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Endocrine Disrupters as Disrupters of Brain Function: A Neurosteroid Viewpoint

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The mechanisms of neurosteroid synthesis in the rat hippocampus were investigated. Metabolism assay demonstrated the pathway of "cholesterol → pregnenolone → dehydroepiandrosterone → androstenedione → testosterone → estradiol." Upon exposure of pups to bisphenol A (BPA) from the embryonic stage until 3 week-old stage, a significant facilitation of the synthesis of estradiol was observed in the hippocampus. The localization of cytochrome P450s (P450scc, P45017α, and P450arom) as well as estrogen receptor alpha (ERα) was observed in pyramidal and granule neurons, using immunohistochemical staining. Furthermore, the synaptic localization of P45017α, P450arom and ERα was demonstrated with immuno-electron microscopic analysis. The acute action of estradiol and endocrine disrupters were then analyzed with an electrophysiological measurement of hippocampal pyramidal neurons. A 30 min preperfusion of diethylstylbesterol (DES) enhanced the induction of long-term potentiation (LTP) by almost an identical magnitude to that obtained by estradiol perfusion. On the other hand, although the application of BPA alone did not affect LTP-induction, the co-perfusion of BPA with estradiol completely suppressed the enhancement effect of LTP by estradiol. The current investigations demonstrate in the hippocampus (1) that locally synthesized estrogen rapidly enhances the synaptic plasticity of neurons, and (2) that BPA and DES modulate the synaptic plasticity as well as the synthesis of estradiol. The probable targets of BPA and DES are ERα and steroidogenic proteins.

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1. Research Strategy

Our research team investigates the disruptive actions of endocrine disrupters (EDs), as well as the action of estrogen on neurotransmission in the hippocampus, the center of learning and memory. In current neuroendocrinology, it is widely believed that steroid hormones are synthesized in the gonads and/or adrenal glands, and reach the brain via the blood circulation.[1] In contrast with this view, (A) we are in progress of demonstrating that neurosteroids (including sex steroids) are synthesized locally by P450s in the brain, and that these steroids act acutely/non-genomically (or chronically/genomically) to modulate neuronal plasticity, as 4th generation neuromessengers.[2,3] Because both the memory-related synaptic plasticity and the neonatal development of neuronal networks are significantly affected by the presence of neuroactive steroids,[1,3–5] it is probable that estrogen-like EDs (*e.g.,* bisphenol A (BPA), diethylstylbesterol (DES), etc.) have disruptive actions on these neuronal functions and properties. One of our primary concerns is the elucidation of these ED actions.

Our present work involves the search for estrogen receptors in the hippocampal neuronal synapses (i.e., "synaptic estrogen receptors"), that play a pivotal role in synaptic plasticity, learning and memory, with an eye towards establishing the biochemical basis for the observed acute ED actions on the synaptic plasticity.

Because other leading laboratories in the field have yet to report the discovery of even classical nuclear estrogen receptors in principal hippocampal neurons,[1] (B) we have also undertaken a vigorous effort in this regard, and have made some progress towards elucidation of the location of the synaptic estrogen receptors and estrogen receptor-dependent signal pathways which either acutely modulate the activity of an NMDA type glutamate receptor (a key factor in learning and memory), or chronically (within several days) increase the number of neuronal synapses (a storage area for short-term memory).[6] This machinery has provided a promising mechanism by which estrogens may play an important role in enhancing the efficiency of synaptic plasticity in the hippocampus. It is therefore quite reasonable to postulate that orally administered estrogen-like EDs might also disrupt synaptic transmission, when reaching neurons via the blood circulation and by crossing the blood-brain barrier. We have developed new techniques of capturing and analyzing the actions of EDs on synaptic estrogen receptor-dependent pathways at the molecular level. We study the action of EDs not only on adult brains (in which neuronal wiring development is completed), but also on fetal/neonatal brains (in which neuronal wiring is still under development).

2. Research Content

Endocrine disrupters can reach the brain via the blood circulation and by crossing the blood-brain barriers. Iguchi and coworkers revealed that BPA, injected into the mother's body (single s.c. injection), was transferred to the brains of both mother and fetus via the blood circulation, within 60 min.[7] The time required for EDs to reach the brain is not significantly different from that required to reach other peripheral organs. In contrast to the efficient detoxification of endocrine disrupters in the liver, detoxification in the brain is

expected to be less efficient, due to the extremely low level of drug-metabolizing enzymes (*e.g.*, cytochrome P450s) in the brain.[2] These findings suggest that endocrine disrupters reach mammalian brains (including human brains) at concentrations sufficient to impact brain function and development.

