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ROLE OF CYTOCHROME P450 IN SYNAPTOCRINOLOGY: ENDOGENOUS ESTROGEN SYNTHESIS IN THE BRAIN HIPPOCAMPUS

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In the hippocampus, the center for learning and memory, cytochrome P450s (P450scc, P450(17α), and P450arom) as well as 17β-, 3β-hydroxysteroid dehydrogenases, and 5α-reductase participate in the synthesis of brain steroids from endogenous cholesterol. These brain steroids include pregnenolone, dehydroepiandrosterone, testosterone, dihydrotestosterone, and 17β-estradiol. Both estrogens and androgens are synthesized in the adult male hippocampal neurons. Although the expression levels of steroidogenic enzymes are as low as 1/200 to 1/50,000 of those in testis or ovary, the levels of synthesized steroids are sufficient for the local usage within small neurons (i.e., intracrine system). This intracrine system contrasts with the endocrine system in which high expression levels of steroidogenic enzymes are necessary in endocrine organs in order to supply steroids to many other organs via blood circulation. Endogenous synthesis of sex steroids in the hypothalamus is also discussed.

Rapid modulation by estrogens and xenoestrogens is discussed concerning synaptic plasticity such as the long-term potentiation, the long-term depression, or spinogenesis. Synaptic expression of P450(17α), P450arom, and estrogen receptors suggests "synaptocrine" mechanisms of brain steroids, which are synthesized at synapses and act as synaptic modulators.

Key Words: Androgen; Estrogen; Hippocampus; Neurosteroid; P450.

INTRODUCTION

This article describes the importance of cytochtrome P450 enzymes in local endogenous synthesis of estrogens and androgens in the mammalian brain, particularly the hippocampus. Because of extremely low levels of expression for P450s in the brain, much lower than 1/100 of their levels in endocrine organs, many scientists had not seriously considered that brain P450s have essential function in either steroid synthesis or drug metabolism.

The hippocampus is essentially involved in learning and memory processes, and is known to be a target for the neuromodulatory actions of sex hormones produced in the gonads. Estrogens and androgens have specific contributions to rapid action on the synaptic plasticity as neuromodulators. 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 (Bi et al., 2000; Foy et al., 1999; Pozzo-Miller et al., 1999; Shibuya et al., 2003; Woolley,

1998; Woolley and McEwen, 1994). The hypothalamus-putuitary-adrenal (HPA) axis and hypothalamus-putuitary-gonadal (HPG) axis are known to be essential circuits for neuroendocrine regulations. Determination of male or female type of the brain depends on sex steroids from gonads in the early developmental stage (postnatal 1–10 days) of the rat and mouse brain.

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; Kawato et al., 2003; Kimoto et al., 2001). Dehydroepiandrosterone (DHEA) has also been found in the mammalian brain at concentrations greater than that in plasma (Baulieu, 1997; Corpechot et al., 1981). 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 has, however, been not successful, 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, and rather were believed to reach the brain exclusively via blood circulation (Baulieu and Robel, 1998). This belief is supported by many reports suggesting the absence of cytochrome P450(17α) in adult mammalian brain (Le Goascogne et al., 1991; Mellon and Deschepper, 1993) and also by the observation of the complete disappearence of testoterone in the brain within 1 day after castration (Baulieu and Robelet, 1998). In particular, sex steroids cannot be synthesized without P450(17α), which converts PREG to DHEA.

STEROIDOGENIC SYSTEMS IN THE ADULT RAT HIPPOCAMPUS

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).

Collectively, 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 that of P450arom expressed at an intermediate level (Table 1).

The concentration of P450scc 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 grand (Table 1). As a result, the presence of P450scc mRNA could be demonstrated only by reverse transcriptase polymerase chain reaction (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). In contrast, 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 et al., 1998; King et al., 2003).

Concerning P450(17α), many attempts to demonstrate the immunohistochemical reactivity in the rat brain had been unsuccessful for almost two decades (Le Goascogne et al., 1991). The mRNAs for P450(17α) had not been detected in adult rat brain by either RNase

Table 1 Comparison of relative mRNA expression level for steroidogenic enzymes in the adult rat (3 mo).

