Neuroscience 138 (2006) 757–764

HIPPOCAMPAL SYNTHESIS OF ESTROGENS AND ANDROGENS WHICH ARE PARACRINE MODULATORS OF SYNAPTIC PLASTICITY: SYNAPTOCRINOLOGY

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Abstract—Hippocampal pyramidal neurons and granule neurons of adult male rats are equipped with a complete machinery for the synthesis of pregnenolone, dehydroepiandrosterone, testosterone, dihydrotestosterone and 17β-estradiol. Both estrogens and androgens are synthesized in male hippocampus. These brain steroids are synthesized by cytochrome P450s (P450scc, P45017α and P450arom), hydroxysteroid dehydrogenases and reductases from endogenous cholesterol. The expression levels of enzymes are as low as 1/300–1/1000 of those in endocrine organs. Synthesis is dependent on the acute Ca^{2+} influx upon neuron–neuron communication via NMDA receptors. Estradiol is particularly important because estradiol rapidly modulates neuronal synaptic transmission such as long-term potentiation via synaptic estrogen receptors. Xenoestrogens may also act via estrogen-driven signaling pathways.

Key words: neurosteroid, P450, hippocampus, estradiol, endocrine disrupter.

This article describes local endogenous synthesis of estrogens and androgens in the mammalian brain, particularly hippocampus, in relation to their rapid action as modulators of the synaptic plasticity. The hippocampus is essentially involved in learning and memory processes, and is known to be a target for the neuromodulatory actions of hormones produced in the gonads. As both estradiol and testosterone may reach the brain via blood circulation, after crossing the blood–brain barrier, extensive studies have been performed to investigate their role in modulating hippocampal plasticity and function (Woolley and McEwen, 1994; Woolley, 1998; Foy et al., 1999; Pozzo-Miller et al., 1999; Bi et al., 2000; Shibuya et al., 2003). In addition to endocrine-derived hormones, recent experiments have demonstrated that hippocampal neurons may also be exposed to locally synthesized brain steroids, such as pregnenolone (PREG) (Baulieu, 1997; Kimoto et al., 2001; Kawato et al., 2003). Dehydroepiandrosterone (DHEA) has also 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, been difficult, due to the extremely low levels of steroidogenic proteins in the brain (Warner and Gustafsson, 1995). Sex steroids had not been considered to be brain-derived steroids, because of many reports suggesting the absence of cytochrome P45017α in adult mammalian brain (Le Goascogne et al., 1991; Mellon and Deschepper, 1993). In particular, since sex steroids cannot be synthesized without P45017α, which converts PREG to DHEA, they are thought to reach the brain via blood circulation (Baulieu and Robel, 1998). On the other hand, frog brain has been reported to synthesize testosterone from PREG (Mensah-Nyagan et al., 1996). There has been an exceptional report suggesting that human brain tissue has an ability of conversion of androstenedione to testosterone (Steckelbroeck et al., 1999).

To date, the term ‘neurosteroids’ has been used to refer to steroids produced both in the brain and in the peripheral nerves and Schwann cells (Morfin et al., 1992; Koenig et al., 1995). Here, ‘brain neurosteroid’ refer to a steroid that is synthesized de novo in the brain by P450 systems.

Localization of steroidogenic systems in the adult rat hippocampus

All experiments were conducted along with the institutional ethical guidelines. All efforts were made to minimize the number of animals per group and any potential suffering of those subjects.

Expression of transcripts for steroidogenic enzymes. Highly sensitive molecular biology investigations are necessary for determination of 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).

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Abbreviations: BPA, bisphenol A; DES, diethylstilbestrol; DHEA, dehydroepiandrosterone; LTP, long-term potentiation; PBR, peripheral benzodiazepine receptor; PREG, pregnenolone; RNase, ribonuclease; StAR, steroidogenic acute regulatory protein; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.

0306-4522/06$30.00+0.00
doi:10.1016/j.neuroscience.2005.09.010

Collectively from many studies, 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), with that of P450arom expressed at an intermediate level.