2.1 *Neurosteroid synthesis in the hippocampus and its disruption by endocrine disrupters (Figs. 1, 2 and 3)*

The metabolic mechanism, by which neurosteroidogenic systems produce estrogens and androgens in the hippocampus, was analyzed by administrating a radioactive steroid substrate to the hippocampal slices. After treatment, metabolic products were extracted using organic solvent and analyzed with HPLC (see Figs. 2A and 2B). Based on this analysis, we reported the existence of two types of steroid synthesis pathways: (1) "cholesterol → pregnenolone → DHEA (dehydroepiandrosterone) → androstenedione", and either (2a) " → testosterone → estradiol", or (2b)"→ estrone → estradiol" (Fig. 1).[2,3,8–10] In addition, it was found that while the synthesis step of "pregnenolone → DHEA" was catalyzed by P45017α, the synthesis steps of "testosterone → estradiol" and "androstenedione → estrone" were both catalyzed by P450 aromatase. This conclusion was confirmed

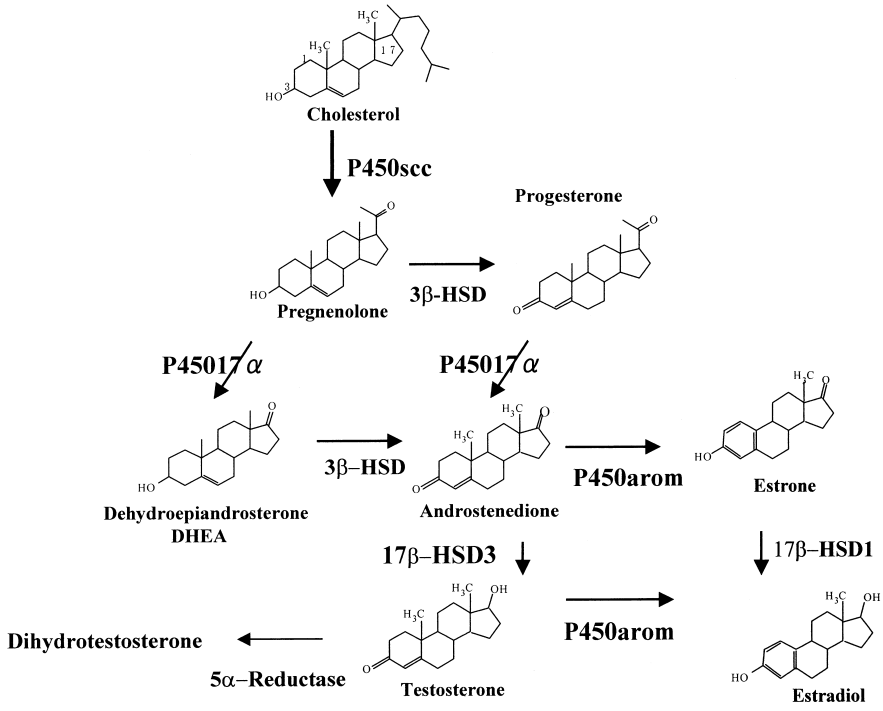
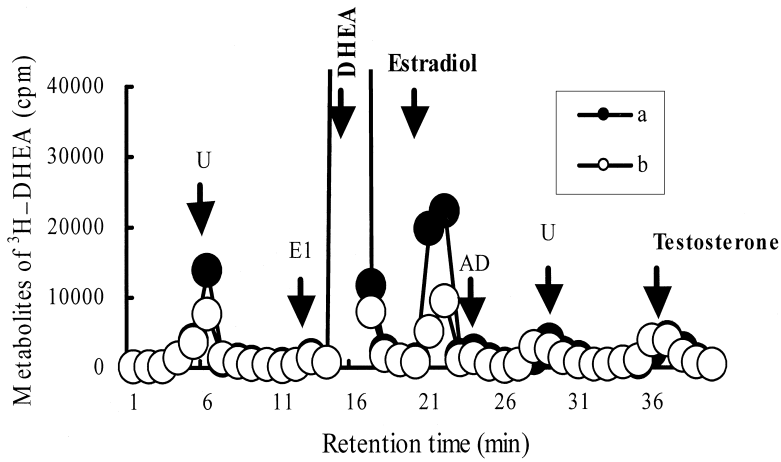


Fig. 1. Schematic illustration of neurosteroid synthesis in rat hippocampus. Structure of neurosteroids and name of enzymes required for the synthesis.

(A)



(B)

