Estrogen synthesis in the brain—Role in synaptic plasticity and memory

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Abstract

Estrogen and androgen are synthesized from cholesterol locally in hippocampal neurons of adult animals. These neurosteroids are synthesized by cytochrome P450s and hydroxysteroid dehydrogenases (HSDs) and Salpha-reductase. The expression levels of enzymes are as low as 1/200–1/50,000 of those in endocrine organs, however these numbers are high enough for local synthesis. Localization of P450(17alpha), P450arom, 17beta-HSD and Salpha-reductase is observed in principal glutamatergic neurons in CA1, CA3 and the dentate gyrus. Several nanomolar levels of estrogen and androgen are observed in the hippocampus.

Estrogen modulates memory-related synaptic plasticity not only slowly but also rapidly in the hippocampus. Rapid action of 17beta-estradiol via membrane receptors is demonstrated for spinogenesis and long-term depression (LTD). The enhancement of LTD by 1–10 nM estradiol occurs within 1 h. The density of spine is increased in CA1 pyramidal neurons within 2 h after application of estradiol. The density of spine-like structure is, however, decreased by estradiol in CA3 pyramidal neurons. ERalpha, but not ERbeta, induces the same enhancement/suppression effects on both spinogenesis and LTD.

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1. Introduction

Sex hormones are synthesized in the gonads, and reach the brain via the blood circulation. In addition, the local endogenous synthesis of estrogens and androgens occurs in the mammalian brain, in areas such as the hippocampus (Kimoto et al., 2001; Kawato et al., 2002, 2003; Hojo et al., 2004; Kretz et al., 2004). A neurosteroid hypothesis was proposed by Baulieu’s group in the 1980s, suggesting that pregnenolone (PREG), progesterone, and dehydroepiandrosterone (DHEA) may be endogenously synthesized in the brain due to the finding that PREG and DHEA has been found in the mammalian brain at concentrations greater than that in plasma (Corpechot et al., 1981; Baulieu, 1997). 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 et al., 1981; Robel et al., 1987).

Direct demonstration of steroidogenesis in the mammalian brain had, however, long been not successful over decades, due to the extremely low levels of steroidalogenic 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(17alpha(DHEA synthase)) in adult mammalian brain (Le Gascoigne et al., 1991; Mellon and Deschepper, 1993) and also by the observation of the complete disappearance of testosterone in the brain within 1 day after castration (Baulieu and Robel, 1998).

Neuromodulatory actions of gonadal sex hormones have been investigated in the hippocampus, because the hippocampus is attractive as a center of learning and memory (Woolley and
McEwen, 1994; Woolley, 1998; Foy et al., 1999; Pozzo-Miller et al., 1999; Bi et al., 2000; Shibuya et al., 2003). Many scientists had, however, not seriously considered that memory formation process might favor hippocampus-derived steroids rather than circulating gonadal steroids. Therefore, many investigations have been focused on the role of slow modulation by sex steroids on spinogenesis and electrophysiological properties, for example, upon s.c. injection of estradiol (in a time scale of days) (Woolley and McEwen, 1994; Woolley, 1998; Foy et al., 1999; Pozzo-Miller et al., 1999; Bi et al., 2000; Shibuya et al., 2003). The rapid effect of estrogen (within 1–2 h) is also observed on modulation of electrophysiological properties of the hippocampal slices (Teyler et al., 1980; Foy et al., 1999; Bi et al., 2000; Mukai et al., 2006a). These rapid modulations favor the hippocampus-derived steroids rather than circulating gonadal hormones which travel over the long distance until they reach the brain. A weak activity of sex steroid production in the hippocampus is sufficient for the local usage within small neurons (i.e., intracrine system). This intracrine system contrasts with the endocrine organs in which high expression levels of steroidogenic enzymes are necessary in order to supply steroids to many other organs via the blood circulation. For brain-derived sex hormones, the rapid modulation of synaptic plasticity and cognitive functions may be their essential functions.

2. Synthesis of estrogen in the hippocampus

In assay of brain steroidogenesis, it is essential to improve the sensitivity of measurements (e.g., immunostaining, Western blot as well as steroid metabolism assay) by 100–1000-fold of those in endocrine organs. Even RT-PCR method is necessary to be improved by specific primer pair design.

