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RESEARCH ARTICLE

Functional changes in dopamine D3 receptors by prenatal and neonatal exposure to an endocrine disruptor bisphenol-A in mice

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Abstract

Bisphenol-A (BPA), one of the most common environmental endocrine disrupters, has been evaluated extensively for toxicity and carcinogenicity. However, little is still known about its action on the central nervous system (CNS). In the previous study, we found that prenatal and neonatal exposure to BPA markedly enhanced the rewarding effect induced by morphine. Here we found that prenatal and neonatal exposure to BPA resulted in the attenuation of dopamine D3 receptor-mediated G-protein activation by 7-OH-DPAT in the mouse limbic forebrain. This treatment also caused a significant decrease in the B_{max} value of [^{3}H]PD128907, a dopamine D3 receptor ligand, in this area. Under these conditions, no change in dopamine D3 receptor mRNA expression in the limbic forebrain and lower midbrain was observed by prenatal and neonatal exposure to BPA. The present data provide further evidence that prenatal and neonatal exposure to BPA leads to the reduction of functional dopamine D3 receptors without affecting the new synthesis of dopamine D3 receptors in the mouse limbic forebrain.

Introduction

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. It has been reported that administration of bisphenol-A (BPA) to pregnant mice on gestation days at a dose that is within the range typical of the environmental exposure of human produces significant changes in the postnatal growth rate and brings on early puberty in these mice.[1] These results imply that BPA may cause the unpleasant toxicity in the developmental process. In contrast, little is known about the neuronal toxicity induced by BPA in the central nervous system (CNS). More recently, we have reported that prenatal and neonatal exposure to BPA enhances the methamphetamine-induced rewarding effect and central dopamine D1 receptor function in mice.[2]

The dopamine D3 receptor cloned by Sokoloff and colleagues has been characterized extensively.[3] The dopamine D3 receptor shows a distinct distribution in limbic areas of the brain, including the nucleus accumbens and olfactory

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tubercle.[3] Several pharmacological studies with dopamine D3 receptor-preferring agonists such as 7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OH-DPAT) suggest that the dopamine D3 receptor regulates the inhibitory effect to produce hyperlocomotion in rodents.[4,5] In addition, we found that the morphine-induced rewarding effect and hyperlocomotion were markedly enhanced in mice lacking dopamine D3 receptor gene.[6] These findings suggest that the dopamine D3 receptor plays a critical role in the psychoeffective functions of dopamine neurotransmission.

The present study was then designed to investigate the changes in the function of dopamine D3 receptors by prenatal and neonatal exposure to BPA in mice.

Materials and method

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. All efforts were made to minimize the number of animals used and their suffering.

Animals

All experiments were performed using male ddY mice (8–12 weeks old) that had been exposed prenatally and neonatally to BPA. Prenatal and neonatal BPA exposure was conducted as described previously.[2] Adult female mice were chronically treated with BPA-admixed powder food containing 0 (B0) and 2 (B2) mg BPA/g of food from mating to weaning. Their pups were prenatally (about 3 weeks) and neonatally (about 2 weeks) exposed to the respective concentration of BPA from their mothers. During the treatment with BPA, animals did not show either weight loss or disrupted maternal behaviours.

Membrane preparations

In the membrane preparation, mice were killed by decapitation and the limbic forebrain was then dissected as described previously.[7] The limbic forebrain was rapidly excised at 4°C, and the tissues were homogenized using a Potter-Elvehjem tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris-Mg^{2+} buffer containing 50 mM Tris-Hcl (pH 7.4), 5 mM $MgCl_2$ and 1 mM EGTA for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 4°C for 10 min at 48,000 g. The pellet was resuspended in ice-cold Tris buffer *or* [^{35}S]GTPγS binding assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4°C for 10 min at 48,000 g. The resultant pellet was resuspended in ice-cold Tris buffer or [^{35}S]GTPγS binding assay buffer and stored at −70°C until used.

[^{35}S]GTPγS binding assay

The membrane homogenate (3–8 μg protein/assay) was incubated at 25°C for 2 h in 1 ml of assay buffer with 10 μM 7-OH-DPAT or a dopamine D_2 receptor agonist N-propylnorapomorphine (NPA), 30 μM guanosine-5′-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$ at 4°C for 2 h. Filters were then washed three times with 5 ml of an ice-cold Tris-HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 ml of Soluene-350 and 4 ml of Hionic Fluor, equilibrated for 12 h and the radioactivity in the samples was determined with a liquid scintillation analyser. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from at least three independent sets of experiments.