The concentration of P450scc mRNA expressed in the brain is reported to be only 10^{-4}-10^{-5} of that in the adrenal gland (Mellon and Deschepper, 1993; Sanne and Krueger, 1995). Our analysis showed approximately $1/10^3$ for P450scc (Ishii, Furukawa and Kawato, unpublished observations). As a result, the presence of P450scc mRNA could be demonstrated only by 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 et al., 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 levels in the adrenal gland (Furukawa et al., 1998; King et al., 2003).

Concerning P45017α, many attempts to demonstrate the immunohistochemical reactivity in the rat brain had been unsuccessful (Le Goascogne et al., 1991). The mRNAs for P45017α had not been detected in adult rat brain by either RNase protection assays or RT-PCR (Mellon and Deschepper, 1993). The expression of the mRNA for P45017α had been reported by many laboratories as only transient, occurring during rat embryonic and neonatal development (Compagnone et al., 1995; Zwain and Yen, 1999a,b). We overcame this difficulty by carefully designing primer pairs which were free from three-dimensional loop formation, using computer calculation (Hojo et al., 2004). In the hippocampal tissues from adult male rats aged 3 months, we observed the P450 transcripts expressed approximately 1/300 for P45017α (Hojo et al., 2004), when compared with those expressed in the testis. It should be noted that approx. 1.5-fold of P45017α transcripts was expressed in the hypothalamus as compared with those in the hippocampus.

The role of P450arom in the hippocampus had also not been well elucidated, primarily because many studies had indicated the absence of P450arom in the adult rat and mouse hippocampus. Recently, however, the significant expression of mRNA for P450arom in the pyramidal and granule neurons of the adult rat hippocampus has been demonstrated using *in situ* hybridization (Wehrenberg et al., 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 the P450 transcripts expressed approximately 1/300 for P450arom (Hojo et al., 2004), when compared with those expressed in the ovary by using carefully designed primer pairs for RT-PCR. Note that approx. 1.5-fold of P450arom transcripts was expressed in the hypothalamus as compared with those in the hippocampus.

The presence of mRNAs for 17β-hydroxysteroid dehydrogenase (17β-HSD) types 1 and 3 has also been demonstrated in the human and rat hippocampus (Beyenburg et al., 2000). We investigated the expression level of mRNA transcripts for 17β-HSD (types 1–4) by using carefully designed primer pairs in the hippocampus from adult male rats. The mRNA level of 17β-HSD transcripts observed was approximately 1/10, relative to the level in the ovary for 17β-HSD (type 1), 1/200–1/300, relative to the level in the testis for 17β-HSD (type 3), respectively (Hojo et al., 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}-10^{-3} of the levels in the adrenal gland) were observed to be localized along the pyramidal cell layer in the CA1–CA3 regions and the granule cell layer in the dentate gyrus of rats (Furukawa et al., 1998) and mice (King et al., 2003).

Glial cells have been considered to play an important role in steroidogenesis, as many reports have indicated the presence of mRNA for P450scc, P45017α, 3β-HSD, and 17β-HSD in cultures of astrocytes and oligodendrocytes from embryonic and neonatal brains (Jung-Testas et al., 1989; Baulieu, 1997; Zwain and Yen, 1999a,b). Although a similar level of P45017α mRNA had been reported to be expressed in both astrocytes and neurons in primary cell cultures from the brain of neonatal rats, neurons had exhibited a much lower metabolic activity than astrocytes for the conversion of PREG to DHEA (Zwain and Yen, 1999a,b).

These extensive investigations are possible because primary glial cell cultures can easily be obtained for embryonic and neonatal brains. As a result, however, direct information is not available from these studies regarding the biosynthesis system of neurosteroids in 'adult' rat brain.