2.1. Neuronal localization of steroidogenic proteins

Which cells are steroidogenic in the hippocampus, neurons or glial cells? In earlier studies, glial cells were thought to be a major place for steroidogenesis, because the white matter including glial cells had been stained with anti-P450scC antisera, throughout the adult rat brain (Le Goascogne et al., 1987). However, this white matter staining of P450scC antisera is likely to be an artifact which is due to the nonspecific adsorption of the non-purified bovine antisera in rat hippocampus.

The role of neurons in steroid synthesis in mammalian brains had long been difficult to determine. The absence of P450(17α) in both neurons and glial cells had been believed due to the fact that many attempts to demonstrate the immunohistochemical reactivity in the rat brain had been unsuccessful for almost two decades.

![Fig. 1. Immunohistochemical staining of P450(17α) (A) and P450arom (B) in the coronal section of the adult male rat hippocampus. (C) Fluorescence dual staining of P450(17α) (green) and neuronal nuclear antigen, a marker for neurons (red). (D) Fluorescence dual staining of P450(17α) (green) and glial fibrillary acidic protein, a marker for astroglial cells (red). (E) Fluorescence dual staining of P450(17α) (green) and myelin basic protein, a marker for oligodendroglial cells (red). In (C)–(E) (CA1 region), superimposed regions of green and red fluorescence are represented by yellow. P450(17α) and P450arom are primarily expressed in neurons, although a weak expression of P450(17α) is associated with astroglial cells. pcl, pyramidal cell layer; so, stratum oriens; sr, stratum radiatum. Scale bar, 800 μm for A and B, and 120 μm for (C)–(E) (modified from Hojo et al., 2004).]
Fig. 2. Immunoelectron microscopic analysis of the distribution of P450(17α) (A1 and A2) and P450arom (B1 and B2) within axospinous synapses, in the stratum radiatum of the hippocampal CA1 region. Gold particles (indicated with arrows) were observed in the presynaptic region (A1 and B1), and the postsynaptic region (A2 and B2) of pyramidal neurons. Scale bar, 200 nm (modified from Hojo et al., 2004).

We overcame difficulties of nonspecific immunostaining by using affinity column-purified antibodies (Shinzawa et al., 1988; Jakab et al., 1993) instead of using non-purified antisera) in order to avoid cross-reaction with IgG with unknown proteins having similar antigen sequences, as well as using fresh frozen slices of hippocampus (instead of using paraffin sections). A significant localization of cytochromes P450scc, P450(17α) and P450arom was observed in pyramidal neurons in CA1–CA3, as well as in granule cells in the dentate gyrus (DG), by means of the immunohistochemical staining of hippocampal slices from adult (12 weeks) and developmental rats (Fig. 1) (Kimoto et al., 2001; Kawato et al., 2002; Shibuya et al., 2003; Hojo et al., 2004). The co-localization of immunoreactivity against P450s and NeuN (marker of neuron) confirmed the presence of P450s in these neurons (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). Astroglial cells were weakly stained with P450(17α) or P450scc antibodies, however, oligodendroglial cells were not stained significantly by these P450 antibodies (Fig. 1) (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). StAR was co-localized with P450s (Zwain and Yen, 1999a; Kimoto et al., 2001). These results imply that pyramidal neurons and granule neurons are equipped with complete steroidogenic systems which catalyze the conversion of cholesterol to PREG, DHEA, testosterone and estradiol. From a weak immunostaining of P450s in glial cells, the activity of neurosteroidogenesis in glial cells is probably much lower than that of neurons.

An immunoelectron microscopic analysis using postembedding immunogold method is 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 and CA3 regions and of granule neurons in DG (Fig. 2). These results suggest ‘synaptic’ synthesis of estrogens and androgens, in addition to classical microsomal synthesis of sex steroids.

The existence of these steroidogenic proteins was confirmed by Western immunoblot analyses (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). The molecular weights obtained for P450scc, P450(17α) and P450arom were identical to those obtained from peripheral steroidogenic organs. The relative levels of these P450s in the hippocampus were approximately 1/1000 (P450scc) and 1/300 (P450(17α) and P450arom) of that in the testis (P450scc and P450(17α)) and the ovary (P450arom), respectively.