Dopamine D_3 receptor binding assay

The dopamine D_3 receptor binding assays were carried out in duplicate with [^{3}H] PD128907 (specific activity, 111 Ci/mmol; Amersham, Arlington Heights, IL) at 0.039 to 5 nM in a final volume of 1.0 ml which contained 50 mM Tris-HCl buffer (pH 7.4), 100 μM guanosine-5′-[β,γ-imido]triphosphate (Gpp(NH)p) and 0.1 ml) of the homogenated membrane fraction. The amount of membrane proteins used in each assay was in the range of 90 to 140 μg, as determined by the method of Bradford.[8] The test tubes were incubated for 2 h at 25°C. The specific binding was defined as the difference in bindings observed in the absence and

presence of 10 μM unlabeled PD128907. The incubation was terminated by collecting the membranes on Whatman GF/B filters using a Brandel cell harvester. The filters were then washed three times with 5 ml Tris-HCl buffer (pH 7.4) at 4°C and transferred to scintillation vials. Then, 0.5 ml of Soluene-350 (Packard Instrument Company, Inc, Meriden, CT) and 4 ml of Hionic Fluor Cocktail (Packard Instrument Company) were added to the vials. After a 12 h equilibration period, the radioactivity in the samples was determined in liquid scintillation analyzer.

RT-PCR assay

Total RNA in the limbic forebrain and lower midbrain were extracted using SV Total RNA Isolation System (Promega, Madison, WI). To prepare first strand cDNA, 1 μg of RNA was incubated in 100 μl of buffer containing 10 mM DTT, 2.5 mM $MgCl_2$, dNTP mix, 200 U of reverse transcriptase II (Gibco-BRL, Grand Island, NY) and 0.1 mM oligo $(dt)_{12-18}$ (Gibco-BRL). The dopamine D_3 receptor was amplified in a 50 μl PCR solution containing 0.8 mM $MgCl_2$, dNTP mix and DNA polymerase with synthesized primers: a sense primer of dopamine D3 receptor, which is at position 391–407 (5′-GCA GTG GTC ATG CCA GTT CAC TAT CAG-3′) of the receptor, and an antisense primer at position 498 to 526 (5′-CCT GTT GTG TTG AAA CCA AAG AGG AGA GG-3′), which were designed according to sequence Accession nos U26915 in GenBankTM.

Samples were heated to 94°C for 2 min, 55°C for 2 min and 72°C for 3 min, and cycled 35 times through 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. The final incubation was 72°C for 7 min. The mixture was run on 1% agarose gel electrophoresis with the indicated markers and primers of the internal standard GADPH. Three independent experiments were performed in this study. Semi-quantitation of the intensity of the bands for dopamine D_3 receptor/GADPH was conducted by using a NIH image.

Results

The attenuation of dopamine D3 receptor-mediated G-protein activation in mice prenatally and neonatally exposed to BPA

The dopamine D3 receptor agonist 7-OH-DPAT (0.001-10 μm) produced a concentration-dependent increase in $[^{35}S]GTP\gamma S$ binding to membranes obtained from the limbic forebrain including the nucleus accumbens of B0 mice.