	Hippo	Hypo	Adrenal/testis/ovary/liver
P450scc	1	3	50,000 (Ad)
P450(17α)	1	3	300 (Te)
P450arom	1	3	600 (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)
5α-Reductase (type 2)	1	2	200 (Pr)
ERα	1	5	15 (Ov)
ERβ	1	4	80 (Ov)

The level in the hippocampus is normalized to be 1.

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

Values of mRNA expression level are approximate values obtained from semiquantitative RT-PCR analyses (Hojo et al., 2004; Ishii and Kawato, unpublished results).

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 et al., 1995; Zwain and Yen, 1999a; Zwain and Yen, 1999b). We overcame this difficulty by carefully choosing the sequence of primer pairs that have high specificity by minimizing Gibbs free energy on recombination of a 3′-primer with cDNA, using computer calculation (Hojo et al., 2004). In the hippocampal tissues from adult male rats age 3 months, we observed the P450(17α) transcripts expressed approximately 1/300 (Hojo et al., 2004), when compared with those expressed in the testis.

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. 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 et al., 2001). The level of the mRNA expression in the adult mouse hippocampus was approximately one-half of that in neonatal stages (Ivanova and Beyer, 2000). We observed the P450arom transcripts expressed approximately 1/300 (Hojo et al., 2004), as compared with those expressed in the ovary by using carefully designed primer pairs for RT-PCR.

The presence of mRNAs for 17β-hydroxysteroid dehydrogenase (17β-HSD) types 1 and 3 has 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) 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 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-hydroxysteroid dehydrogenase (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–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 because 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 et al., 1989; Zwain and Yen, 1999a; Zwain and Yen, 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 brain 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; Zwain and Yen, 1999b).

These extensive data are available on primary glial cell cultures, which are easily prepared from embryonic and neonatal brains. However, information regarding the biosynthesis system of neurosteroids in adult rat brain is not directly available from these studies.

Neuronal Localization of Enzymes Investigated with Immunostaining

The role of neurons in steroid synthesis has not yet been clearly determined in mammarian brain, although some reports suggested the expression of several steroidogenic enzymes in nonmammalian brains (Mensah-Nyagan et al., 1999) and rat brain neurons (Koenig et al., 1995; Tsutsui et al., 2000). We overcame many difficulties of nonspecific immunostaining by using affinity column-purified antibodies (instead of using nonpurified 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), P450(17α) (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) (Hojo et al., 2004; Kawato et al., 2002; Kawato et al., 2003; Kimoto et al., 2001). The colocalization of immunoreactivity against P450s and NeuN (marker protein of neuronal nuclei) confirmed the presence of P450s in these neurons (Hojo et al., 2004; Kawato et al., 2002; Kimoto et al., 2001). StAR was colocalized with P450s (Kimoto et al., 2001; King et al., 2003). 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 postembedding immunogold was performed in order 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 and the postsynaptic region 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 synthesis of estrogens and androgens, in addition to classical 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 P450 (Hojo et al., 2004; Kawato et al., 2002; Kimoto et al., 2001). 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.