2.2. mRNA expression of steroidogenic enzymes

From many molecular biological investigations (Warner and Gustafsson, 1995), the relative level of mRNA expressed in the hippocampus has been suggested to be lowest for cytochrome P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD), and highest for steroidogenic acute regulatory protein (StAR) and 5α-reductase, with those of P450(17α) and P450arom expressed at an intermediate level (Table 1). The expression level of cytochrome P450scc (CYP11A1) mRNA is extremely low, preventing many scientists to believe the physiological significance of neurosteroid synthesis. P450scc is 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 (Table 1) (Murakami et al., 2006b). 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 32P-labelled rat P450scc antisense riboprobe, however, yielded no detectable specific hybridization signals (Furukawa et al., 1998). On the other hand, STAR is most abundant, therefore, not only the PCR-amplification but also the RNase protection assay demonstrated the presence of STAR transcripts with an expression level of approximately 1/300 of that in neonatal rat ventral prostate tissues (Ivanova and Beyer, 2000). We observed the P450arom mRNA in cultures of mixed glial cells, or astrocytes and neurons from rat hippocampal slices by means of careful HPLC analysis (Kawato et al., 2002; Hojo et al., 2004). The expression of the mRNA for P450(17α) (astrocytes and neurons in primary cell cultures from the brain of neonatal rats, a much lower metabolic activity is observed in neurons than astrocytes for the conversion of PREG to DHEA (Zwain and Yen, 1999a.b). The very low metabolic activity of neurons might be caused by cytosine arabinoside applied to cultures in order to suppress the proliferation of glial cells. 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 ‘adult’ rat brain is not directly available from these cell culture studies, because we cannot culture adult neurons.

### Table 1
Comparison of relative mRNA expression level for steroidogenic enzymes in the adult rat (12 weeks)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Hippo</th>
<th>Hypo</th>
<th>Adrenal/Testis/Ovary/Liver/Prostate</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450scc</td>
<td>1b</td>
<td>3</td>
<td>50,000 (Ad) 1000 (Te)</td>
</tr>
<tr>
<td>P450(17α)</td>
<td>1</td>
<td>3</td>
<td>300 (Te)</td>
</tr>
<tr>
<td>P450arom</td>
<td>1</td>
<td>3</td>
<td>300 (Ov)</td>
</tr>
<tr>
<td>17β-HSD (type 1)</td>
<td>1</td>
<td>3</td>
<td>200 (Ov)</td>
</tr>
<tr>
<td>17β-HSD (type 3)</td>
<td>1</td>
<td>3</td>
<td>300 (Te)</td>
</tr>
<tr>
<td>3β-HSD (type 1)</td>
<td>3</td>
<td>5</td>
<td>5,000 (Ov)</td>
</tr>
<tr>
<td>3β-HSD (types 2–4)</td>
<td>N.D.</td>
<td>D4</td>
<td>D4 (Ov)</td>
</tr>
<tr>
<td>5α-Reductase (type 1)</td>
<td>1</td>
<td>2</td>
<td>5 (Li)</td>
</tr>
<tr>
<td>5α-Reductase (type 2)</td>
<td>1</td>
<td>1/3</td>
<td>200 (Pro)</td>
</tr>
<tr>
<td>ERα</td>
<td>1</td>
<td>5</td>
<td>35 (Ov)</td>
</tr>
<tr>
<td>ERβ</td>
<td>1</td>
<td>4</td>
<td>80 (Ov)</td>
</tr>
</tbody>
</table>

a Hippocampus (Hippo), hypothalamus (Hypo), adrenal gland (Ad), testis (Te), ovary (Ov), liver (Li), and prostate (Pro) are compared.

b The level in the hippocampus is normalized to be 1. The level of mRNA expression is approximate value obtained from semiquantitative RT-PCR analyses.

c 3β-HSD (types 2–4) were not detectable, even after 50 cycles of PCR amplification. For 3β-HSD (type 1), 40 cycles were used for PCR. For Reverse-Transcription, 200 ng of total RNAs were used for 3β-HSD (types 1–4), though 50 ng of total RNAs were always used for other steroidogenic enzymes examined.

d 3β-HSD (types 2–4) were expressed at roughly the same level as 3β-HSD (type 1).

