— **Minireview** —

Brain Disrupting Actions of Prenatal Diethylstilbestrol Exposure in Mice — Behavioral, Neurochemical and Electrophysiological Studies

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In addition to effects on the reproductive system, endocrine disruptors are thought to have brain-disrupting actions based on epidemiological and animal studies. In this review, we discuss the current state of studies on the brain-disrupting actions of diethylstilbestrol (DES), which is a prototype endocrine disruptor that has potent estrogen-like action, focusing on our own findings. The major findings concerning the brain-disrupting action of DES are as follows: Exposure to a minute amount of DES during the late stage of gestation, but not the late stage of the lactation period, caused neurobehavioral changes such as impairment of the passive avoidance response in mice offspring. There is a difference in effect between male and female offspring of DES exposure on dendritic arbolization of hippocampal pyramidal neurons. In 6-week-old male offspring, prenatal DES exposure did not affect the responsiveness of *N*-methyl-D-aspartate (NMDA)- or $GABA_A$-receptors in the hippocampal pyramidal neurons, but drastically increased the level of phosphorylated Ca^{2+}/calmodulin-dependent protein kinase II in the hippocampus. These findings suggest that, in mice, DES has brain-disrupting actions, with a critical period of exposure. Further studies are needed to clarify the primary site and mechanism of these actions, using various methods and animal models such as estrogen-receptor 'knock-out' and/or 'knock-in' mice.

Key words —— endocrine disruptor, diethylstilbestrol, estrogen receptor, Ca^{2+}/calmodulin-dependent kinase II, passive avoidance response, hippocampus

INTRODUCTION

Based on the results of epidemiological and animal studies, endocrine disruptors have received considerable attention for their disruptive effects not only on reproductive organs, but also on the developing brain. There is increasing evidence that sex hormones[1,2] and thyroid hormone[3,4] play important roles in the development of the brain, controlling gene expression *via* binding to their specific nuclear receptors. This suggests that perinatal exposure to endocrine disruptors affects brain development *via* effects on these hormone systems. Also, increasing evidence suggests that polychlorinated biphenyl (PCB) and its metabolites, which are organic-chloride compounds, exert brain-disrupting action *via* interaction with the thyroid hormone system.[5–7] In this review, we discuss the current state of studies on brain-disrupting actions of diethylstilbestrol (DES), which is a prototype endocrine disruptor with potent estrogen-like action, focusing on our own findings.

Our strategy for studies of DES involved the following considerations: It is very important to elucidate the primary action or critical period of brain-disrupting actions of environmental chemicals, since the brain actively develops during the perinatal period through the expression of many genes, and endocrine hormones are involved in this process. If environmental chemicals have primary action on the developing brain during the prenatal stage, the effect would appear later as behavioral changes such as deficits of emotional, reproductive and/or learning behaviors, and/or as changes in sensory func-

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tions. Elucidation of a critical period of brain-disrupting action, a primary site of action, and an endpoint of brain-disrupting action (*e.g.*, behavioral change), would facilitate studies on mechanisms of the brain-disrupting actions of endocrine disruptors. Elucidation of mechanisms of the brain-disrupting action of DES should contribute to the development of a method for the assessment of endocrine disruptors possessing brain-disrupting effects. Employing this strategy, we selected DES as the experimental agent because it is a prototype endocrine disruptor with potent estrogen-like action.

Effects on Behavior

It seems to be important to know how exposure to a minute amount of endocrine disruptor during the fetal and neonatal period influences various behaviors of offspring, when considering endpoints of brain-disrupting action of endocrine disruptors. We studied the effects of prenatal DES exposure on various behaviors of mice using an "open field" (OF) test, "fighting behavior" (FB) test and passive avoidance response (PAR) test. Pregnant mice (ddY strain) were purchased from SLC in Shizuoka, Japan. In one group, 0.1 μg/animal DES was given orally once a day from the 11th to 17th days of gestation. In another group, administration of the same dose of DES was continued until the 21st day of lactation. DES exposure had no influence on the number of offspring, male/female ratio of offspring, or body weight gain after birth.

In an OF test performed 1 month after birth, spontaneous ambulation for 2 min was significantly decreased in male offspring in both DES exposure groups, compared with corn oil-treated mice. No such effect was observed in female offspring. In an FB test performed using 2-month-old offspring exposed to DES during the prenatal and lactation periods, there was a difference in effects between male and female offspring: the number of fighting behaviors for 2 min significantly increased and decreased in DES-exposed male and female offspring, respectively, compared to the control mice.