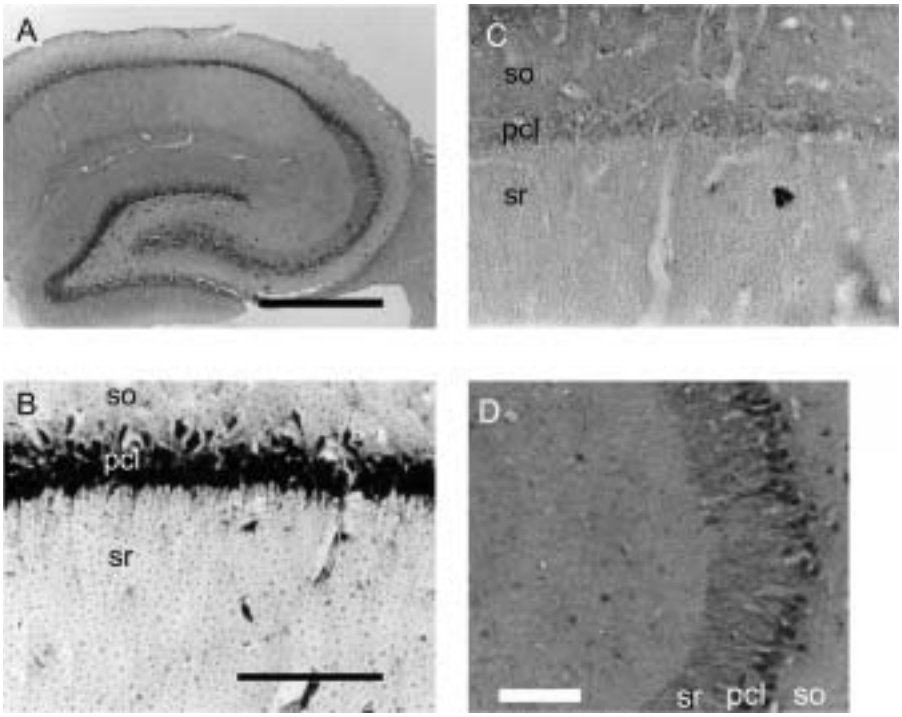

Figure 1 Immunohistochemical staining of P450arom in the hippocampus of an adult male rat. (A) The coronal section of the whole hippocampus. (B) The CA1 region. (C) The CA1 stained with P450arom IgG preadsorbed with purified P450arom. (D) The CA3, where not only cell bodies, but also processes of neurons, are densely stained. so, stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. Scale bar 800 μm for (A) and 120 μm for (B–D). (Taken from Hojo et al., 2004.)

For decades, neurosteroidogenesis had been extensively studied in glial cells. This line of investigations was motivated by the absorption of antibovine P450scc antibodies by white matter, which was believed to be rich in glial cells, throughout the rat brain (Le Goascogne et al., 1987), and by the many reports that indicated the presence of steroidogenic proteins in astrocytes, oligodendrocytes, and white matter (Jung-Testas et al., 1989; Kimoto et al., 1997; Le Goascogne et al., 1987). 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, P450(17α), and P450arom (Hojo et al., 2004; Kimoto et al., 2001). This indicates that the majority of P450-containing cells are neither astroglial cells nor oligodendroglial cells.

SYNTHESIS OF ESTROGENS AND ANDROGENS IN THE HIPPOCAMPUS

A direct demonstration of the neuronal synthesis of DHEA and estradiol in adult mammals was for the first time reported by our group (Hojo et al., 2004; Kawato et al., 2002). 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 has been reported in a number of studies (Baulieu and Robel, 1998;