Neuronal localization of enzymes investigated with immunohistochemical and Western immunoblot analysis. The role of neurons in steroid synthesis had not yet been clearly determined in mammalian brain, although there had been some reports indicating the expression of several steroidogenic enzymes in non-mammalian brains (Mensah-Nyagan et al., 1994) and rat brain neurons (Guennoun et al., 1995; Tsutsui et al., 2000). We overcame many difficulties of nonspecific immunostaining by using purified antibodies (instead of using non-purified antisera) with a slightly higher Triton X-100 concentration (0.5%) in order to obtain a good penetration of IgG, as well as using fresh frozen slices of hippocampus (instead of using paraffin sections) from adult male rats. A significant localization of cytochromes P450scc (CYP11A1), P45017α (CYP17A) and P450arom (CYP19) was observed in pyramidal neurons in the CA1-CA3 regions, as well as in granule cells in the dentate gyrus, by means of the immunohistochemical staining of hippocampal slices (Fig. 1) (Kimoto et al., 2001; Kawato et al., 2002, 2003; Hojo et al., 2004). The co-localization of immunoreactivity against P450s and NeuN confirmed the presence of P450s in these neurons (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). StAR

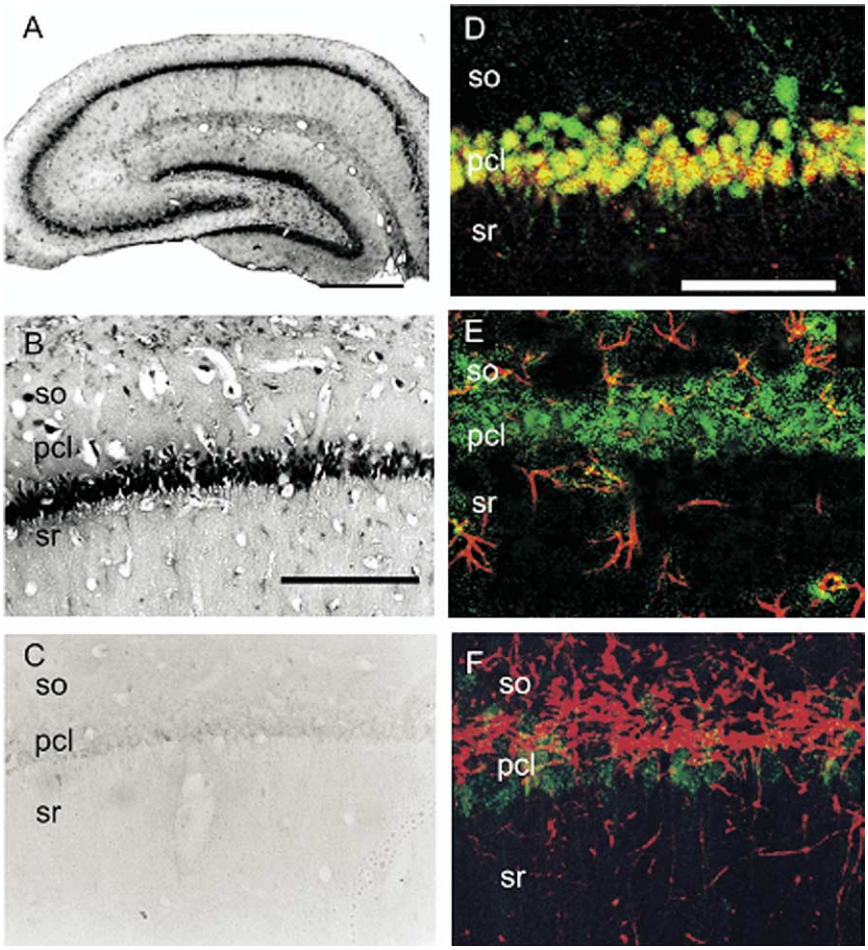

Fig. 1. Immunohistochemical staining of cytochrome P45017α in the hippocampal slices of adult male rat. (A) The coronal section of the whole hippocampus. (B) The CA1. (C) The CA1 stained with anti-P45017α IgG presaturated with P45017α protein. (D) Fluorescence dual staining of P45017α (green) and NeuN (red). (E) Fluorescence dual staining of P45017α (green) and GFAP (red). (F) Fluorescence dual staining of P45017α (green) and myelin basic protein (red). Superimposed regions of green and red fluorescence are represented by yellow. pcl, pyramidal cell layer; so, stratum oriens; sr, stratum radiatum. Scale bar=800 μm for A, and 120 μm for B–F. [Taken from Hojo et al., 2004.]

was co-localized with P450s (Kimoto et al., 2001; King et al., 2003). These results, taken together, imply that pyramidal neurons and granule cells are equipped with complete steroidogenic systems which catalyze the conversion of cholesterol to PREG, DHEA, testosterone and estradiol.