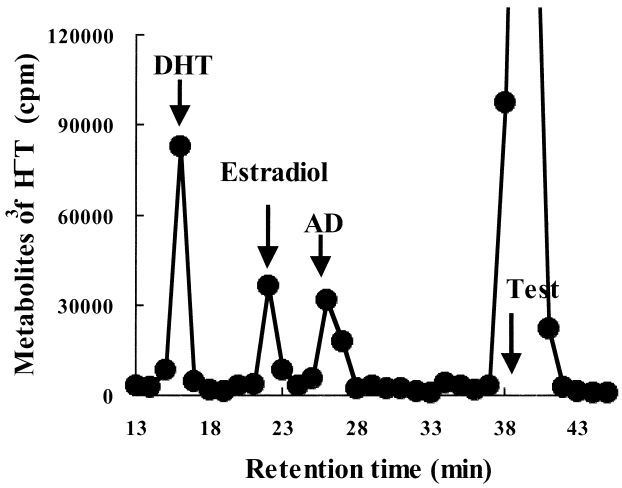


Fig. 2. HPLC analysis of steroid metabolism in adult rat hippocampal slices. Slices were incubated with tritiated precursor steroids for 5 h. Panel A: Profiles of ^{3}H-DHEA metabolites, in the absence of an inhibitor (curve a) or in the presence of fadrozole (curve b). Panel B: HPLC profiles of ^{3}H-testosterone metabolites using elution solvent. Slices were incubated for 5 h with ^{3}H-testosterone. The arrows designate the elution peak positions of the standard ^{14}C-steroids with abbreviations: DHEA, AD (androstenedione), E1 (estrone), Test (testosterone), and DHT (dihydrotestosterone). 'U' designates unknown metabolites. The vertical axis indicates ^{3}H radioactivity (cpm). (Taken from Ref. 10)

by an observed reduction in the production of either DHEA or estradiol, attendant upon application of either P45017α's or P450arom's inhibitor, prior to the initiation of steroid metabolism. Moreover, both "androstenedione → testosterone" and "estrone → estradiol" steps were found to be catalyzed by 17β-HSD (types 1 and 3).[10] There was almost no sexual

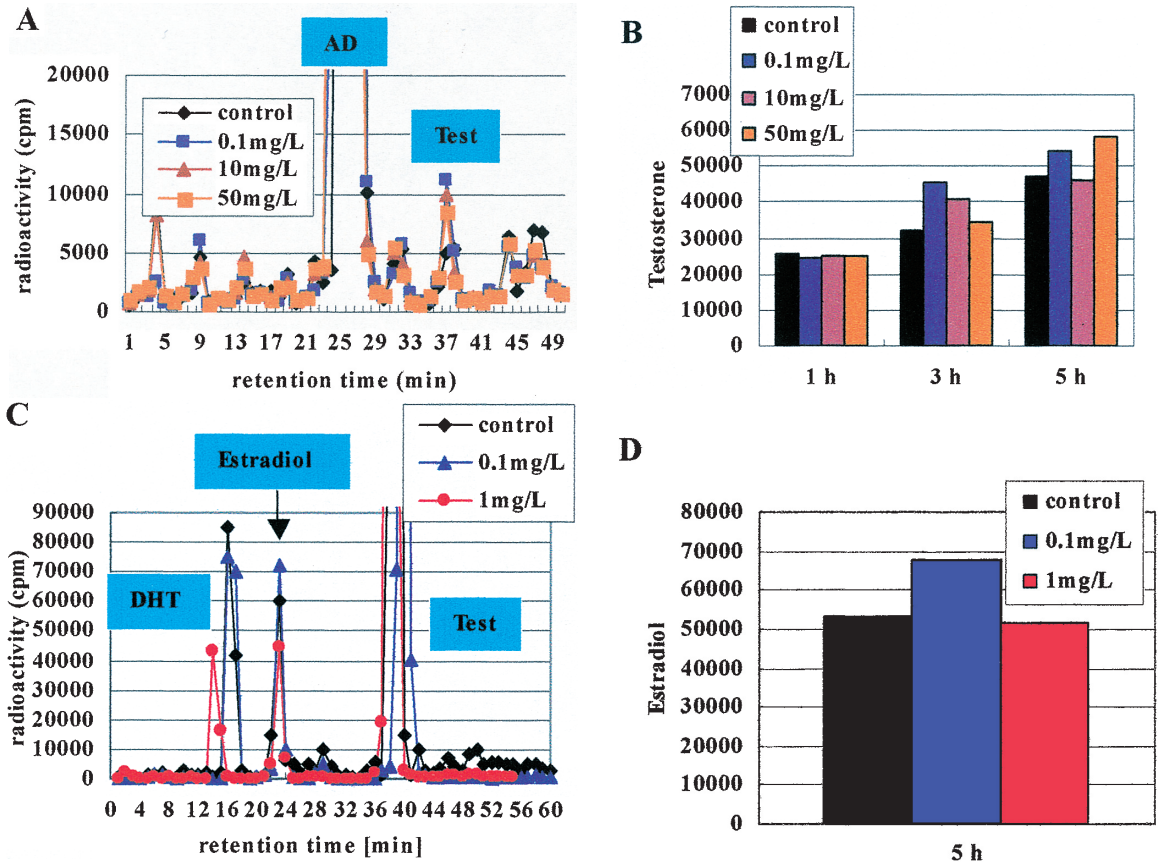
dimorphism in the synthesis of these sex steroids in the hippocampus, (i.e., such that the male also synthesizes estrogens, and the female also synthesizes androgens). The hippocampus, which is responsible for higher-order cognitive functions, may not have significant sexual dimorphism.