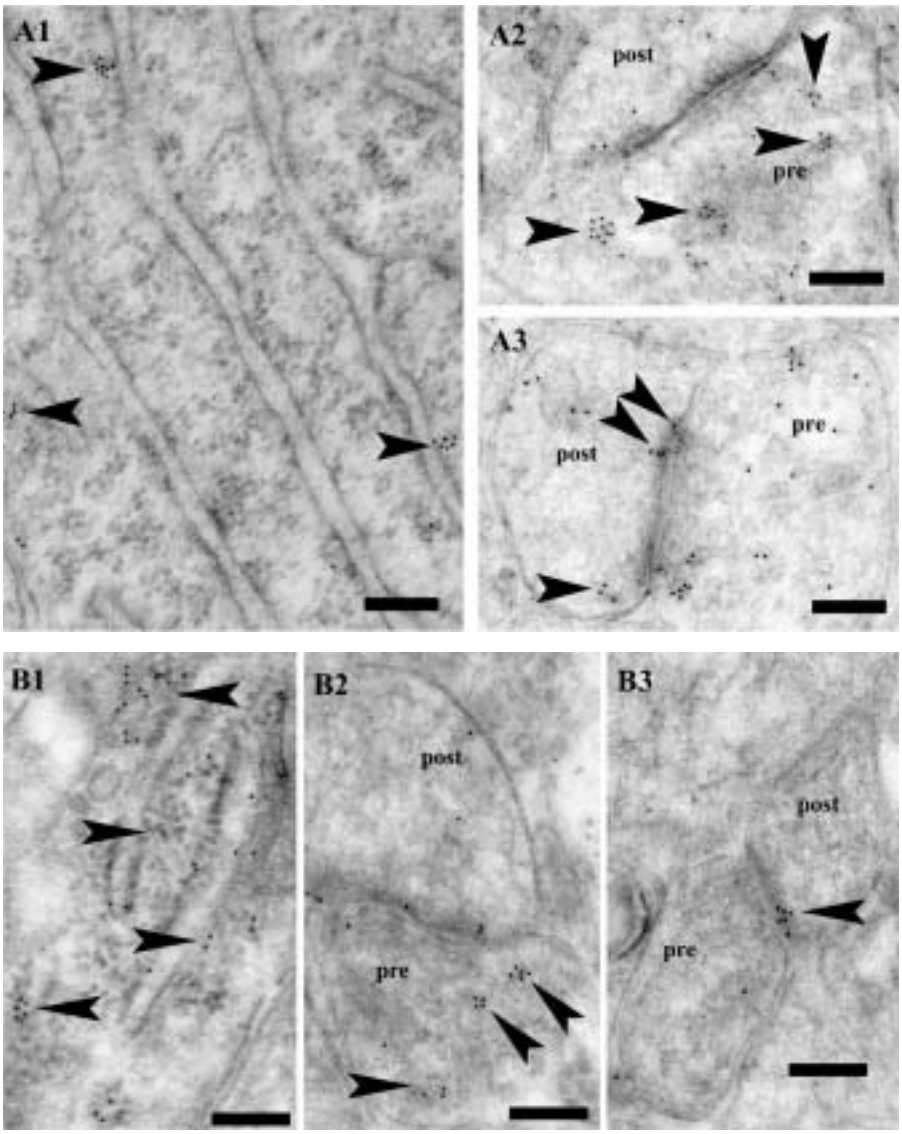

Figure 2 Immunoelectron microscopic analysis of the distribution of P450(17α) (A1–A3) and P450arom (B1–B3) within axospinous synapses in the stratum radiatum of the hippocampal CA1 region. Gold particles (indicated with arrowheads) were localized in the endoplasmic reticulum (A1 and B1), the presynaptic region (A2 and B2), and the postsynaptic region (A3 and B3) of pyramidal neurons. In dendritic spines, gold particles were found within the head of the spine (A3 and B3), and in some cases, gold particles were affiliated within the postsynaptic density. In the axon terminal (A2 and B2), gold particles were associated with small synaptic vesicles (A2 and B2). In dendrites, gold particles were distributed within the cytoplasm of the head of the spine (data not shown). pre, presynaptic region; post, postsynaptic region; scale bar 200 nm. (Taken from Hojo et al. 2004.)

Kibaly et al., 2005; Le Goascogne et al., 1991; Mensah-Nyagan et al., 1999). 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 attempted to demonstrate the synthesis of DHEA, testosterone, and estradiol in the hippocampal slices by means of high-performance liquid chromatography (HPLC) analysis (Hojo et al., 2004; Kawato et al., 2002). 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 (Hojo et al., 2004; Kimoto et al., 2001; Wang et al., 1997). The significant 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 (Fig. 3) (Hojo et al., 2004). 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. In contrast, dihydrotestosterone was rapidly converted to 3α,5α-androstanediol.

We determined the concentration of DHEA and estradiol, as well as PREG, in the acute hippocampal slices from adult male rats by means of radioimmunoassay (RIA) or mass spectroscopy after careful purification of steroids with HPLC (Hojo et al., 2004; Kawato et al., 2002; Kimoto et al., 2001). 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 (Hojo et al., 2004; Kimoto et al., 2001). To demonstrate the rapid net production of neurosteroids on synaptic stimulation, the NMDA-induced production of PREG and estradiol was investigated in hippocampal slices (Hojo et al., 2004; Kimoto et al., 2001). Upon stimulation with NMDA for 30 min, the hippocampal level of PREG and estradiol increased to approximately twofold that 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

