-7 cells (Razandi and others 1999; Pedram and others 2006). Association is demonstrated by binding of [^{3}H]-17β-estradiol to purified plasma membranes in combination with treating by antisera against ERβ. Several investigations of immunostaining of ERβ have suggested extranuclear expression of ERβ, including dendritic appearance in the hippocampal principal neurons. However, subcellular immunostaining patterns of these reports may reflect relatively minor expression of ERβ and major expression of unknown proteins, due to multiple reactivity of

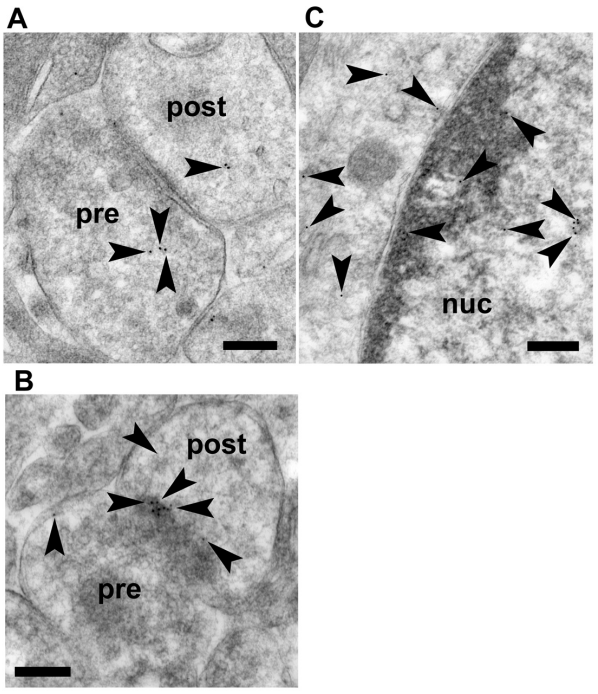

Fig. 4. Immunoelectron microscopic analysis of the distribution of ERα within axospinous synapses, in the stratum radiatum of the hippocampus. (*A*) Gold particles (arrowheads) were lo1calized in the pre- and postsynaptic regions. (*B*) In dendritic spines, gold particles were found within the spine head and, in some cases, were associated with postsynaptic density (PSD) regions. (*C*) Gold particles were also localized in the nuclei. Pre, presynaptic region; Post, postsynaptic region; Scale bar: 200 nm.

nonpurified ERβ antisera to several unknown proteins in the Western blot analysis of hippocampal tissues (Kawato and others, unpublished results). The purity of commercially available ERβ antisera was much worse than that of ERα antisera, as judged from the Western blot analysis (Kawato and others, unpublished results).

Recently, transmembrane G-protein-coupled estrogen receptor GPR30 has been identified in the plasma membrane of SKBR3 breast cancer cells that lack ERα and ERβ (Thomas and others 2005), as well as in endoplasmic reticulum membranes of COS7 after genetic expression of GPR30 fused with green fluorescent protein (Revankar and others 2005). Because expression of GPR30 has been suggested in the hippocampal neurons (O'Dowd and others 1998), particularly at synapses (Fujiwara and Kawato, unpublished results), intensive investigations should be performed to reveal its contribution to rapid estradiol modulation of synaptic plasticity.

Modulation of Synaptic Plasticity by Androgens

The hippocampus is a putative site of action for androgen's antianxiety effect. Supplement of testosterone or dihydrotestosterone has been shown to have antianxiety effect on behavior when androgen has been injected subcutaneously (s.c.) to castrated male rats in vivo (Edinger and Frye 2005; Fernandez-Guasti and Martinez-Mota 2005). The supplement of testosterone or dihydrotestosterone in castrated male rats increases the density of CA1 spines 48 hours after the application, resulting in recovery of spines to the level of the wild rat (Leranth and others 2003). Interestingly, a low dose of estradiol application (10 μg/animal, single s.c. injection) to castrated rats is less potent than a high dosage of testosterone (500 μg/animal) or dihydrotestosterone (500 μg/animal). Androgen receptor-immunoreactivity has been reported to be localized significantly in CA1 pyramidal neurons and weakly in CA3 and DG neurons. Overall, much less is known about androgens than estrogens regarding their effect on synaptic plasticity; therefore, further extensive investigations should be performed.