The ability of BPA to modulate the synthesis of testosterone and/or estradiol in the hippocampus was examined, in collaboration with Dr. Nakamura in Tsutsumi's CREST team. BPA was administrated to fetal rats (*in utero*) and newborn rats via their mother's oral route. Mother rats were fed water containing 0.1, 1, 10, and 50 mg/L BPA, beginning at the 11th day of pregnancy (day 0 = day of plug), until their pups had reached an age of 3 weeks. The metabolism of sex steroids in the hippocampus of the pups was then measured at age 4 weeks. Figure 3 illustrates the metabolites of ^{3}H-steroid substrates in HPLC analysis. The synthesis step of "androstenedione → testosterone" was facilitated most strongly by exposure to 0.1 mg/L BPA (the lowest concentration of BPA) (Fig. 3B). This concentration was also observed to have the most significant effect on the conversion, "testosterone → estradiol" (Fig. 3D).

2.2 *Neuronal localization of steroidogenic enzymes in the hippocampus*

The localization of neurosteroidogenic enzymes, the probable target of ED action, was also investigated. A significant localization of steroidogenic acute regulatory (StAR) protein and cytochromes (P450scc, P45017α, P450arom) was observed in pyramidal neurons of the CA1–CA3 and granule cells of the dentate gyrus (DG), using immunohistochemical staining with antibodies against the enzymes (Figs. 4 and 5).[2,3,8–10] No significant staining was observed in astroglial cells or oligodendroglial cells. Rigorous Western immunoblot analysis indicated the molecular weights of these enzymes to be nearly identical to those obtained from peripheral steroidogenic organs (adrenal cortex, testis, and ovary).[8–10] Most of the StAR belonged to the 37 kDa full-length type. Upon NMDA stimulation, StAR was truncated to a 30 kDa form, following an NMDA receptor-mediated Ca^{2+} influx.[8] We also succeeded in identifying the mRNAs of these enzymes using RT-PCR. Our RT-PCR analysis also demonstrated the existence of mRNAs for 17β-HSD (types 1 and 3) in the hippocampus.[10] These mRNAs were present in the hippocampus at levels approximately 1/300–1/1000 of those in peripheral steroidogenic organs (adrenal cortex, testis, and ovary), in qualitative agreement with results obtained via Western immunoblot analysis.

Post-embedding immunogold electron microscopy[11] was also employed, to determine the subcellular localization of these steroidogenic proteins in neurons, in collaboration with Morrison's group at Mt. Sinai School of Medicine. We demonstrated that P45017α and P450arom were localized in the synapses (axon terminals and spines) of pyramidal neurons in the CA1–CA3 regions and in granule neurons in the DG region, (Fig. 6).[10] This result clearly demonstrates sex steroids to be locally synthesized within neuronal synapses, the site of memory storage, and suggests that EDs probably act to modulate synaptic transmission. Previously, a number of studies had reported the absence of P45017α in the adult mammalian brain. Many attempts to demonstrate the immunohistochemical reactivity for P45017α in the rat brain had been unsuccessful.[12] RT-PCR had failed to detect mRNAs for P45017α in adult rat brain.[13] Moreover, a direct demonstration of the actual synthesis of


A
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50mg/L
Testosterone
1 h
3 h
5 h
C
Estradiol
DHT
Test
control
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Fig. 3.

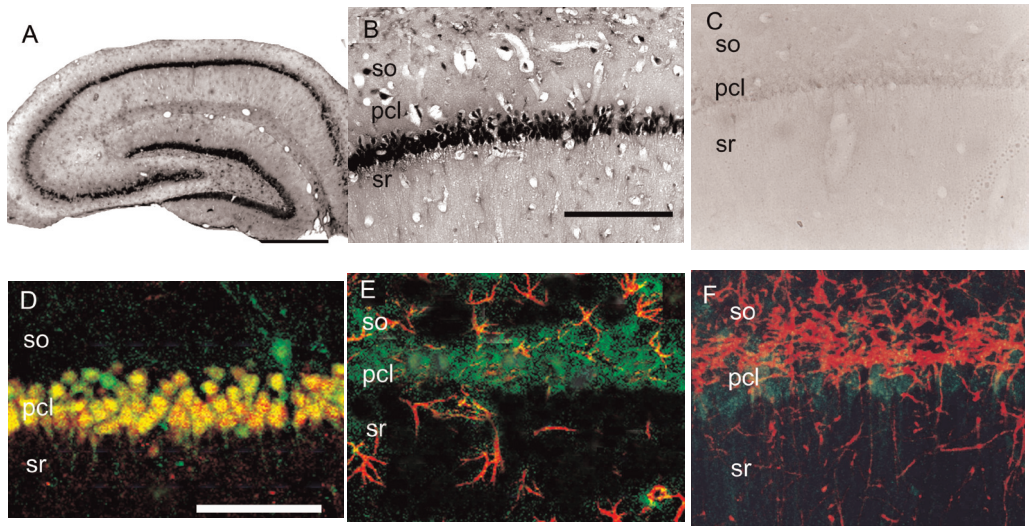
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Fig. 4.