Also, we performed a PAR test using the standard method. As shown in Fig. 1, prenatal exposure to a relatively low dose of DES caused impairment of PAR at 3 months after birth in both sexes of mice.[8,9] There were no effects on motor or sensory functions, as determined by interval of steps and a tail-flick test. Significant impairment of PAR was also observed 8 months after birth in male offspring with prenatal exposure to DES. A similar effect was found in female offspring exposed to DES. This suggests that impairment of higher brain functions such as PAR by prenatal exposure to DES may be long lasting, although a longer observation period is needed to determine whether the impairment is irreversible. No impairment of PAR was found in male offspring exposed to the same dose of DES from day 1 to 18 of the lactation period.[9] Furthermore, perinatal exposure to the same dose of DES from the 11th day of gestation to the 18th day of the lactation period caused impairment of PAR in female offspring, but not in male offspring. These results suggest that there may be a critical period for the brain-disrupting actions of DES, such as on PAR impairment. It also demonstrates gender-based differences.

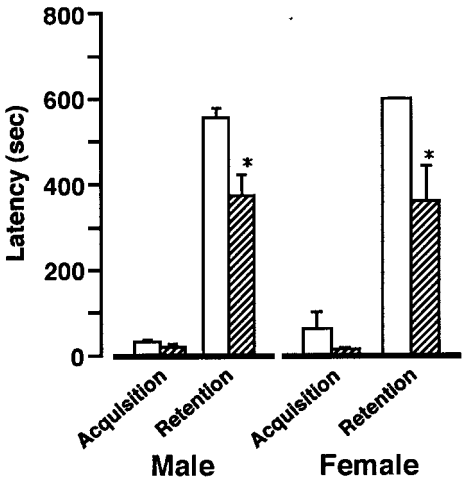

Fig. 1. Effect of Prenatal Exposure to a Low Dose of DES on Passive Avoidance in Mice[9]

DES at 0.1 μg/animal was given orally once a day on days 11 through 17 of gestation. Passive avoidance responses were tested at 3 months after birth. A retention test was carried out one day after the acquisition test. Open column: control, hatched column: DES-exposed. Data are expressed as the mean ± S.E.M. $*p < 0.05$.

DES has potent estrogen-like action. If the brain-disrupting actions of DES are produced *via* activation of an estrogen receptor (ER), it is important to know when and where ERα and ERβ are expressed in the developing brain. Auto-radiography using [^{3}H] moxestrol has shown that the number of ERs in the brain is drastically increased during the period from the later days of pregnancy to immediately after birth.[10] A recent study using *in situ* hybridization found that ERα and ERβ mRNA is expressed in the brain at embryonic day (ED) 16.5 and ED 10.5, respectively,[11] although regional distribution in the brain was not determined. We confirmed this finding using *in situ* hybridization,[12] determined regional

distribution and observed developmental changes in the levels of ERα and ERβ mRNA in the brain.[12] ERβ mRNA was strongly expressed in the medial amygdala, paraventricular nucleus of the hypothalamus and the bed nucleus of the stria terminalis from ED 15 to postnatal day 21 in mice. The magnitude of the ERβ mRNA signal was greater than that of the ERα mRNA signal for the above period. In the hippocampus and cerebral cortex of ED 15 mice, both ERα and ERβ mRNA signals were detected. These results indicate that ERα and ERβ mRNA is expressed in the later period of gestation, during which we exposed pregnant mice to DES. Although little information is available about developmental changes in expression levels of ERα and ERβ mRNA in the brain, we have preliminarily found that ERα and ERβ mRNA is stably expressed in the hippocampus and cerebral cortex after birth, as indicated by results of reverse transcription-polymerase chain reaction (RT-PCR). Given these findings, together with the above-mentioned finding that impairment of PAR did not occur in offspring exposed to DES during the lactation period, we should be careful when concluding that the brain-disrupting action of DES such as PAR impairment may be produced *via* changes in the ER system. A recent study using a micro-array showed that there is not always coincidence between DES and estradiol in the expression pattern of genes stimulated.[13]

Effect on Dendritic Arborization

Impairment of PAR indicates deficits of higher brain functions. ERs are involved in the formation of synapses and the control of synaptic functions. Several studies have shown that estrogen has growth- or neurite-promoting properties for its target neurons within the developing brain.[14] This action of estrogen is not seen in the normal adult female brain, but it has been observed under conditions of estrogen deprivation such as ovariectomy.[14] These findings suggest that the morphology of brain neurons may be changed when the estrogen system is disrupted by endocrine disruptors such as DES, which has estrogen-like activity, although estradiol treatment had little effect on the total length and arborization of dendrites of brain neurons. However, little is known as to whether perinatal exposure to endocrine disruptors with estrogen-like action (such as DES) causes changes in the morphology of neurons, although it has been reported that decreased arborization of Purkinje cell dendrites was produced by perinatal hypothyroidism,[6] which may be mimicked by perinatal exposure to PCB.