An immunoelectron microscopic analysis using postembedding immunogold was performed in order to determine the intraneuronal localization of P45017α in the hippocampal neurons of adult male rats. Surprisingly, we observed that both P45017α and P450arom were localized not only in the endoplasmic reticulum but also in the presynaptic as well as the postsynaptic regions of pyramidal neurons in the CA1–CA3 regions and of granule neurons in the dentate gyrus (Fig. 2). These results suggest a possibility of 'synaptocrine' mechanisms of estrogens and androgens in addition to blood-derived sex steroids.

The expression of these steroidogenic proteins was confirmed by Western immunoblot analysis. A single protein band was observed for each of these P450s (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). The resulting molecular weights obtained for P450scc, P45017α and P450arom were almost 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 (P45017α and P450arom) of that in the testis (P450scc and P45017α) and the ovary (P450arom), respectively.

For decades, neurosteroidogenesis had been extensively studied in glial cells. This line of investigations was motivated by the absorption of anti-bovine P450scc antibodies by white matter throughout the rat brain (Le Goascogne et al., 1987), and by the many reports which indicated the presence of steroidogenic proteins in astrocytes, oligodendrocytes and white matter (Le Goascogne et al., 1987; Jung-Testas et al., 1989; Kimoto et al., 1997). From our observation in adult hippocampus, the distributions of astroglial cells and oligodendroglial cells, however, displayed very different patterns from those characteristics of the cells containing P450scc, P45017α and P450arom (Kimoto et al., 2001; Hojo et al., 2004). This indicates that the majority of P450-containing cells are neither astroglial cells nor oligodendroglial cells.