Fig. 3. HPLC analysis of the effect of BPA exposure on sex steroid synthesis. Male fetal/newborn rats were exposed to BPA from embryonic 11th day to postnatal 21st day, via their mother's oral route. Sex steroid synthesis was measured at age 4 weeks. Panel A: HPLC profiles of ^{3}H-AD metabolites. Slices were incubated for 3 h. Panel B: Effect of BPA concentration on ^{3}H-testosterone production obtained from slices incubated for 1, 3 and 5 h with ^{3}H-AD. After 3 h incubation, the synthesis step of "androstenedione → testosterone" was facilitated most strongly by exposure to 0.1 mg/L BPA. Panel C: HPLC profiles of ^{3}H-testosterone metabolites. Panel D: Effect of BPA concentration on ^{3}H-estradiol production obtained from slices incubated for 5 h with ^{3}H-testosterone. BPA exposure at 0.1 mg/L was also observed to have the most significant effect on the conversion of "testosterone → estradiol." The vertical axis indicates ^{3}H radioactivity (cpm). AD (androstenedione), Test (testosterone), and DHT (dihydrotestosterone).

Fig. 4. Immunohistochemical staining of cytochrome P45017α in the hippocampal slices of an adult male rat. (A) the coronal section of the whole hippocampus; (B) the CA1 region; (C) the CA1 region stained with anti-P45017α IgG preadsorbed with purified P45017α; (D) fluorescence dual staining of P45017α (green) and neuronal nuclear antigen (red); (E) fluorescence dual staining of P45017α (green) and glial fibrillary acidic protein (GFAP; red); (F) fluorescence dual staining of P45017α (green) and myelin basic protein (MBP; red). In A–C, immunoreactive cells are visualized by means of diaminobenzidine-nickel. In D, E and F, the distribution of P45017α-positive cells is visualized using Oregon Green 488 fluorescence, and the distribution of neuronal and glial cell markers is visualized with Cy3 fluorescence. Superimposed regions of green and red fluorescence are represented by yellow. Abbreviation: so, stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. Scale bar: 800 μm for A, and 120 μm for B–F. (Taken from Ref. 10)

DHEA in adult mammalian brain has not previously been reported, although the presence of significant amounts of DHEA had been noted.[4] It has therefore been assumed that DHEA and the sex steroids such as estradiol are synthesized in the ovary or testis, and supplied to the brain via the blood circulation.[14] Our current observation of the local synthesis of sex steroids is therefore a breakthrough discovery.

2.3 *Localization of estrogen receptors in the neuronal synapses*

We have endeavored to determine the localization of estrogen receptors, the most probable target of EDs, in rat hippocampus. Although hippocampal neurons are known to be significantly susceptible to estrogens,[1,15] no laboratory has reported finding even the classical nuclear estrogen receptors (ERα) in hippocampal principal neurons. In contrast, the hypothalamus and amygdala, already known as neuroendocrinological brain regions, have been clearly shown to express ERα.[16] This difference in ERα expression has been considered to be one of the most significant differences between the brain regions responsible for higher-order cognitive function (*e.g.*, the hippocampus) and the neuroendocrinological brain regions (*e.g.*, the hypothalamus and amygdala, centers of "emotion and instinct"). As a result of our research, we recently found that this view has been based on several misunderstandings, resulting mainly from poor experimental methods adopted by the researchers. In particular, several famous antibodies against ERα (*e.g.*, MC-20), which have been used for the determination of ERα localization, are "antisera," which inevitably contain impure antibodies. The reactivity of these antisera was examined using Western immunoblotting and electron microscopy with immunogold labeling. Results indicated unexpected, nonspecific binding to unknown 63 kDa or 97 kDa proteins in brain regions such