Accordingly, we determined the effects of perinatal DES exposure on dendritic arborization of cerebral cortical and hippocampal pyramidal neurons in mice. *Sholl* ring analysis of Golgi-Cox staining preparations showed differences in the degree of dendritic arborization of cortical and hippocampal pyramidal neurons between male and female mice exposed to DES perinatally. Among male offspring, the degree of dendritic arborization in the cortex and hippocampus was significantly greater in DES-exposed mice than in controls. Specifically, in hippocampal pyramidal neurons, the degree of arborization increased in dendrites 50 to 120 μm from the soma. In cortical pyramidal neurons, arborization increased in dendrites 140 to 180 μm from the soma. Surprisingly, the reverse was true in female DES-exposed mice: in hippocampal pyramidal neurons, the degree of arborization was significantly decreased in dendrites 20 to 200 μm from the soma; in cortical neurons, arborization was significantly decreased in dendrites 20 to 180 μm from the soma. These sex-related differences in effects of DES on dendritic arborization in the hippocampus and cortex suggest that DES-induced PAR impairment may be due to mechanisms other than the effects of DES on dendritic arborization, because PAR was impaired equally in male and female offspring exposed to DES. In previous studies, perinatal exposure to endocrine disruptors changed nuclear volume in the sexual dimorphic nucleus of the preoptic area[15] and locus ceruleus,[16] but perinatal DES exposure had little influence on the number of hippocampal pyramidal neurons. Dendritic spines appear and disappear under physiological conditions; *e.g.*, the density of dendritic spines fluctuates even during the estrus cycle of 4 to 5 days.[17,18] It has been reported that the density of dendritic spines in hippocampal CA1 pyramidal neurons decreases after ovariectomy.[19] This decreased level is recovered by treatment with estradiol.[20] In a preliminary study using a confocal microscope, we found no significant difference in the number of dendritic spines of hippocampal pyramidal neurons between control and DES-exposed mice.

Effects on Responsiveness of NMDA and $GABA_A$ Receptors

Electrophysiological examination has shown that the dendritic spines newly formed after estradiol treatment have synaptic function. Estradiol treatment caused persistent excitatory postsynaptic potentials

in hippocampal CA1 pyramidal neurons, and increased the frequency of repetitive neuronal firing caused by afferent stimulation.[21,22] It has been reported that *N*-methyl-D-aspartate (NMDA) receptors may be involved in these changes in hippocampal neurons.[23] Estradiol increases the affinity of agonists to NMDA receptors[23] and the number of NMDA receptors in the CA1 region of the hippocampus.[24] NMDA and $GABA_A$ receptors are involved in higher brain functions. Therefore, we examined whether the responsiveness of NMDA and $GABA_A$ receptors in hippocampal pyramidal neurons is altered by prenatal DES exposure in mice. Using the patch-clamp technique, we found no significant differences between control and DES-exposed mice in concentration-response curves of NMDA- and GABA-induced currents, nor in the inhibitory ratio of NMDA-induced currents due to Mg^{2+} block, or potentiation of GABA-induced currents by diazepam and zopiclon.[25] These results suggest that exposure to a minute amount of DES during fetal life does not affect the responsiveness of NMDA or $GABA_A$ receptors in the hippocampal pyramidal neurons of mice.