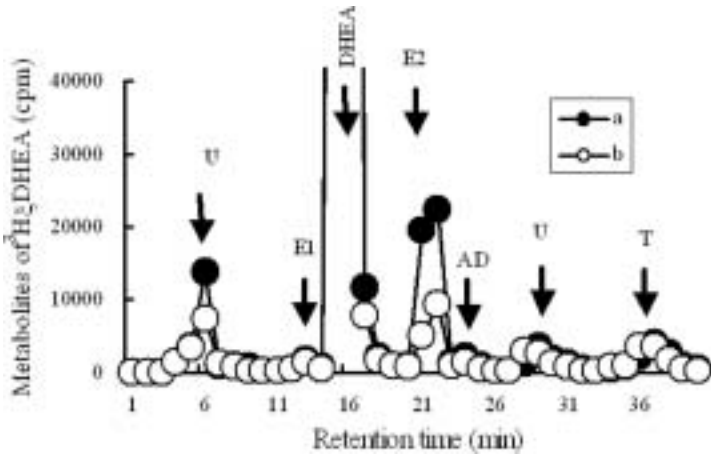

Figure 3 Synthesis of estradiol and testosterone in adult hippocampal slices. HPLC analysis shows the profile of ^{3}H–DHEA metabolites in the absence (line a) or in the presence (line b) of fadrozole (inhibitor of P450arom) after incubation of slices for 5 h at 30°C. E2 (estradiol), T (testosterone), AD (androstenedione), E1 (estrone), and U (unknown metabolites). The vertical axis indicates ^{3}H radioactivity (cpm). (Taken from Hojo et al., 2004.)

(Kretz et al., 2004). Recently, DHEA synthesis from PREG has also been demonstrated in the spinal cord of adult rat (Kibaly et al., 2005).

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 et al., 2003; Liere et al., 2004; Liu et al., 2003). In many previous publications, PREG sulfate or DHEA sulfate has been determined indirectly (i.e., measuring PREG or DHEA after solvolysis of water soluble fractions that may contain some PREG derivatives different from sulfated steroids) (Baulieu, 1997; Corpechot et al., 1981; Kimoto et al., 2001; Liere et al., 2000; Liu et al., 2003). Because a numerous publications report that sulfated steroids are very effective for neuromodulation, careful considerations should be performed (Baulieu and Robel, 1998; Vallee et al., 1997; Wu et al., 1991).

It is necessary to consider whether the local concentration of brain neurosteroids is sufficiently high to allow 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 to 10 nM. These levels are sufficient to allow estradiol to act as local mediators that modulate synaptic transmission (Bi et al., 2000; Foy et al., 1999; Gu and Moss, 1996; Ito et al., 1999; 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 a low concentration of blood-derived estradiol whose level changes dependent on circadian rhythm, while endogenous synthesis of estradiol is a transient event occurring mainly during synaptic transmission, which drives Ca^{2+} influx (Hojo et al., 2004).

LOCALIZATION OF STEROIDOGENIC SYSTEMS IN THE HYPOTHALAMUS

The hypothalamus plays an essential role in the HPG axis, which controls reproductive systems. The hypothalamus has been considered to be a main target of gonadal steroids because of its feedback function mediated by circulating steroids. The blood–brain barrier of the hypothalamus could be much looser/more leaky than that in other brain regions such as the hippocampus, allowing circulating steroids to penetrate into the hypothalamus. The role of hypothalamus in the reproduction and sexual dimorphism has been extensively investigated. One of the essential role of estrogen in reproductive signaling is that estrogen treatments stimulate the progesterone receptor synthesis in ovariectomized females (Bayliss and Millhorn, 1991; Chappell et al., 2000). During development, estrogen or androgen, aromatized to estrogen, is essential for neuronal organization and sexual differentiation of the brain, particularly in the hypothalamus (Kawata, 1995; Resko and Roselli, 1997).

Because no reports have demonstrated endogenous sex steroid synthesis in the adult rat hypothalamus, we investigated a possibility of local synthesis of estrogen and androgen in the hypothalamus. A significant expression of mRNAs for several essential steroidogenic enzymes was observed in the hypothalamus. The level of transcripts for P450(17α), P450arom, and 17β-HSDs (types 1 and 3) in the hypothalamus were approximately two- to threefold of those in the hippocampus (Hojo et al., 2004) (Table 1). A significant immunostaining for steroidogenic enzymes was also observed. Both StAR and

P450scc immunoreactive neurons were localized to the hypothalamus, including the preoptic area (King et al., 2003). We demonstrated significantly higher levels (two- to three-fold) of steroid synthesis such as testosterone and estradiol from ^{3}H-DHEA in the hypothalamus than those in the hippocampus, by means of HPLC analysis (Hojo and Kawato, unpublished results). Because endogenous steroid syntheses has also been observed in other regions of the brain such as cerebellum (Tsutsui et al., 2000), further investigations are encouraged in order to obtain a whole view of steroidogenesis in the mammalian brain.