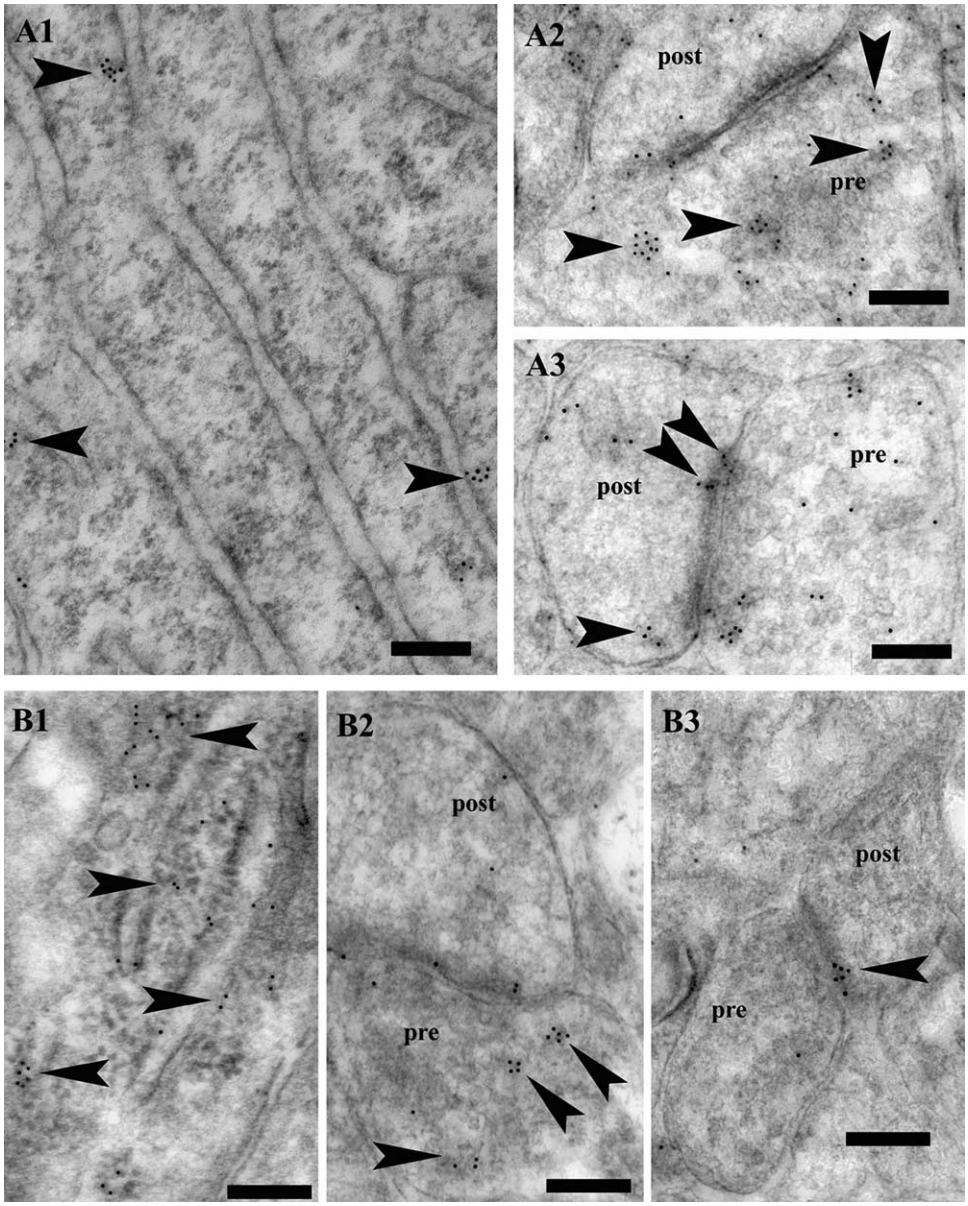

Fig. 2. Immunoelectron microscopic analysis of the distribution of P45017α (A1–A3) and P450arom (B1–B3) within axospinous synapses, in the strata radiatum of the hippocampal CA1 region at the central region of the rostrocaudal level. Gold particles (indicated with arrowheads) were observed to be localized in the endoplasmic reticulum (A1 and B1), the presynaptic region (A2 and B2), and the postsynaptic region (A3 and B3) of pyramidal neurons. In the axon terminal (A2 and B2), gold particles were associated with small synaptic vesicles (A2 and B2). In dendritic spines, gold particles were found within the head of the spine (A3 and B3). Post, postsynaptic region; Pre, presynaptic region. Scale bar=200 nm. [Taken from Hojo et al., 2004.]

Synthesis of estrogens and androgens

A direct demonstration of the neuronal synthesis of DHEA in adult mammals was for the first time reported by our group (Kawato et al., 2002; Hojo et al., 2004). It had been assumed that DHEA and the sex steroids are supplied to the brain such as hypothalamus, via the blood circulation, where they are converted to estradiol by P450arom (Baulieu, 1997; Baulieu and Robel, 1998). The absence of P45017α activity in the brain of adult mammals has 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 [^{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 ^{3}H-DHEA (Baulieu and Robel, 1998).

We challenged to demonstrate the synthesis of DHEA, testosterone and estradiol in the hippocampal slices by means of HPLC analysis (Kawato et al., 2002; Hojo et al., 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 (Wang et al., 1997; Kimoto et al., 2001; Hojo et al., 2004). The signifi-

cant conversion from [^{3}H]-PREG to [^{3}H]-DHEA, from [^{3}H]-DHEA to [^{3}H]-androstenediol, [^{3}H]-androstenedione, [^{3}H]-testosterone and [^{3}H]-estradiol was observed after incubation with the slices for 5 h at 20 °C. 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 inactivated.

We determined the concentration of DHEA and estradiol as well as PREG in the acute hippocampal slices from adult male rats by means of RIA or mass spectroscopy after careful purification of steroids with HPLC (Kimoto et al., 2001; Kawato et al., 2002; Hojo et al., 2004). The basal concentrations of PREG, DHEA and estradiol in the male rat hippocampus were approximately 18, 0.3 and 0.6 nM which were six to 10 times greater than those typical of plasma (Kimoto et al., 2001; Hojo et al., 2004). 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; Hojo et al., 2004). Upon stimulation with NMDA for 30 min, the hippocampal level of PREG and estradiol increased to approx. two-fold 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' treatment with letrozole, the amount of estradiol released into the medium was significantly decreased (Kretz et al., 2004). Recently DHEA synthesis from PREG has also been demonstrated in the spinal cord of adult rat (Kibaly et al., 2005). Considering our observation of P45017α, P450arom and 17β-HSD (types 1, 3) in the hypothalamus and the cerebral cortex (Hojo et al., 2004), further investigations are encouraged on endogenous synthesis of estrogens and androgens in different regions of the mammalian brain.