### 2.3. Synthesis of 17β-estradiol

A direct demonstration of the neuronal synthesis of PREG, DHEA, testosterone and 17β-estradiol in adult mammals is for the first time reported by our group (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). It had been assumed that testosterone is supplied to the male brain such as hypothalamus, via the blood circulation, where testosterone is 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 et al., 1991; Baulieu and Robel, 1998; Mensah-Nyagan et al., 1999; Kibaly et al., 2005). Incubations of 3H-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 succeeded in demonstration of the synthesis of DHEA, testosterone and estradiol in the adult (12 weeks) hippocampal slices by means of careful HPLC analysis (Kawato et al., 2002; Hojo et al., 2004). The purification of neurosteroids from very fatty brain tissues requires the combination of several sophisticated methods, which included purification with organic solvent, solid column chromatography, and normal phase HPLC (Wang et al., 1997; Kimoto et al., 2001; Hojo et al., 2004). The significant conversion from 3H-PREG to 3H-DHEA, from 3H-DHEA to 3H-androstenediol, 3H-testosterone and 3H-estradiol was observed after incubation with the slices for 5 h (Fig. 3) (Hojo et al., 2004). The rate of production for 3H-estradiol from 3H-testosterone was very slow, and the production rate of 3H-dihydrotestosterone from 3H-testosterone was much more rapid than that of estradiol. These activities were abolished by the application of specific inhibitors of cytochrome P450s. Surprisingly, 3H-estradiol was extremely stable and not significantly converted to other steroid metabolites.
such as estrone. On the other hand, dihydrotestosterone was rapidly converted to 3α,5α-androstanediol.

We determined the concentration of DHEA and 17β-estradiol as well as PREG in hippocampal slices from adult male rats by means of RIA, after careful purification of steroids with normal phase 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 6–10 times greater than those typical of plasma (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). Recently we very much improved the determination of steroids by using liquid chromatography–tandem-mass spectrometry (LC–MS/MS) which has a high specificity and accuracy. A much higher value of 8 nM was obtained for estradiol (Kawato et al., 2007). The concentration of testosterone in the hippocampus (17 nM) was only slightly higher than that of circulating testosterone (15 nM) in male rats. To demonstrate the rapid net production of neurosteroids upon synaptic stimulation, the NMDA-induced production of PREG and estradiol was investigated in hippocampal slices (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). Upon stimulation with NMDA for 30 min, 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 is also demonstrated in cultured hippocampal slices from neonatal rats in the absence and presence of letrozole, an inhibitor of P450arom. After 4 days treatment with letrozole, the amount of estradiol released into the medium was significantly decreased (Kretz et al., 2004).

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

Is the local concentration of brain neurosteroids sufficiently high to allow action as local mediators? Based on RIA determination, the concentration of estradiol detected in the hippocampus was only about 0.6 nM (basal) and 1.3 nM after the NMDA-stimulation, respectively (Hojo et al., 2004). However, from accurate LC–MS/MS analysis, basal level of estradiol was determined to be roughly 8 nM (Hojo et al., 2006). This level is sufficient to allow estradiol to act as local mediators that modulate synaptic plasticity (Gu and Moss, 1996; Foy et al., 1999; Ito et al., 1999; Bi et al., 2000; Shibuya et al., 2003).

Functional differences for estradiol produced from circulating testosterone and estradiol produced from hippocampus-derived testosterone may be differences in the time-dependence of their levels. Brain is filled with circulating testosterone (for male), or estradiol (for female) whose level slowly changes depending on the circadian rhythm, while the endogenous synthesis of estradiol (for both male and female) is a transient event depending on neural activity (Hojo et al., 2004).