Modulation of Synaptic Plasticity by Activin

Endogenous synthesis and rapid action of activin in the hippocampus is an interesting target, but little is known about this mechanism at this time. The significant role of activin in synaptic plasticity has been suggested by the increase of inhibin β_A mRNA within three hours after the LTP induction of the dentate gyrus upon stimulation of the perforant pathway in the adult rat hippocampus in vivo (Inokuchi and others 1996). We recently investigated in vitro the modulation by activin A of the spine density and morphology in the stratum radiatum of the CA1 region of hippocampal slices from 12-week-old male rats (Mitsuhashi, Turugizawa, Mukai, and Kawato, unpublished results). The same single spine imaging method was employed as that used for the estradiol effect (Tsurugizawa and others 2005; Mukai and others 2006). Spines along the apical dendrites of Lucifer Yellow–injected neurons were analyzed. A two-hour treatment of slices with activin A increased the total spine density to 1.20 spines/μm (10 ng/mL activin) from 0.99 spines/μm (control, no activin). Blocking of 10 ng/mL activin A by 100 ng/mL follistatin, a specific activin inhibitor, completely suppressed the enhancing effect of activin A on the spine density (0.90 spines/μm). PD98059, an Erk MAP kinase inhibitor, abolished the activin effect (1.05 spines/μm). Therefore, the activin A–induced modulation of spinogenesis drives Erk MAP kinase-dependent processes, which are probably smad-independent processes (Derynck and Zhang 2003). The morphological changes in spines induced by a two-hour treatment were also assessed. Upon activin A treatments, the density of the thin spine increased significantly, from 0.84 spines/μm to 0.98 spines/μm, whereas the density of other types of spines (filopodium, mushroom spine, and stubby spine) was not significantly altered. As a functional site of activin A, an expression of type IB activin receptors at synapses was demonstrated by a postembedding immunogold electron micrograph using antibody XALK4 (Fukui and others 2003). The presence of the type II activin receptor, which forms an active receptor complex with type I receptor upon activin A

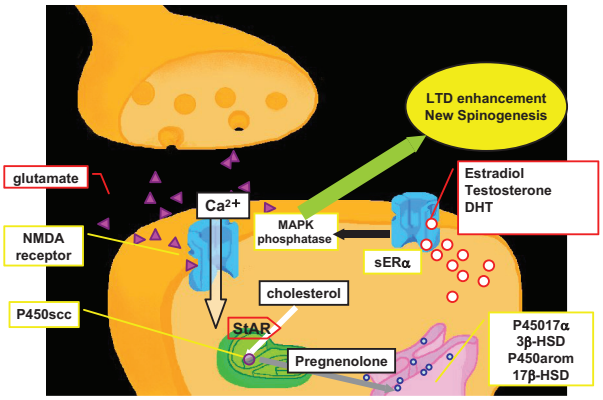

Fig. 5. Schematic illustration for the synaptic synthesis of sex hormones and the modulation of the synaptic plasticity of neurons by estradiol. The AMPA type of glutamate receptors is omitted for clarity. Steroidogenic acute regulatory protein (StAR), and P450scc are present in the mitochondria. P450(17α), 3β hydroxysteroid dehydrogenases (HSD), 17β-HSD, and P450arom are localized in the membranes in the synaptic compartment. The site of rapid action for estradiol is synaptic ERα. Synaptic ERβ might also function. The site of delayed action for estradiol is ERα present in the cytoplasm and nuclei. Synthesis and action of activin A probably also occur at synapses. NMDA, *N*-methyl-D-aspartate; MAPK, mitogen activated protein kinase; LTD, long-term depression.

binding, has been demonstrated with immunohistochemical staining in hippocampal neurons of the rat (Funaba and others 1997).

Hypothetical Model of Synaptocrinology

Based on experimental observations, we illustrate in Figure 5 a hypothetical model for the synaptic synthesis of brain steroid and the modulation of the synaptic plasticity of neurons by brain steroid. According to this model, 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 or peripheral benzodiazepine receptor (Papadopoulos 1993) to transport cholesterol into the mitochondria, where P450scc converts cholesterol to pregnenolone. After reaching the endoplasmic reticulum, the conversion of pregnenolone → DHEA → androstenediol → testosterone → estradiol or testosterone → dihydrotestosterone → androstandiol is performed by P450(17α), 3β-HSD, 17β-HSD, P450arom, and 5α-reductase. Produced estradiol binds to synaptic ERα and drives the signaling pathway, including phosphatase, MAP kinase and so on, finally resulting in the modulation of AMPA receptors or NMDA receptors. Consideration of these mechanisms might contribute to a better understanding and a possible improvement of the effect of estrogen replacement therapy for patients with Alzheimer's disease.

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