ACTION OF ESTROGENS AND XENOESTROGENS (ENDOCRINE DISRUPTERS) ON SYNAPTIC PLASTICITY

Over decades, 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; MacLusky et al., 2005b; Woolley et al., 1997). In vitro investigations, using hippocampal slice cultures, have also demonstrated chronic modulation of spinogenesis by estradiol and xenoestrogens (Kretz et al., 2004; Murphy and Segal, 1996; Pozzo-Miller et al., 1999). The essential contribution of endogenous estradiol is reported by Rune and coworkers, who have demonstrated that the suppression of endogenous estradiol synthesis by letrozole has decreased the spine density of the CA1 region in cultured slices (Kretz et al., 2004).

Evidence is emerging, however, that estrogens exert a rapid influence on the excitability of adult rat hippocampal neurons, as demonstrated by means of electrophysiology (Foy et al., 1999; Gu and Moss, 1996; Ito et al., 1999; Shibuya et al., 2003; Teyler et al., 1980). In case of enhancement of long-term potentiation (LTP) by 1 to 10 nM estradiol in CA1 pyramidal neurons, an immediate increase by approximately 20% has been observed on the onset of estradiol perfusion in the initial slope of the excitatory postsynaptic potential (EPSP), which has been attendant on a further approximately 130% increase on high-frequency tetanic stimulation of Schaffer collaterals (Bi et al., 2000; Foy et al., 1999; Mukai et al., 2006). However, it should be noted that if we subtract the 20% immediate increase of EPSP slope on the onset of estradiol perfusion before the tetanic stimulation, the enhancement by estradiol is not significant about the pure tetanic stimulation-induced LTP. In other words, the magnitude of tetanic stimulation-induced LTP is nearly the same between in the presence and in the absence of estradiol. In contrast, we demonstrated a significant enhancement of the long-term depression (LTD) by 1 to 10 nM estradiol perfusion in rat hippocampal CA1, CA3, and dentate gyrus (DG) using multielectrode probes (MED64, Panasonic) (Mukai et al., 2006). By using the multielectrode system, stimulation of Schaffer collaterals in the CA1 (recording at CA1 pyramidal neurons), recurrent collateral fibers in the stratum radiatum of CA3 (recording at CA3 pyramidal neurons), and the medial perforant pathways in the molecular layer of DG (recording at DG granule cells) can be performed. In the LTD enhancement by estradiol, an immediate increase of EPSP by the onset of estradiol purfusion was not accompanied.

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 reported (Bi et al., 2000). These findings have led researchers to postulate the existence of so-called membrane or nongenomic estrogen effects. Kawato and coworkers recently demonstrated

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; Mukai et al., 2006; Tsurugizawa et al., 2005).

Recently, an issue of endocrine disrupters or xenoestrogens, which are artificial substances whose chemical structure resembles natural estrogen, has emerged as a social and environmental problem. Rapid action of xenoestrogens has, therefore, been investigated in the adult brain using electrophysiology. So far, high levels of xenoestrogens had often been observed to induce estrogenic/antiestrogenic effects in electrophysiological investigations. High levels of diethylstilbestrol (DES) at 3 to 10 μM inhibited the current evoked by 100 μM kainate in the hippocampal CA1 pyramidal neurons acutely dissociated from the mice (Ishibashi et al., 2000). The presence of a high-level DES at 10 μM induced an increase in the amplitude of the population spikes measured in the pyramidal layer without modifying the EPSP in mice hippocampal slices (SanMartin et al., 1999). In contrast, low levels of xenoestrogens also induced estrogenic/antiestrogenic effects (Kawato, 2004). The estradiol (10 nM)-induced enhancement of the LTP in CA1 on tetanic stimulation was considerably suppressed by the coperfusion with 100 nM bisphenol A (BPA), although the perfusion of BPA alone did not alter the LTP induction (Fig. 4) (Kawato, 2004). DES at 10 nM,