3. Modulation of synaptic plasticity by estrogen

3.1. Spinogenesis

Brain-derived estradiol may rapidly modulate several different types of synaptic plasticity of neurons. One is spinogenesis, and another one is synaptic transmission such as LTD or LTP. Spinogenesis includes not only spine-synapses (spines forming synapses) but also free spines (spines without forming synapses), whereas LTD and LTP probe the characteristics of preformed synapses. Modulation of spinogenesis is essential action of estrogen in memory processes, involving production of new spines that creates sites for new neuronal contacts. We demonstrated that dendritic spines were rapidly modulated upon estradiol application, using single spine analysis of Lucifer Yellow-injected neurons in hippocampal slices from adult male rats (3 months) (Komatsuzaki et al., 2005; Tsurugizawa et al., 2005; Mukai et al., 2006b; Murakami et al., 2006a). Following a 2-h treatment with estradiol in the stratum radiatum of 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) (Fig. 4) (Mukai et al., 2007). Propyl-pyrazole-tris-phenol (PPT, ER \(\alpha\) agonist) (Harrington et al., 2003) induced a significant enhancement of the spine density to 1.20 spines/μm. However, diarylpropionitrile (DPN, ER \(\beta\) agonist) (Harrington et al., 2003) increased the spine density only slightly (0.95 spines/μm). Blocking of ER\(\alpha\) by ICI 182,780 completely suppressed the enhancing effect of estradiol on the spine density. Blocking of phosphorylation of Erk MAP kinase by PD98059 or U0126 completely prevented the estradiol-induced spinogenesis
Fig. 4. Changes in the density and morphology of spines in CA1 or thorns in CA3 pyramidal neurons upon treatments of 17β-estradiol (E2) and drugs in hippocampal slices from adult male rats. Spines/thorns were analyzed along the dendrites of pyramidal neurons. Upper left panel (CA1 neuron), a whole image of Lucifer Yellow-injected pyramidal neuron, vertical bar 100 μm. (Control and E2) Maximal intensity projections onto XY plane from z-series confocal micrographs, showing spines along the dendrites in stratum radiatum without drugs (Control) or with 1 nM E2 (E2). Horizontal bar 2 μm. Upper right panel (CA3 neuron), a whole image of Lucifer Yellow-injected pyramidal neuron, vertical bar 100 μm. (Control and E2) Maximal intensity projections onto XY plane from z-series confocal micrographs, showing thorns in the stratum lucidum without drugs (Control) or with 1 nM E2 (E2). Horizontal bar 5 μm. Lower panel (CA1 stratum radiatum), effect of drug treatments on the total spine density of CA1 neurons.
Together, the enhancement of the spine density is probably induced by activation of Erk MAP kinase via estradiol and ERα at the basal low Ca²⁺ concentration of around 0.1–0.2 μM in resting neuronal synapses (Ishii et al., 2007). When the Ca²⁺ concentration in spines was further decreased by blocking NMDA receptors with MK-801, the enhancing effect by estradiol was completely suppressed. Function of estradiol-bound ERα therefore needs the basal level of Ca²⁺ concentration of around 0.1–0.2 μM. The morphological changes in CA1 spines occurred by 2-h estradiol treatments. In control slices (0 nM estradiol), the relative population of spines was approximately 24% for mushroom spine, 62% for thin spine, 1% for filopodium, and 13% for stubby spine. Upon 1 nM estradiol treatment, the density of thin spine was selectively increased, from 0.57 spines/μm to 0.97 spines/μm, while the density of mushroom and stubby was not significantly altered.

The spine density is not always increased but in some cases decreased by the estradiol treatment. The estradiol-induced spine density 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 originated from granule cells) decreased dramatically to approximately 70% upon a 2-h treatment of 1 nM estradiol (Fig. 4) (Tsurugizawa et al., 2005). PPT significantly decreased the density of thorns from 2.19 to 1.66 thorns/μm. When the density of thorns was decreased from 2.19 to 1.66 thorns/μm, but DPN did not significantly change the density of thorns (Fig. 4). Blocking of Erk MAP kinase by PD98059 completely prevented the estradiol-induced decrease of thorns. Taken together, in the stratum lucidum of CA3, the decrease of the thorn density is probably induced by activation of Erk MAP kinase by estradiol-bound ERα at the basal Ca²⁺ concentration of around 0.1–0.2 μM. When the Ca²⁺ concentration was decreased to below the basal level by blocking AMPA receptors with CNQX, or by changing the outer medium to Ca²⁺-free ACSF, the suppression effect of estradiol was completely inhibited (Fig. 4). These results suggest that the decrease of thorns requires the basal Ca²⁺ concentration which is kept by spontaneous postsynaptic Ca²⁺ fluctuation via voltage-activated calcium channels depending upon AMPA receptor-mediated spontaneous voltage fluctuations, because the spontaneous Ca²⁺ influx within thorny excrescences occurs mainly via voltage-activated calcium channels (Monaghan et al., 1983; Baude et al., 1995; Fritschy et al., 1998; Reid et al., 2001; Reid, 2002). Note that blocking of NMDA receptors by MK-801 did not prevent the estradiol-induced decrease of thorns. This may be due to much smaller contribution of NMDA receptors to the spontaneous Ca²⁺ influx within thorns than that of voltage-activated calcium channels. Function of ERα therefore needs the basal level of Ca²⁺ concentration around 0.1–0.2 μM.

We always use isolated hippocampal slices in order to examine the direct effect of estradiol on glutamatergic neurons within slices. Results from in vivo investigations using whole rat may reflect not only direct but also indirect effects of estradiol on glutamatergic neurons via cholinergic or serotonergic neurons, projecting to the hippocampus (Leranth et al., 2000; MacLusky et al., 2005).