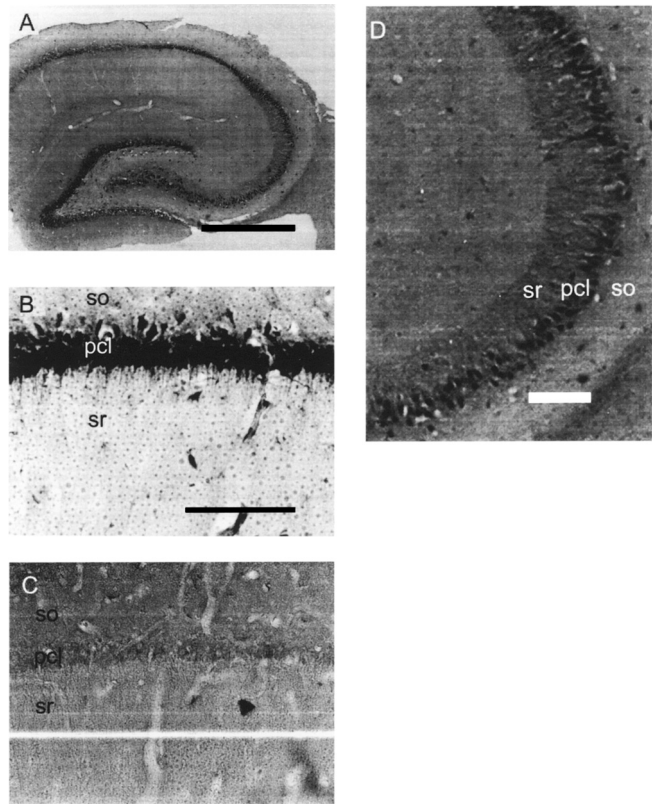

Fig. 5. Immunohistochemical staining of cytochrome P450arom in the hippocampal slices of an adult male rat. (A) The coronal section of the whole hippocampus. (B) The CA1 region; (C) The CA1 region, stained with anti-P450arom IgG preadsorbed with purified P450arom; (D) The CA3 region. Note staining of both cell bodies and dendrites of pyramidal neurons. Immunoreactive cells are visualized by means of diaminobenzidine-nickel. Abbreviaton: so, stratum oriens; pcl, pyramidal cell layer; sr, stratum radiatum. Scale bar: 800 μm for A, and 120 μm for B–D. (Taken from Ref. 10)

as the hippocampus and cortex, where ERα is very weakly expressed. In contrast, these antibodies are observed to bind significantly to 67 kDa ERα in the ovary and hypothalamus, where the ERα expression is very strong.

Many publications have therefore induced serious misunderstandings regarding the immunohistochemistry of hippocampal slices,[15,17] immunoelectron microscopy,[11] and the immunostaining of cultured hippocampal neurons.[18]

To overcome this difficulty, we employed an affinity-column purified antibody RC-19 against ERα prepared by Kominami and coworkers, resulting in the identification of true ERα protein.[19] This RC-19 reacted with the same 67 kDa ERα in both the synaptosomal (membrane) fractions and nuclear fractions of the hippocampus in Western immunoblotting. Principal neurons in CA1–CA3 and DG were stained with RC-19 in immunohistochemical analysis of the hippocampal slices. Glial cells reacted very weakly with RC-19.

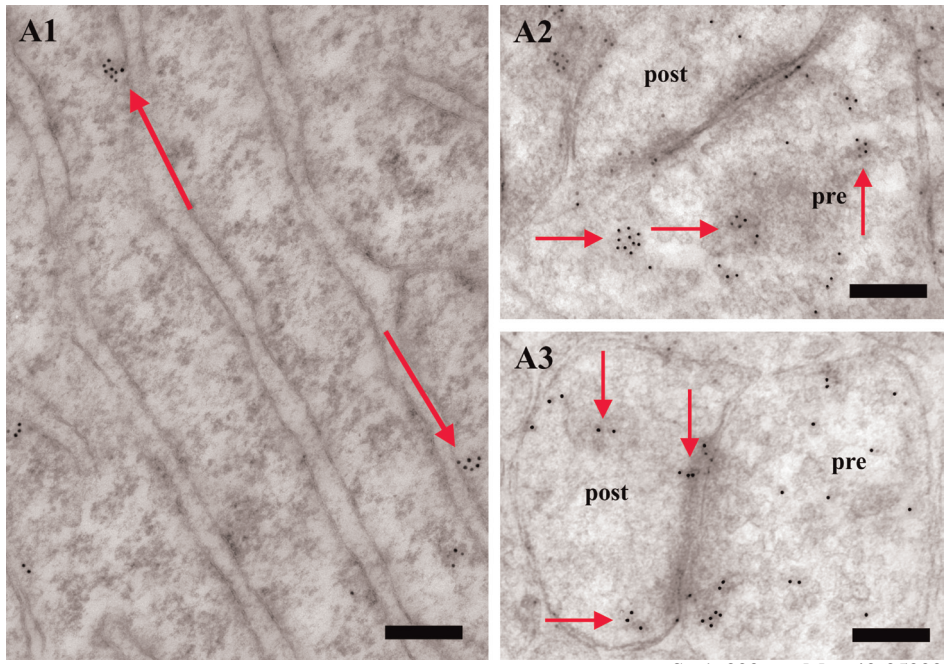

Fig. 6. Immunoelectron microscopic analysis of the distribution of P45017α (A1–A3) and P450arom (B1–B3) within axospinous synapses, in the stratum radiatum of the hippocampal CA1 region. Gold particles were observed to be localized in the endoplasmic reticulum (A1 and B1), the presynaptic region (A2 and B2), and the postsynaptic region (A3 and B3) of pyramidal neurons. In the axon terminal, 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). Abbreviation: Pre, presynaptic region; Post, postsynaptic region; Scale bar: 200 nm. (Taken from Ref. 10)

Postembedding immunogold electron microscopic analysis was applied to determine the subcellular localization of ERα. Results clearly showed that ERα immunoreactivity was localized in pre- and postsynapses as well as nuclei in hippocampal principal neurons. In several images, ERα was localized at the membrane structures of synapses, suggesting the existence of membrane ERα, as has been proposed for cells in peripheral organs.[20] We also demonstrated that mRNA for ERα was strongly expressed in the hippocampus (at least 1/10 of that expressed in the ovary). Rigorous RT-PCR analysis was performed to examine a possible existence of splice variants of ERα mRNA. Analysis demonstrated that no splice variant lacking any of exons 1–8 was expressed in the hippocampus. Rather, only a single full-length mRNA of classical ERα was expressed in the hippocampus.