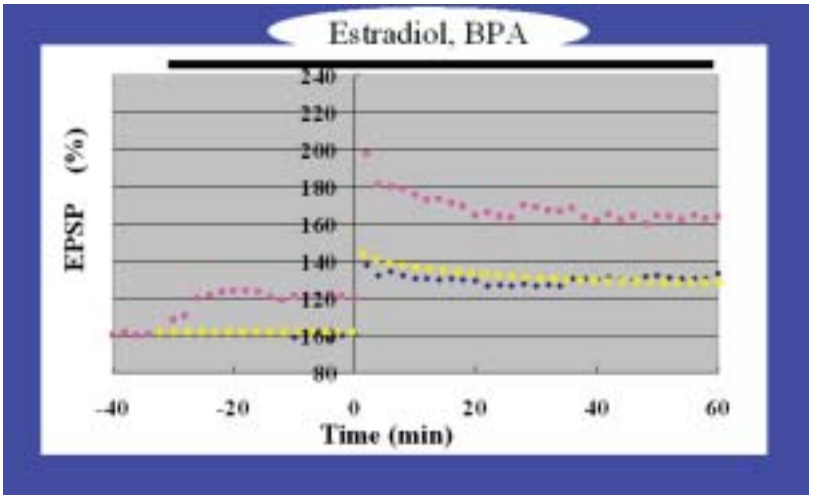

Figure 4 Rapid suppression by BPA of estradiol-induced enhancement of LTP in the hippocampal CA1 region. Preperfusion of 10 nM estradiol for 30 min at 30°C immediately increased the slope of the EPSP slope to approximately 120% (paired pulse facilitation value of 1.76 ± 0.05). On tetanic stimulation (100 Hz, 1 sec, 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, $n = 6$). The enhancement by estradiol is mainly due to an immediate 20% increase in EPSP slope at the onset of estradiol perfurion. When 100 nM BPA was copurfused 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 errors (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) using a glass microelectrode (3–5MΩ filled with ACSF) for recording, DIGIDATA 1320A and pClamp 9 for data analysis. (Taken from Mukai et al., 2006.)

however, enhanced the LTP by an almost identical magnitude to that obtained by 10 nM estradiol. We also observed that the effect of BPA, DES, nonylphenol (NP), and octylphenol (OP) was clearly detectable using the LTD analysis in the rat hippocampal CA1, CA3, and DG using multielectrode probes. LTD was induced pharmacologically by the transient application (3 min) of NMDA. The effect of endocrine disrupters on LTD was classified into two types, BPA/DES type and NP/OP type. BPA and DES induced the LTD enhancement in CA1 and CA3. NP and OP induced the LTD suppression in CA1 and the LTD enhancement in CA3 (Ogiue-Ikeda and Kawato, unpublished results).

Estrogens also significantly affect the density and morphology of dendritic spines. The density of dendritic spines in the CA1 pyramidal neurons is modulated in vivo by supplement of estrogens in ovariectomized animals (Gould et al., 1990; Leranth et al., 2000; Leranth et al., 2002; Woolley et al., 1990; Woolley and McEwen, 1992) and androgens in castrated animals (Leranth et al., 2003), both increasing/recovering the number of spines after several day's treatments. In vitro investigations have also shown that spine density is increased following several days of treatment using cultured hippocampal slices with estradiol (Murphy and Segal, 1996; Pozzo-Miller et al., 1999).

The rapid effect of estrogens and xenoestrogens has also been observed. Leranth, MacLusky, and coworkers have demonstrated that the estradiol-induced increase in the spine-synapse density was inhibited by the simultaneous application of BPA (40 μg/kg body weight) and estradiol (60 μg/kg) in ovariectomized rats for 30 min (MacLusky et al., 2005a). It has been also demonstrated that 300 μg/kg BPA alone suppressed the spine-synapse density in the CA1 region of the hippocampus in ovariectomized rats (MacLusky et al., 2005a). In CA3 pyramidal neurons, the total density of thorns of thorny excrescences (spine-like postsynaptic structures in CA3), having contacts with mossy fiber terminals originated from granule cells, decreased dramatically to approximately 70% on 2 h application of 1 nM estradiol (Tsurugizawa et al., 2005). 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.