The rapid effect of estrogen has also been observed in vivo. Leranth and co-workers have demonstrated that the estradiol (60 μg/kg) increases the spine-synapse density due to synaptic rearrangements in ovariectomized adult rats as rapid as after 30 min of estradiol injection using electron micrographic analysis (MacLusky et al., 2005). On the other hand, the slow genomic effects (1–4 days) of estradiol on spine plasticity, have been extensively investigated in vivo from the viewpoint of estrogen replacement therapy. For example, supplement of estrogens in ovariectomized adult female rats (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992; MacLusky et al., 2005), increase the density of spines in the stratum radiatum of CA1 pyramidal neurons, resulting in recovery of spines to the level of intact rat. These effects of enhancement in spineogenesis have also been observed as rapid as at 4.5 h after s.c. injection of estrogen (MacLusky et al., 2005). 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 et al., 1999). The contribution of hippocampus-derived estradiol has been reported by Rune and co-workers who demonstrated that the suppression of endogenous estradiol synthesis by letrozole treatments for 4 days significantly decreased the density of spines, spine-synapses, spinophilin (spine marker) and synaptophysin (presynaptic marker) in the stratum radiatum of CA1 region in cultured slices (Kretz et al., 2004). No increase in the density of spines, spine-synapses and spinophilin expression was seen after exogenous application of 100 nM estradiol to the medium of slice cultures that had not been treated with letrozole. Application of 100 nM estradiol, however, induced rescue effect which restored the synaptophysin expression that had been once decreased by letrozole.

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

Estradiol-induced modulation of LTD or LTP occurs only in pre-existent synapses, because newly generated spines by estradiol treatments do not form new synapses within 2 h, as judged from no increase in the baseline magnitude of excitatory postsynaptic potential (EPSP) signal during 2 h of estradiol perfusion (Mukai et al., 2007).

Evidence is emerging that estradiol exerts a rapid influence (0.5–1 h) on synaptic transmission of hippocampal slices from adult rats (3 months), as demonstrated by electrophysiology (Teyler et al., 1980; Gu and Moss, 1996; Foy et al., 1999; Ito et al., 1999; Shibuya et al., 2003).

In memory processing, not only LTP (memory forming mechanism) but also LTD is essential. Mutant mice, which show enhanced LTD and suppressed LTD, have shown impaired learning of Morris water maze (Migaud et al., 1998). This suggests that LTD may be required to correct wrong memories formed by initial LTP processes, which store not only correct information but also wrong information. We found that LTD was very sensitive to 17β-estradiol treatments in hippocampal slices from adult male rats. We demonstrated, for the first time, a significant rapid enhancement of LTD by 1–10 nM estradiol perfusion in CA1, CA3 and DG (Fig. 5) (Mukai et al., 2007). Recordings were 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 was induced pharmacologically by the transient application (3 min) of 30 μM NMDA. This LTD was
Fig. 5. Rapid modulation of LTD by 17β-estradiol and agonists in hippocampal slices from adult male rats. (Upper CA1, CA3 and DG) Time dependence of maximal EPSP amplitude in CA1 (CA1), CA3 (CA3) and DG (DG). Estradiol concentration was 0 nM (open circle), 10 nM (red closed diamond), 100 nM PPT (yellow closed triangle) and 100 nM DPN (blue closed square), respectively. (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 40 min prior to NMDA stimulation, irrespective of the test condition. LTD was induced by 30 μM NMDA perfusion at time t=0–3 min (closed red bar above the graph). Hatched bar above the graph indicates period of time during which estradiol was administered. The LTD enhancement was reproducibly observed in more than 90 slices out of 100 slices. (Lower CA1, CA3 and DG) Comparison of modulation effect of LTD by 17β-estradiol and agonists in the CA1 (CA1), CA3 (CA3) and DG (DG) of hippocampal slices. Vertical axis is 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-estradiol (Estradiol), PPT and DPN at indicated concentrations. Note that co-perfusion of 1 μM ICI with 10 nM 17β-estradiol did not suppress the enhancing effect of LTD by estradiol (data not shown). The significance of the estradiol effect was confirmed at 60 min via statistical analysis using ANOVAs (*p<0.05; **p<0.01) (modified from Mukai et al., 2007).
induced by the activation of phosphatase due to a moderate Ca\(^{2+}\) influx through NMDA receptors (Lee et al., 1998). LTD is effectively induced by the transient application of NMDA to adult hippocampus, whereas low-frequency stimulation cannot induce LTD in adult slices. Low-frequency stimulation can induce LTD in slices from animals younger than 2 weeks. The plateau EPSP amplitude at 60 min after NMDA application was 80.4% (CA1), 88.8% (CA3) and 95.1% (DG), respectively. A 30 min pre-perfusion of 10 nM estradiol significantly enhanced LTD resulting in the residual EPSP amplitude of 59.7% (CA1), 79.1% (CA3) and 92.2% (DG) (Mukai et al., 2007). Investigations using specific estrogen agonists indicated that the contribution of ER\(_{\alpha}\) (but not ER\(_{\beta}\)) was essential to these estradiol effects. PPT at 100 nM exhibited a significant LTD enhancement in CA1, while DPN did induce a suppression of LTD in CA1, implying that the contribution of ER\(_{\beta}\) was opposite to that of ER\(_{\alpha}\) in the estradiol effect on LTD. Taken collectively, estradiol-bound ER\(_{\alpha}\) may activate phosphatase at the moderate Ca\(^{2+}\) concentration of around 0.7–1 \(\mu\)M induced upon 30 \(\mu\)M NMDA application (Lisman, 1989), and facilitated dephosphorylation of AMPA receptors may induce enhancement of LTD. On the other hand, estradiol-bound ER\(_{\alpha}\) is not functional in LTP modulation at the transiently high Ca\(^{2+}\) concentration of around 5–12 \(\mu\)M under tetanic stimulation (Lisman, 1989; Yang et al., 1999; Mukai et al., 2006b; Ogiue-Ikeda et al., 2008), because phosphorylation of AMPA receptors by CaM kinase II is a dominant process at the high Ca\(^{2+}\) concentration.