Interestingly it has been reported that PREG sulfate and DHEA sulfate are absent in the rat brain as measured by direct mass spectroscopic analysis, although cholesterol sulfate is present (Higashi et al., 2003; Liu et al., 2003). In many previous publications, PREG sulfate or DHEA sulfate has always been suggested by indirect methods, i.e. measuring PREG or DHEA after solvolysis of water soluble fractions which may contain some PREG-derivatives 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 report that sulfated steroids are very effective for neuromodulation, careful considerations should be performed (Wu et al., 1991; Vallee et al., 1997; Baulieu and Robel, 1998).

It is necessary to consider whether the local concentration of brain neurosteroids is sufficiently high to allow

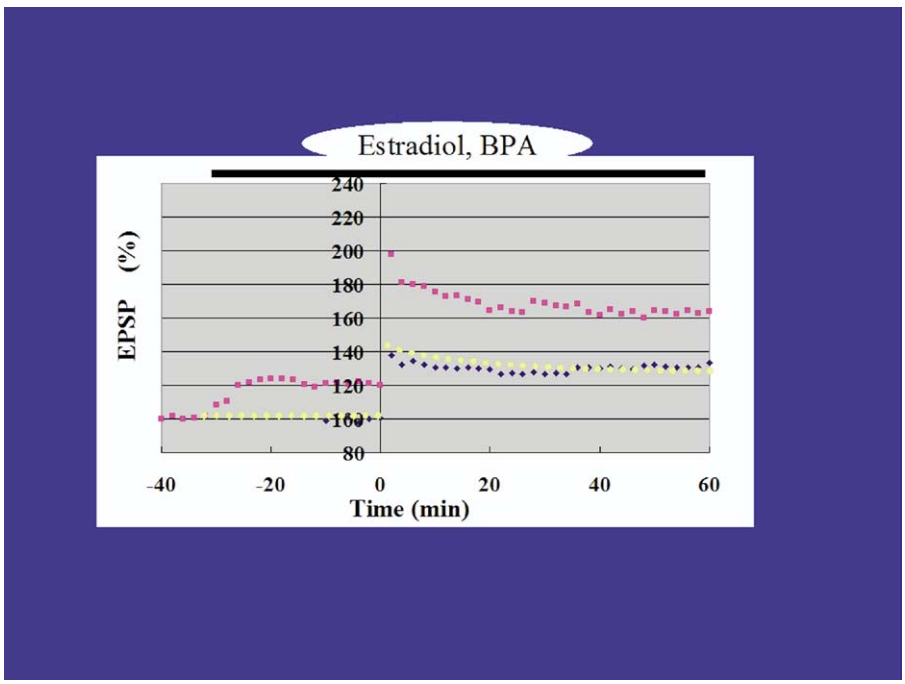

Fig. 3. Rapid suppression by BPA of estradiol-induced enhancement of LTP in the hippocampal CA1 region. Pre-perfusion of 10 nM estradiol for 30 min at 30 °C immediately increased the slope of the excitatory postsynaptic potential (EPSP) slope to approximately 120% (paired pulse facilitation value of 1.76±0.05). Upon tetanic stimulation (100 Hz, 1 s, at t=0) of the Schaffer collaterals, EPSP slope was significantly increased (LTP-induction). The final level of EPSP slope was 164.4±12.6% in the case of perfusion with 10 nM estradiol (pink square, n=5), and 132.1±7.8% in control experiments (blue diamond, n=6). When 100 nM BPA was co-perfused with 10 nM estradiol, EPSP enhancement was suppressed to 132.1±5.4% (yellow circle, n=6). Only 100 nM BPA perfusion showed an almost identical LTP-induction to that obtained in control experiments, within experimental error (data not shown, n=5). Statistical significance of * P<0.05 was obtained by ANOVAs. Young adult male Wistar rats aged 4 weeks were investigated with a conventional electrophysiological setup (Axon Instruments, USA).