Taken together, these results should be taken as significant progress towards understanding the actions of estradiol on hippocampal neurons, which have, to date, remained largely unrevealed. The clear identification of the existence of a synaptic estrogen receptor would aid in discussing the disruptive actions of EDs on neurotransmission and learning and memory at the molecular level. We show in the following sections that this synaptic ERα is the binding site of BPA and DES.

2.4 *Analysis of signal transduction in hippocampal neurons by Ca^{2+} imaging*

The acute actions of estradiol and BPA on hippocampal neurons were analyzed using Ca^{2+} imaging. In cultured rat hippocampal neurons, in which intracellular Ca^{2+} concentrations were optically measured using fluorescent dyes, both BPA and estradiol (at 10–100 nM) acutely (within 10 s) induced Ca^{2+} signals.[2,3,21] Ca^{2+} signals were abolished by the pre-application of ICI 182,780, an inhibitor of classical ERα. The rapid character of these Ca^{2+} responses indicates that they should be catalized by membrane ERα and non-genomic signal transduction, rather than by slow, genomic processes via nuclear ERα. That the same concentration range of estradiol and BPA (10–100 nM) was observed to induce the same type of acute Ca^{2+} signals indicates that BPA actions via membrane ERα are considerably stronger than BPA actions via nuclear ERα, since nuclear ERα-mediated BPA actions (appearing approximately at 10,000 nM) require a much higher concentration of estradiol than synaptic ERα-mediated estradiol actions (appearing approximately at 1–10 nM).[3,22,23] These results indicate that the action of BPA via membrane ERα in neurons is more disruptive and much stronger than that via nuclear ERα in the peripheral organs. An increasing number of results have indicated that membrane ERα and nuclear ERα may be the same protein. In particular, genetically expressed ERα was transported to the cell membranes as well as to nuclei.[20]

2.5 *Analysis of actions concerning learning-and-memory-related synaptic plasticity (long-term potentiation) in the hippocampus*

Electrophysiology is one of the most sensitive methods for detecting the acute effect of neurosteroids on synaptic transmission.[24] The acute actions of estradiol, BPA and DES were analyzed by measuring the long-term potentiation (LTP) induced upon tetanic stimulation of the CA1 pyramidal neurons in rat hippocampal slices. A 30 min pre-perfusion with 10 nM estradiol enhanced LTP in 4- and 12-week-old rats (Fig. 7). Application of 10 nM DES had an enhancing effect on LTP which was almost identical to that obtained by

estradiol. Both effects were completely suppressed by ICI 182,780, an ERα inhibitor. On the other hand, application of BPA alone, even at 100 nM, did not significantly affect LTP, although coapplication of 100 nM BPA and 10 nM estradiol considerably suppressed the estradiol-induced enhancement of LTP. These findings suggest that EDs have significant disrupting actions on learning and memory, and that these enhancing/suppressive effects are individual ED specific.

2.6 *Analysis of neuronal development in the cerebellum*

The cerebellar Purkinje neuron is an experimental system that is very appropriate for analyzing the impact of EDs on neuronal development via nuclear estrogen receptors, because the neuronal dendrites continue growing for 20 days after birth, as judged by the development of the neonatal brain.[25–27] The actions of estradiol, octylphenol, and BPA on the neuronal development of Purkinje neurons were analyzed in one-week-old rats. Estradiol and EDs were injected directly into the fluid surrounding the cerebellum. Several days later, extracted cerebellar slices were immunohistochemically stained with an antibody against calbindin, to analyze the dendritic growth of the Purkinje neurons. Application of either estradiol, octylphenol, or BPA increased both dendritic growth and the number of spines.[28] It is noteworthy that the BPA and octylphenol concentrations were required to be 100 times higher than estradiol concentrations, in order to facilitate the same level of dendritic growth. It should also be noted that all of the facilitating actions of EDs are blocked by tamoxifen, an inhibitor of ERβ which is a major estrogen receptor in the cerebellum.

2.7 *Summary*

In Fig. 8 we schematically illustrate the synaptic synthesis of neurosteroids, and the modulation of the synaptic plasticity of neurons by neurosteroids. Neurosteroid 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 to transport cholesterol into the mitochondria, where P450scc converts cholesterol to pregnenolone. After reaching the microsomes, the conversion, "pregnenolone → DHEA → androstenedione → testosterone → estradiol" is performed by P45017α, 3β-HSD, 17β-HSD and P450arom, respectively (see Fig. 1). The produced estradiol binds to synaptic ERα and may drive signal transfer via Src/MAPK, finally resulting in modulation of NMDA receptors. In this manner, estradiol modulates the synaptic transmission. EDs such as DES and BPA also modulate the synaptic transmission via binding to ERα. In Fig. 8, only the postsynaptic (spine-localized) synthesis/action is illustrated; however, the observation of P450s and ERα in the presynapses indicates that presynaptic synthesis/action also occurs for neurosteroids (see Fig. 6).