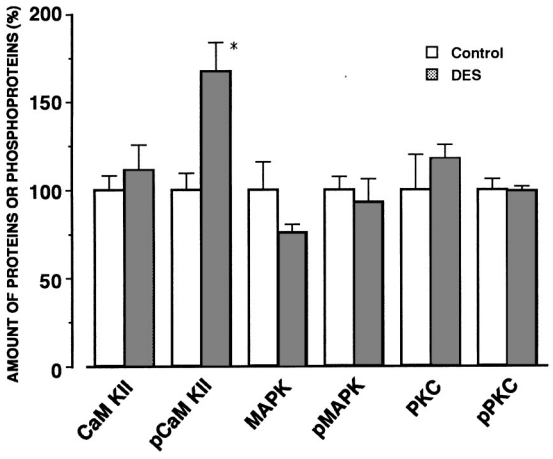

Fig. 2. Effect of Prenatal Exposure to DES on Levels of Substances Involved in Synaptic Functions in the Hippocampus of Male Mice[9]

DES at 0.1 μg/animal was orally given once a day on days 11 through 17 of gestation. The animals exposed to DES were sacrificed at the age of 6 weeks. Levels of each substance are expressed as percentages of the control. Data were expressed as the mean ± S.E.M. of 4–6 animals. $*p < 0.05$. CaM KII: Ca^{2+}/calmodulin-dependent protein kinase II, pCaM KII: phosphorylated CaM KII, MAPK: mitogen activated protein kinase, pMAPK: phosphorylated MAPK, PKC: protein kinase C, pPKC: phosphorylated PKC.

Effects on Ca^{2+}/Calmodulin-Dependent Kinase II

It is important to note whether cellular signal transduction in the synapse is changed, when we study mechanisms of deficit in higher brain functions such as memory and learning. Many substances, including Ca^{2+}/calmodulin-dependent protein (CaM) kinase II, are known to be involved in such transduction systems. Therefore, we determined the effect of prenatal DES exposure on levels and activities of CaM kinase II and related proteins in the hippocampus and cerebral cortex of mice. The protocol of DES treatment was the same as that used in our behavioral study. For immunoblot analysis, Triton X-100-soluble fractions of hippocampi from 6- to 9-week-old mice exposed to DES were compared with those of the controls. In the male hippocampus, DES exposure did not affect the total amount of CaM kinase II, but increased levels of autophosphorylated CaM kinase II[9,26] (Fig. 2). These changes were not found in female offspring exposed to DES. Similar results were observed in the cerebral cortex, although the level of autophosphorylated CaM kinase II β isoform did not increase.

CaM kinase II is involved in synaptic functions.[27] An increase in the level of phosphorylated CaM kinase II facilitates synaptic transmission, and a decrease inhibits it.[27–29] Therefore, it is surprising that prenatal DES exposure, which causes impairment of PAR (a learning and memory behavior), increased the level of phosphorylated CaM kinase II in the hippocampus and cerebral cortex, which are involved in higher brain functions such as memory and learning. However, a recent study using transgenic mice suggested that an increase in the level of phosphorylated CaM kinase II in the hippocampus is not always linked to the expression of long term potentiation[30] and acquisition of learning behaviors.

To verify the correlation between PAR performance and the level of active CaM kinase II in the hippocampus, we performed an experiment in which DES-exposed offspring were divided into an 'impaired PAR group' and an 'unimpaired PAR group.' We found that the level of phosphorylated CaM kinase II in the hippocampus was significantly higher in the 'impaired PAR group' than in the 'unimpaired PAR group.' These results suggest a link between the increased level of active CaM kinase II in the hippocampus and impairment of PAR observed in male offspring prenatally exposed to a minute amount of DES.

Concluding Remarks

Although we focused on our own study of brain disrupting actions of DES in this review, it has been determined, based on recent advances in endocrine

disruptor research, that several endocrine disruptors such as bisphenol A and PCBs may have brain disrupting actions, although it is unknown whether levels in the environment of such endocrine disruptors cause brain disrupting actions in wild animals and humans. Reports suggest that there may be a critical period of exposure for this action. However, the mechanism of this action is unclear. Prenatal exposure to DES caused brain disruption, as indicated by PAR impairment and a marked increase in phosphorylated CaM kinase II in the hippocampus. Although DES has potent estrogen-like action, and ERα/β mRNA signals are expressed during the late period of gestation in mice, it would be premature to conclude that the brain-disrupting actions of DES are caused by changes in ER systems. Clarification of the primary site and mechanisms of the brain-disrupting actions of endocrine disruptors, including DES, requires further studies using various methods and animal models such as 'knock-out' and 'knock-in' mice of various receptors, including ER. In addition, it is important to elucidate the physiological roles of ER in each stage of brain development. Brain development involves the expression of thousands of genes, finely tuned by many hormones and neurotransmitters. Consequently, the brain is particularly vulnerable to chemical disruption. Therefore, it is also important to identify physiological substances and/or genes that are affected by endocrine disruptors in the developing brain.

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