action as local mediators. The concentration of estradiol detected in the hippocampus is about 0.6 nM (basal) and 1.3 nM after the NMDA-stimulation, respectively. The local concentration of estradiol immediately after the 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 1–10 nM. These levels are sufficient to allow estradiol to act as local mediators that modulate synaptic transmission (Gu and Moss, 1996; Foy et al., 1999; Ito et al., 1999; Bi et al., 2000; Shibuya et al., 2003). Functional differences between blood-derived estradiol (reproductive modulator) and brain-synthesized estradiol (neuronal modulator) may be due to the time-dependence of their levels. Brain is filled with low concentration of blood-derived estradiol which level is very slowly changed, while endogenous synthesis of estradiol is a transient event occurring mainly during synaptic transmission which drives Ca^{2+} influx (Hojo et al., 2004).

Action of estrogens and xenoestrogens (endocrine disrupters) on synaptic plasticity

Evidence is emerging that estrogens exert a rapid influence on the excitability of adult rat hippocampal neurons, as demonstrated by means of electrophysiology (Teyler et al., 1980; Gu and Moss, 1996; Foy et al., 1999; Ito et al., 1999; Shibuya et al., 2003). Although the intracellular signaling pathway, from putative membrane estrogen receptors to NMDA receptors for rapid estradiol action (within 30 min), has not been well elucidated, the involvement of Src tyrosine kinase and MAP kinase has been indicated (Bi et al., 2000). These findings have led researchers to postulate the existence of so-called membrane or non-genomic estrogen effects. We demonstrated recently the synaptic localization of ERα in hippocampal pyramidal and granule neurons with immunoelectron microscopic analysis using novel purified anti-ERα antibody (Mukai et al., 2004). Xenoestrogens also act rapidly in the adult brain. We recently demonstrated the modulatory action of endocrine disrupters, bisphenol A (BPA) and diethylstilbestrol (DES) which are xenoestrogens (Kawato, 2004). The estradiol (10 nM) -induced enhancement of the long-term potentiation (LTP) in CA1 upon tetanic stimulation was considerably suppressed by the co-perfusion with 100 nM BPA, although the perfusion of BPA alone did not alter the LTP-induction (see Fig. 3) (Kawato, 2004). DES at 10 nM, on the other hand, enhanced the LTP by an almost identical magnitude to that obtained by 10 nM estradiol. It should be noted that endocrine disrupters can reach the brain via the blood circulation and by crossing the blood–brain barriers. It has been indicated that BPA, injected into the mother's body (single s.c. injection), is transferred to the brains of both mother and fetus via the blood circulation within