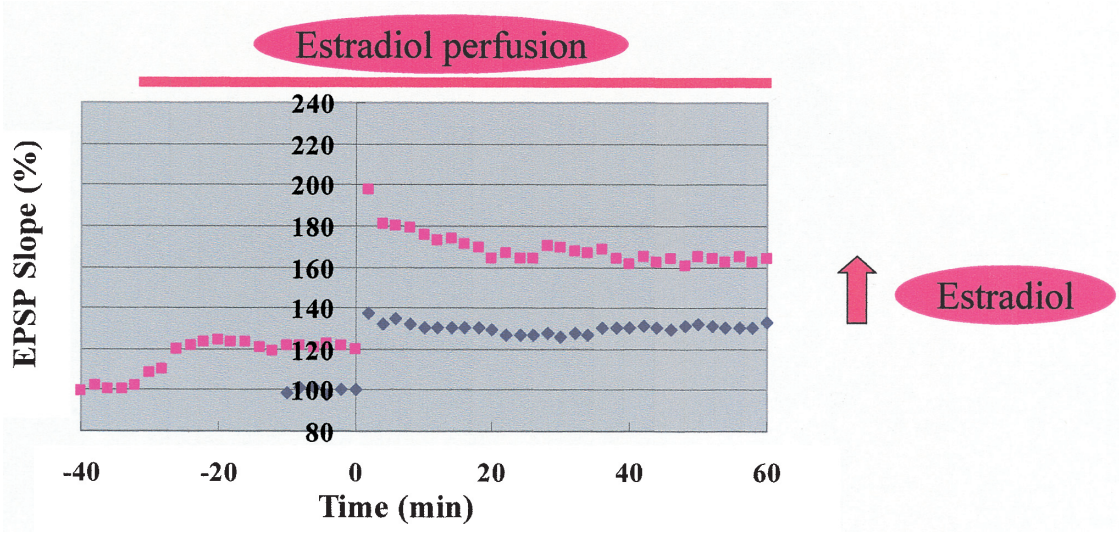

Fig. 7. Acute effect of estradiol on LTP in the hippocampal CA1 region. Upon tetanic stimulation (100 Hz, 1 s, at $t = 0$) of the Schaffer collaterals, the slope of the excitatory postsynaptic potential (EPSP) was enhanced (LTP-induction). This enhancement was 135% in control experiments (blue diamond) and 165% in the case of pre-perfusion with 10 nM estradiol (pink square) for 30 min at 30°C. Note that pre-perfusion with estradiol immediately increased the EPSP slope to 120%. Adult male Wistar rats aged 12 weeks were used.

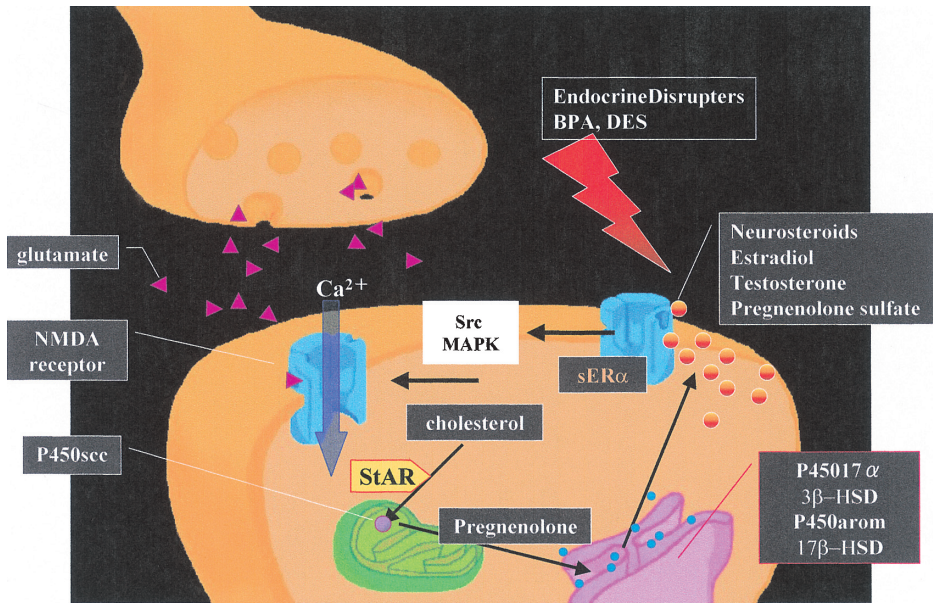

Fig. 8. Schematic illustration of the synaptic synthesis of neurosteroids, with their modulating actions on neuronal synaptic transmission. Note: AMPA type of glutamate receptors are omitted for clarity. StAR and P450scc are present in the mitochondria. P45017α, 3β-HSD, 17β-HSD and P450arom are localized in the membrans. The site of EDs action is at the synaptic ERα.

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