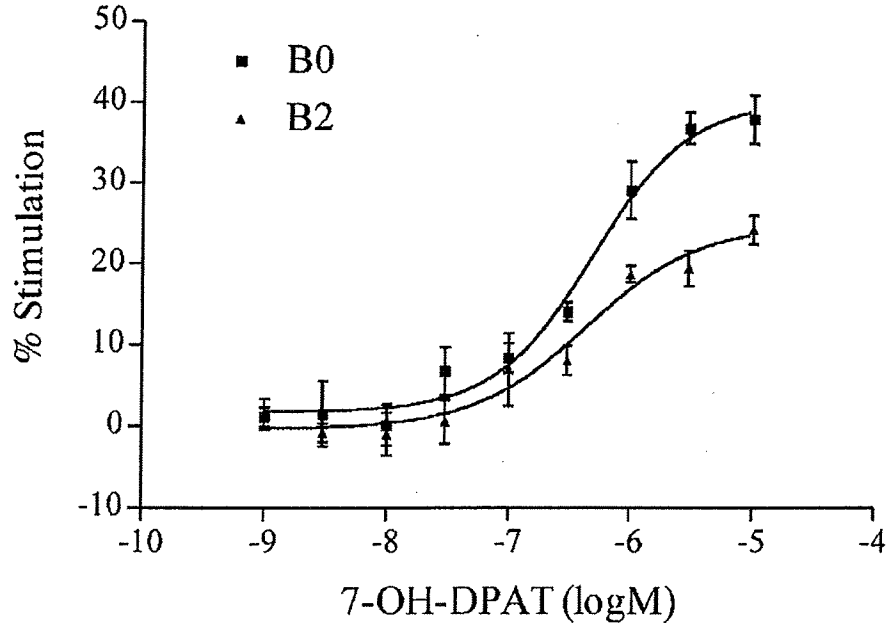

Figure 1. *Comparison of the stimulation of $[^{35}S]GTP\gamma S$ binding by dopamine D_3 receptor agonist 7-OH-DPAT to membranes of the limbic forebrain obtained from control (B0: square) and BPA-treated (B2: triangle) mice. Membranes were incubated with $[^{35}]GTP\gamma S$ (50 pM) and GDP (30 μM) with 7-OH-DPAT (10^{-9}–10^{-5} M). The values are expressed as percentage increase of the value in B0 mice. Data are expressed as the mean ± SEM of three independent samples.*