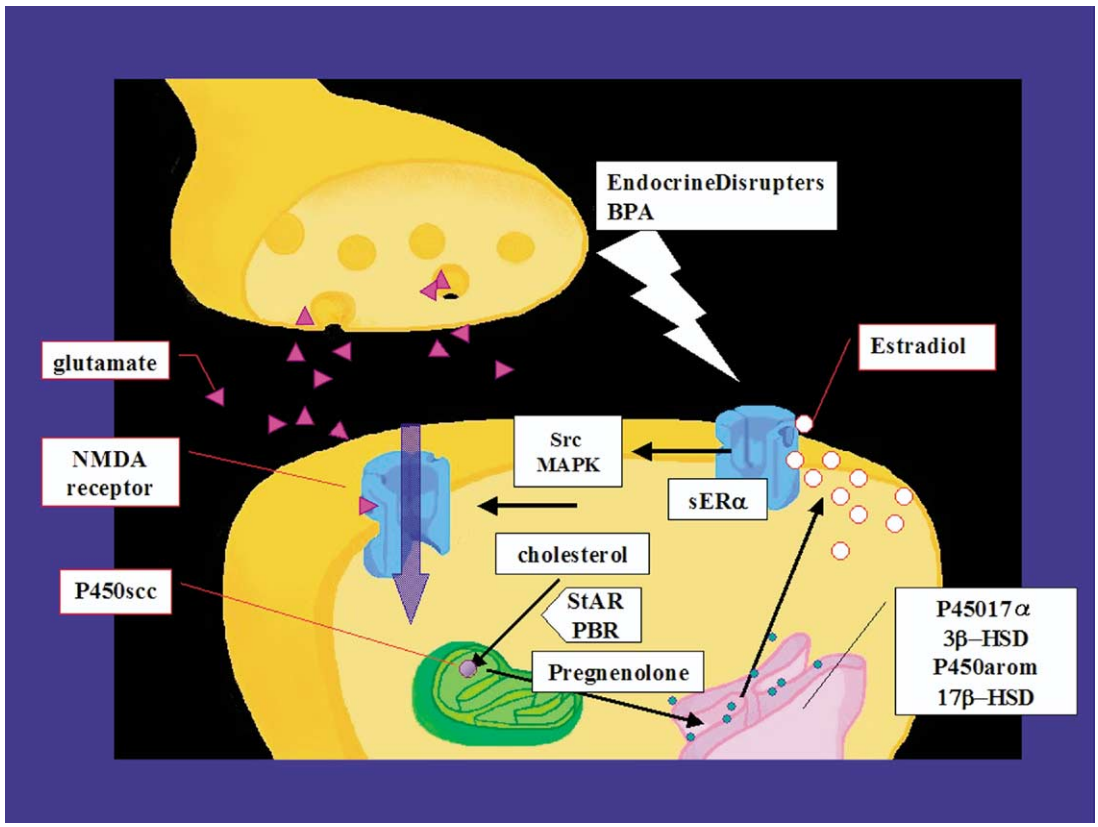

Fig. 4. Schematic illustration for the synaptic synthesis of neurosteroids, and the modulation of the synaptic transmission of neurons by neurosteroids. AMPA type of glutamate receptors are omitted for clarity. StAR, PBR and P450scc are present in the mitochondria. P45017α, 3β-HSD, 17β-HSD and P450arom are localized in the membranes in the synaptic compartment. The site of action for estradiol and BPA is synaptic ERα.

60 min (Uchida et al., 2002). The time required for BPA to reach the brain is 0.5–1 h which is not significantly different from that required to reach peripheral organs such as the placenta, uterus, and liver. In contrast to the efficient detoxification of endocrine disrupters in the liver, detoxification in the brain is much less efficient due to the extremely low level of drug metabolizing enzymes (e.g. cytochrome P450s) in the brain. These findings suggest that endocrine disrupters reach mammalian brains at concentrations sufficient to impact brain function and development.

The chronic genomic effects of estradiol on synaptic plasticity have been extensively investigated. For example, the dendritic spine density in CA1 pyramidal neurons is sensitive to experimentally-induced estrogen depletion and replacement *in vivo* (Gould et al., 1990; Woolley et al., 1997; MacLusky et al., 2005). *In vitro* investigations, using hippocampal slice cultures, have also demonstrated chronic modulation of spinogenesis by estradiol and xenoestrogens (Murphy and Segal, 1996; Pozzo-Miller et al., 1999; Kretz et al., 2004). The essential contribution of endogenous estradiol is reported by Kretz et al. (2004) who have demonstrated that the suppression of endogenous estradiol synthesis by letrozole has decreased the spine density of the CA1 region in cultured slices.

Summary

Based on experimental observations, we illustrate in Fig. 4, a hypothetical model for the synaptic synthesis of brain steroid and the modulation of the synaptic transmission of neurons by brain steroid. 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 and peripheral benzodiazepine receptor (PBR) (Papadopoulos, 1993) to transport cholesterol into the mitochondria, where P450scc converts cholesterol to PREG. After reaching the microsomes, the conversion of "PREG→DHEA→androstenedione→testosterone→estradiol" is performed by P45017α, 3β-HSD, 17β-HSD and P450arom. Produced estradiol binds to synaptic ERα and drives signaling pathway including MAP kinase etc., finally resulting in modulation of NMDA receptors. Endocrine disrupters such as BPA also modulate the synaptic transmission via binding to ERα. Here, only the postsynaptic (spine-localized) synthesis/action is illustrated. The presynaptic synthesis/action, however, also occurs for brain steroid, because P450s and ERα were also observed in the presynapses (Hojo et al., 2004; Mukai et al., 2004).

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(Accepted 8 September 2005)
(Available online 28 November 2005)