The enhancement of LTP has been occasionally observed by 1–10 nM estradiol in CA1 pyramidal neurons. In this case, a baseline increase by 20–30% has always been observed upon the onset of 10 nM estradiol perfusion in the initial slope of EPSP, which has been attendant upon a further increase to approximately 160% upon high-frequency tetanic stimulation of Schaffer collaterals of hippocampus from adult rat (3 months) (Foy et al., 1999; Bi et al., 2000; Kawato, 2004). However, without this 20–30% baseline increase in EPSP slope (before the tetanic stimulation), the enhancement of LTP by estradiol 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 between the presence and the absence of 10 nM estradiol (Ito et al., 1999; Sato et al., 2004). It should be noted that in 3–4 weeks puberty rats, 10 nM estradiol even suppressed LTP-induction down to the same level as that for adult rats (Ito et al., 1999; Shibuya et al., 2007).
al., 2003). Estradiol effects on LTP are strongly dependent on the age of rats.

3.3. Synaptic estrogen receptors

What is the receptor of 17β-estradiol that mediates rapid actions (1–2 h) on synaptic plasticity in the hippocampus? Putative synaptic membrane estrogen receptors remain poorly defined. Many attempts have been made to identify membrane estrogen receptors. At the present stage, the most probable candidates for synaptic estrogen receptors may be ERα, ERβ and GPR30.

Classical nuclear-type receptors ERα and ERβ are candidates for synaptic estrogen receptors. Because ICI do not suppress estradiol-induced rapid modulation of electrophysiological properties such as LTD, LTP, and kainate-induced currents, classical estrogen receptors are suggested to be not involved in these modulations (Gu and Moss, 1996). 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 dimerization of ERα and ERβ. If dimerization processes are not involved in rapid modulation of electrophysiological phenomena, then ICI cannot block these phenomena. On the other hand, rapid enhancement of spinogenesis via ERα was significantly blocked by ICI (Fig. 4) (Mukai et al., 2007), therefore dimerization processes occur for synaptic ERα in spinogenesis.