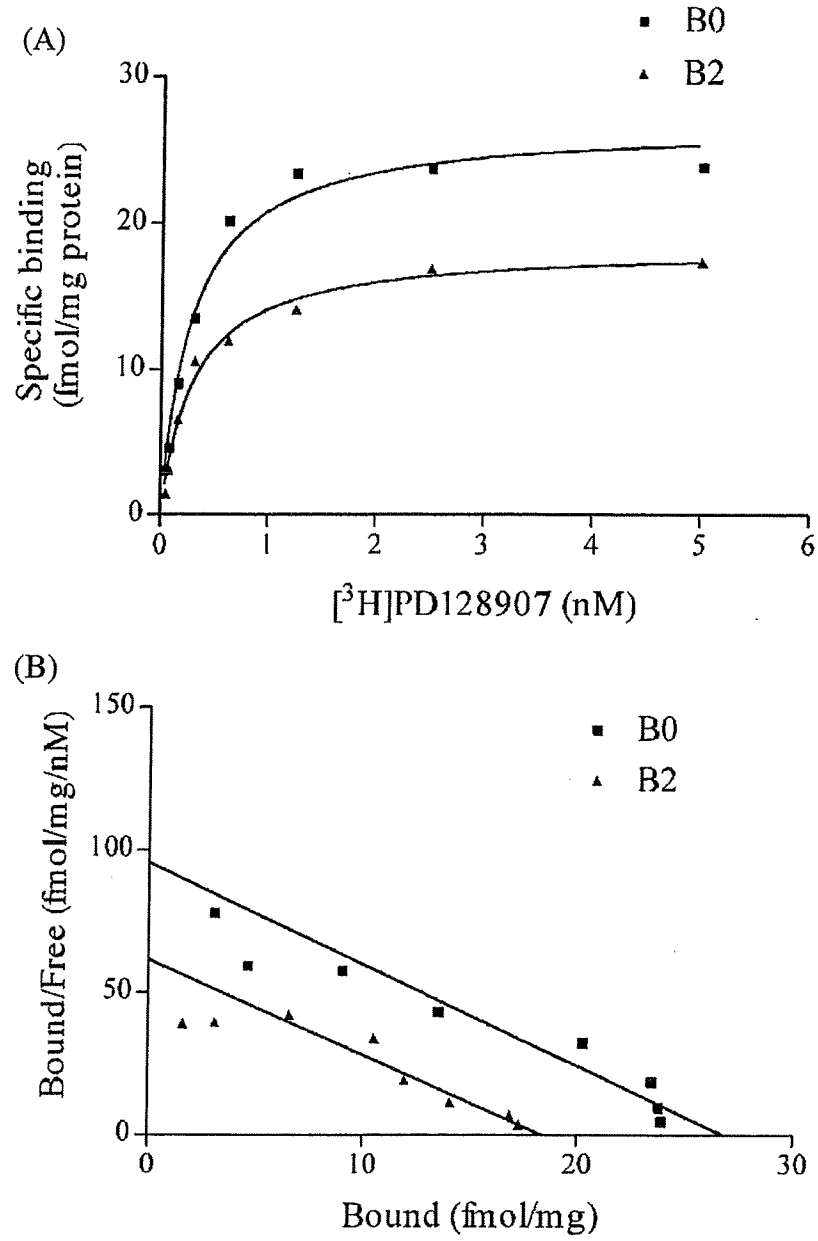

Figure 2. *Decrease of dopamine D3 receptor density in the limbic forebrain obtained from mice prenatally and neonatally exposed to BPA. Saturation binding (A) and Scatchard analysis (B) were performed by receptor binding assay using [^{3}H]PD128907. Membranes were incubated with [^{3}H]PD128907 (0.039–5.0 nM) and Gpp(NH)p (100 μm).*

The key finding of the present study was that the stimulation of [^{35}S]GTPγS binding induced by 7-OH-DPAT was attenuated markedly in B2 mice ($F_{1,\ 174} = 30.45$, $p < 0.001$; Fig. 1). Under these conditions, the G-protein activation induced by the selective dopamine D_2 receptor agonist N-propylnorapomorphine in the limbic forebrain was not affected by prenatal and neonatal exposure to BPA (only 3% increase; figure not shown).

Decrease of dopamine D3 receptor density in the limbic forebrain obtained from mice prenatally and neonatally exposed to BPA

To evaluate the population of dopamine D3 receptors in the mouse brain, we performed the saturation-binding analysis using [^{3}H] PD128907. The saturation-binding curve and Scatchard analysis of [^{3}H]PD128907 are shown in Fig. 2. The B_{max} and apparent K_d value are shown in Table 1. We found that prenatal and