Only a few additional investigations have been reported for xenoestrogen effects on synaptic plasticity. As an example, a low-dose BPA at 10 to 100 nM transiently increased the intracellular Ca^{2+} level of hippocampal neurons via activation of nongenomic pathway, including estrogen receptor (Tanabe et al., 2006). As a mechanism of these events, it was discussed that BPA might disturb the intracellular Ca^{2+} signaling system via binding to activation of membrane-associated ERα stradiol (Mukai et al., 2006; Tsurugizawa et al., 2005). However, the binding affinity of BPA to water soluble ERα has been reported to be much lower (approximately 1/2000) than that of 17β-estradiol (Kuiper et al., 1997). The ligand-binding affinity of BPA to ERα has been shown to be 1/100 to 1/1000 of that of 17β-estradiol (Morohoshi et al., 2005). These reports, however, do not conflict the reported low-dose effect of BPA at nanomolar level, if we take into account the significant concentration processes of BPA in the membrane and the presence of membrane-bound form of estrogen receptors.

Although it had not been clear whether endocrine disrupters could reach the brain via the blood circulation by crossing the blood–brain barriers, it has recently been indicated that BPA, injected into the mother's body (single subcutaneous injection), is transferred to the brains of both mother and fetus via the blood circulation within 60 min (Uchida et al., 2002). The time required for BPA to reach the brain is 0.5 to 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 would be 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 and stay in mammalian brains at concentrations sufficient to impact brain function and development.

HYPOTHETICAL MODEL OF SYNAPTOCRINOLOGTY

Based on experimental observations, we illustrate in Fig. 5, 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) (Guarneri et al., 1994) 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 is performed by P450(17α), 3β-HSD, 17β-HSD, P450arom, and 5α-reductase. 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 and DES also modulate the synaptic transmission via binding to ERα. Here,

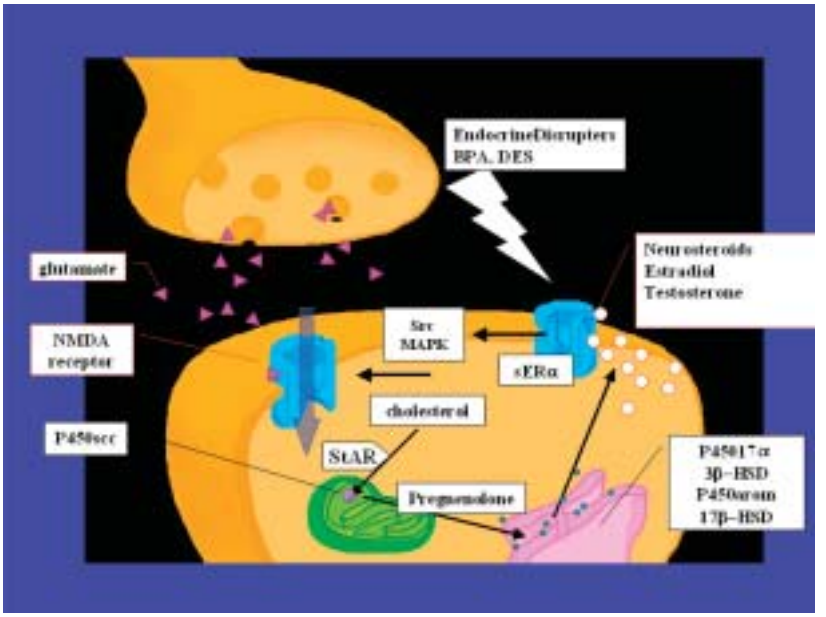

Figure 5 Schematic illustration for the synaptic synthesis of neurosteroids and the modulation of the synaptic transmission of neurons by neurosteroids. AMPA type of glutamate receptors are omitted for clarity. StAR, PBR, and P450scc are present in the mitochondria. P450(17α), 3β-HSD, 17β-HSD, and P450arom are localized in the membranes in the synaptic compartment. The site of action for estradiol, BPA, and DES is synaptic ERα. (Taken from Kawato, 2004.)

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, Tsurugizawa et al., 2005).

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