We 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 affinity-column purified anti-ERα antibody RC-19 (C-terminal antibody) (Mukai et al., 2007). Attention must be paid that non-purified ERα antisera often react significantly with unknown proteins, resulting in wrong staining different from real ERα distribution. A post-embedding immunogold electron microscopic analysis demonstrated the specific localization of ERα in the glutamatergic neurons in CA1, CA3 and DG (Fig. 6). ERα was also localized in the nuclei. Western blot analysis demonstrated that ERα (67 kDa) and Erk MAP kinase were tightly associated with postsynaptic density fractions (PSD) (Fig. 7). On the other hand, ERα was not expressed at dendritic raft (Fig. 7). Because the estradiol-induced modulation of LTD and spine density appeared 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 processes. Interestingly, a significant accumulation of ERα at PSD was observed by a 3-min stimulation with 30 μM NMDA used for the LTD induction, implying that ERα may be dynamically movable in spines (Fig. 7). Note that specific binding of purified RC-19 antibody to real ERα (67 kDa) in the hippocampus was verified using MALDI-TOF mass-spectrometric analysis of RC-19 reacted proteins as well as the absence of reactivity of RC-19 with ERα KO mice hippocampus (Mukai et al., 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 et al., 2007). AS409, another frequently used antisera did mainly react with unknown proteins different from real ERα (Mukai et al., 2007). Non-purified antisera may largely react with proteins having amino acid sequences similar to the real antigen in the hippocampus in which extremely low level of ERα is expressed as compared with that in the ovary. Surprisingly, ERα antisera are often examined for their reactivity only in endocrine organs such as the ovary in which ERα is highly expressed. Therefore, staining of interneurons and no staining of primary neurons with non-purified antisera (such as MC-20 or AS409) probably do not show real ERα distribution in the hippocampus. Antisera should be purified before application to the hippocampus.

ERα knock-out mice may be useful to investigate the participation of ERα in modulation of synaptic plasticity. However, so far no data are available for real ERα knock-out mice. Electrophysiological investigations are performed by using knock-down mice (not knock-out mice) by Moss and co-workers (Gu and Moss, 1996; Gu et al., 1999). They have reported no essential contribution of ERα to estradiol-induced rapid enhancement of the kainate currents of CA1 neurons. They reach this conclusion due to the observation of very small difference in estradiol effect on the kainate currents of CA1 neurons. They reach this conclusion due to the observation of very small difference in estradiol effect on the kainate currents of CA1 neurons. They reach this conclusion due to the observation of very small difference in estradiol effect on the kainate currents of CA1 neurons. They reach this conclusion due to the observation of very small difference in estradiol effect on the kainate currents of CA1 neurons.
investigations. Therefore, it is necessary to investigate real ERα knock-out mice which are, for example, deleted in the whole exon 2 of the mouse ERα gene (Dupont et al., 2000). Note that nomenclature of ERα exon changes recently, and the current exon 1 and exon 2 (Kos et al., 2002; Pedram et al., 2006). Several investigations of immunostaining of ERβ have suggested extranuclear expression of ERβ including dendritic appearance in the hippocampal principal neurons (Milner et al., 2005). ERβ is, however, not yet identified as synaptic membrane receptors. Subcellular immunostaining patterns of these reports might reflect relatively minor expression of ERβ and major expression of unknown proteins, due to multiple reactivity of non-purified ERβ antisera to several unknown proteins in Western blot analysis. The purity of commercially available ERβ antisera may be worse than that of ERα antisera as judged from Western blot analysis.

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 et al., 2005) as well as in endoplasmic reticulum membranes of COS7 after genetic expression of GPR30 fused with green fluorescent protein (Revankar et al., 2005). Because expression of GPR30 has also been observed in the hippocampal neurons (Brailoiu et al., 2007), further investigations may reveal its contribution to rapid estradiol modulation of synaptic plasticity.

4. Synaptocrinology and intracrinology

Based on experimental observations, we illustrate in Fig. 8, a hypothetical model for the synaptic synthesis of brain steroid (synaptocrine mechanisms) and the modulation of the synaptic plasticity of neurons by brain steroid. Brain steroid synthesis proceeds in the following manner. First, glutamate release from the presynapse induces a Ca2+ influx through the NMDA receptors. The Ca2+ influx drives StAR (Kimoto et al., 2001) to transport cholesterol into the mitochondria, where P450scc converts cholesterol to pregnenolone. The conversion of pregnenolone to androstenedione, androstenedione to testosterone, and testosterone to dihydrotestosterone is performed at spines in addition to endoplasmic reticulum by P450(17α), 3β-HSD, 17β-HSD, P450arom, 5α-reductase and 3α-HSD.Produced estradiol binds to synaptic ERα and drives signaling pathway including kinases (such as Erk MAP kinase) or phosphatases, finally resulting in modulation of AMPA receptors or NMDA receptors. Note that brain steroids are synthesized also in endoplasmic reticulum and mitochondria in cell body of neurons. Genomic pathway via nuclear ERα receptors also functions in delayed estradiol effects such as neuroprotection, spinogenesis, keeping homeostasis, etc. (intracrine mechanisms).

References