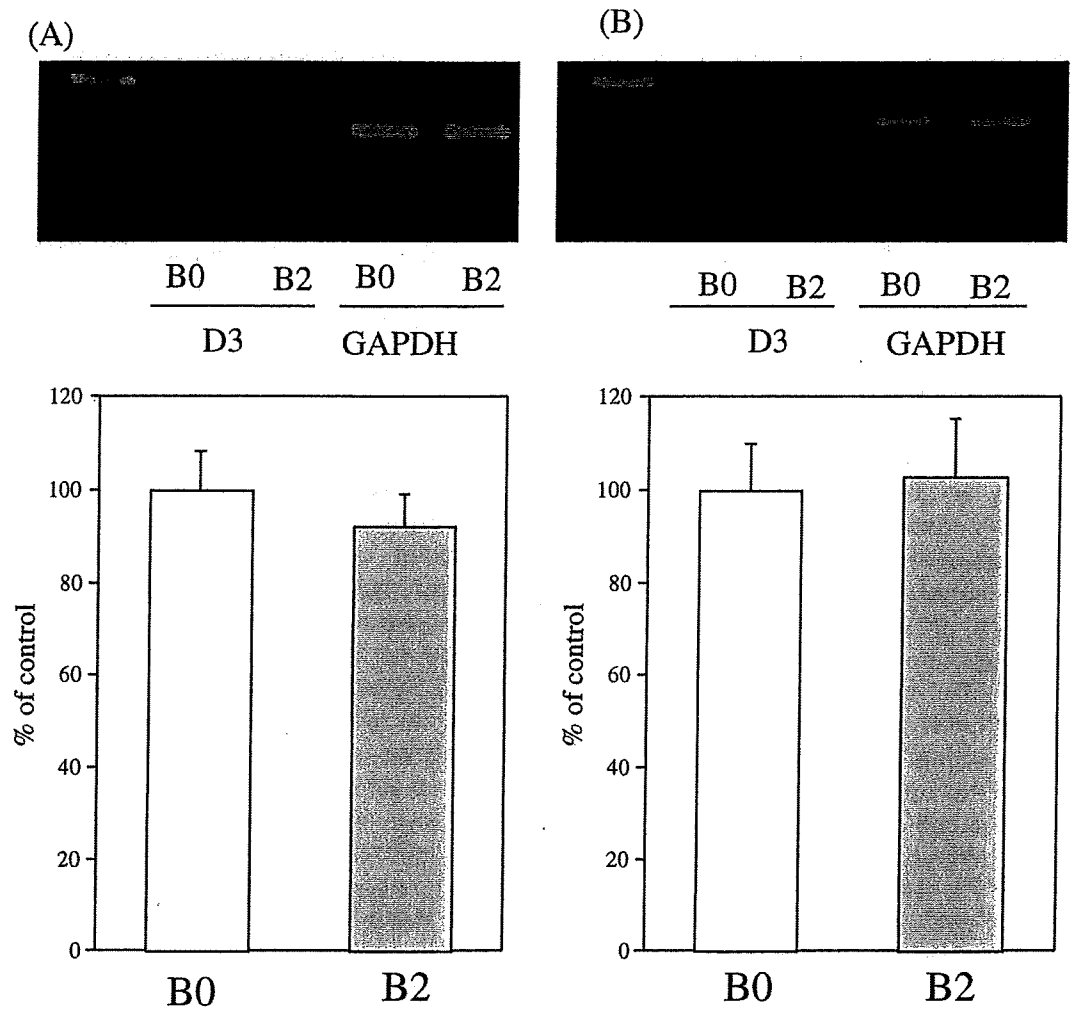

Figure 3. *No changes in the expression of dopamine D3 receptor mRNAs in the (A) limbic forebrain and (B) lower midbrain obtained from B0 and B2 mice. Upper panel: representative reverse transcription-polymerase chain reaction for dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain obtained from B0 and B2 mice. Lower panel: no changes in the expression of dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain obtained from B2 mice (hatched bar) compared to that from B0 mice (open bar). The values are expressed as a percentage of the value in B0 mice. Each column represents the mean ± SEM of three independent samples.*

Table 1. *Dopamine D_3 receptor density and affinity performed by [^{3}H]PD128907 binding to the mouse brain membrane preparation from control (B0) and bisphenol A treated (B2) mice*

	Bmax (fmol/mg of protein)	Kd (nM)
B0	28.24 ± 1.57	0.28 ± 0.04
B2	18.60 ± 0.22*	0.29 ± 0.03

Each value represents the mean with SEM of three samples. *$p < 0.05$ vs. B0 group

neonatal exposure to BPA caused the decrease of dopamine D3 receptor density in the mouse limbic forebrain.

No change in the expression of dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain obtained from mice prenatally and neonatally exposed to BPA

We also found that the expression of dopamine D3 receptor mRNA in the limbic forebrain and lower midbrain was not affected by prenatal and neonatal exposure to BPA (Fig. 3).

Discussion

In the previous study, we demonstrated that prenatal and neonatal exposure to BPA enhanced the methamphetamine-induced rewarding effect associated with the moderate upregulation of

central dopamine D1 receptor function in mice.[2] In the present study, we found that the stimulation of [^{35}S]GTPγS binding induced by 7-OH-DPAT was markedly attenuated in B2 mice. Under these conditions, the G-protein activation induced by the selective dopamine D_2 receptor agonist N-propylnorapomorphine in the limbic forebrain was not affected by prenatal exposure to BPA.

To evaluate the population of dopamine D3 receptors in the membrane surface of the mouse brain, we performed a saturation-binding analysis using [^{3}H]PD128907. In the present study, the density of dopamine D3 receptor was significantly decreased by chronic exposure to BPA. In addition, we found that the expression of dopamine D3 receptor mRNA in the limbic forebrain was not affected by prenatal and neonatal exposure to BPA. These findings suggest that chronic exposure to BPA caused the increase in dopamine D3 receptor turnover without any changes in newly synthesized receptors.

The dopamine D3 receptor is highly distributed in the nucleus accumbens, the terminal sites of the mesolimbic dopaminergic system.[3,9] This can be supported by the present finding that the dopamine D3 receptor expression was predominantly observed in the limbic forebrain. The limbic system-selective expression of the dopamine D3 receptor has led to particular interest in this receptor as a potential mediator of some of the psychoeffective functions of dopamine neurotransmission.[10–13] In the previous study, we demonstrated that the dopamine D3 receptor knockout mice exhibit the enhancement of morphine-induced rewarding effect and hyperlocomotion.[5] Furthermore, prenatal and neonatal exposure to BPA enhances the morphine-induced rewarding effect and hyperlocomotion without direct changes in the μ-opioid receptor function.[14] Thus, these findings provide the possibility that the dramatic suppression of postsynaptic dopamine D_3 receptor function in the nucleus accumbens may be, at least in part, involved in the enhancement of morphine-induced rewarding effect followed by chronic exposure to BPA.

The desensitization of G-protein-coupled receptors is mediated by the phosphorylation of serine and threonine residues within the intracellular domains of receptors.[15] Both second messenger-dependent kinases, such as protein kinase A and protein kinase C, and G-protein-coupled receptor kinases (GRKs) have been shown to contribute to the desensitization and internalization of activated dopamine receptors.[16–18] It has been reported recently that dopamine D3 receptor desensitization can be regulated by GRK3.[19] In the present study, we clearly observed the increase in dopamine D3 receptor turnover without any changes in newly synthesized receptors after chronic treatment with BPA. Taken together, a hypothesis would be advanced that, although further study is required, prenatal and neonatal exposure to BPA may lead to the changes in activities of these kinases associated with the turnover of dopamine D3 receptors.

In conclusion, the present study suggests that chronic exposure to BPA causes the inhibition of dopamine D3 receptor-mediated G-protein activation associated with the decrease in its receptor density in the limbic forebrain of mice. This phenomenon may, at least in part, contribute to the enhancement of morphine-induced rewarding effect by chronic exposure to BPA in mice. Public attention and research efforts are being driven by an understanding of the ever-increasing problems and magnitude of substance abuse. Our findings warn that prenatal and neonatal exposure to BPA in females may predispose their children to the psychological dependence of morphine.

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Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice

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Abstract

Bisphenol-A (BPA), one of the most common environmental endocrine disrupters, has been extensively evaluated for toxicity and carcinogenicity. However, little is still known about its action on the CNS. Here we found that prenatal and neonatal exposure to BPA resulted in the enhancement of the rewarding effect and hyperlocomotion induced by morphine in mice. Under these conditions, no change in the G-protein activation by morphine and μ-opioid receptor expression in the lower midbrain was observed by prenatal and neonatal exposure to BPA. These results suggest that chronic exposure to BPA produces the supersensitivity of the morphine-induced rewarding effect and hyperlocomotion without direct changes in μ-opioid receptor function in the lower midbrain. The present data provide further evidence that prenatal and neonatal exposure to BPA can directly influence the development of the central dopaminergic system.

Keywords: Bisphenol-A; Morphine; Rewarding effect; Hyperlocomotion; μ-Opioid receptor; G-Protein activation

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. It has been reported that administration of bisphenol-A (BPA) to pregnant mice on gestation days at a dose that is within the range typical of the environmental exposure of humans produces significant changes in the postnatal growth rate and brings on early puberty in these mice [6]. These results imply that BPA may cause unpleasant toxicity in the developmental process. In contrast, little is known about the neuronal toxicity induced by BPA in the CNS. More recently, we have reported that prenatal and neonatal exposure to BPA enhances the methamphetamine-induced rewarding effect and central dopamine D_1 receptor function in mice [19].

Many studies have suggested that the mesolimbic dopaminergic system which projects from the ventral tegmental area (VTA) to the nucleus accumbens is critical for the initiation of opioid reinforcement and hyperlocomotion [7,16,20]. Either DAMGO- or morphine-induced place preference can be blocked by dopamine antagonists receptor [13,14]. As well as the rewarding effect, it has been well recognized that hyperlocomotion induced by morphine can be blocked by treatment with dopamine receptor antagonists in the nucleus accumbens [4,8]. These findings indicate that the dopamine-containing neurons of the midbrain VTA, which has a high density of μ-opioid receptors, play a critical role in the rewarding effects and hyperlocomotion by μ-opioids. In terms of dopamine receptor involvement at the terminal site of the mesolimbic dopamine system, the rewarding effect of abused drug has been shown to be mediated by dopamine D_1 receptors [10,14,17], which could be directly affected by prenatal and neonatal exposure to BPA [19]. The aim of the present study was to investigate whether prenatal and neonatal exposure to BPA in mice could affect the rewarding and locomotor-enhancing effects induced by morphine.

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. All efforts were made to minimize the number of animals used and their suffering. All experiments were performed using male ddY mice that had been prenatally and neonatally exposed to BPA. Prenatal and neonatal BPA exposure was conducted as previously described [19]. Adult female mice were chronically treated with BPA-admixed powder food containing 0 (B0), 0.002

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(B0.002), 0.5 (B0.5) and 2 (B2) mg BPA/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of BPA from their mothers. During the treatment with BPA, animals did not show weight loss and disrupted maternal behaviors.

Place conditioning was conducted as previously described [18]. The apparatus was a shuttle box (15 × 30 × 15 cm: w × l × h) which was made of acrylic resin board and divided into two equal-sized compartments. One compartment was white with a textured floor, and the other was black with a smooth floor to create equally preferred compartments. For conditioning, mice were confined to one compartment after drug injections and to the other compartment after saline injections. The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. A day after these conditioning sessions, the animal was placed in the test apparatus without any confinements.

The locomotor activity of mice was measured by an ambulometer as described previously [11]. Briefly, a mouse was placed in a tilting-type round activity cage 20 cm in diameter and 19 cm high. Any slight tilt of the activity cage, which was caused by horizontal movement of the mouse, was detected by three microswitches. Total activity counts were automatically recorded for 3 h following the injection of saline (10 ml/kg, s.c.) or morphine (10 mg/kg, s.c.). Total activity was counted for 3 h after the treatment.

In the membrane preparation, mice were killed by decapitation and the lower midbrain was then dissected as described previously [12]. The lower midbrain was rapidly excised at 4 °C, and the tissues were homogenized using a Potter-Elvehjem tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris–Mg^{2+} buffer containing 50 mM Tris–HCl (pH 7.4), $MgCl_2$ and 1 mM EGTA for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 4 °C for 10 min at 48,000 × *g*. The pellet was resuspended in ice-cold [^{35}S]GTPγS binding assay buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4 °C for 10 min at 48,000 × *g*. The resultant pellet was resuspended in ice-cold [^{35}S]GTPγS binding assay buffer and stored at −70 °C until used. The membrane homogenate (3–8 μg protein/assay) was incubated at 25 °C for 2 h in 1 ml of assay buffer with 0.001–10 μM morphine, 30 μM guanosine-5′-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$ at 4 °C for 2 h. Filters were then washed three times with 5 ml of an ice-cold Tris–HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 ml of Soluene-350 and 4 ml of Hionic Fluor, equilibrated for 12 h and the radioactivity in the samples was determined with a liquid scintillation analyzer. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from at least three independent sets of experiments.

Total RNA in the whole brain was extracted using an SV Total RNA Isolation System (Promega, Madison, WI). To prepare first strand cDNA, 1 μg of RNA was incubated in 100 μl of buffer containing 10 mM DTT, 2.5 mM $MgCl_2$, dNTP mix, 200 units of reverse transcriptase II (Gibco-BRL, Grand Island, NY) and 0.1 mM oligo (dT)12-18 (Gibco-BRL). The μ-opioid receptor was amplified in a 50 μl PCR solution containing 0.8 mM $MgCl_2$, dNTP mix and DNA polymerase with synthesized primers: a sense primer of μ-opioid receptor, which is at position 299–320 (5′-AGACTGCCACCAACATCTACAT-3′) of the receptor, and an antisense primer at position 623–643 (5′-TGGACCCCTGCCTGTATTTTG-3′). Samples were heated to 94 °C for 2 min, 55 °C for 2 min and 72 °C for 3 min, and cycled 35 times through 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min. The final incubation was 72 °C for 7 min. The mixture was run on 1% agarose gel electrophoresis with the indicated markers and primers of the internal standard GAPDH. Three independent experiments were performed in this study. Semi-quantitation of the intensity of the bands for μ-opioid receptor/GAPDH was conducted by using a NIH image.

Data represent the mean counts with SEM. Statistical analyses were performed using two-way ANOVA with Bonferroni/Dunnett's test.

In the previous study, we found that prenatal and neonatal exposure to BPA enhances the methamphetamine-induced pharmacological actions [19]. Here, we investigated the influence of prenatal and neonatal exposure to BPA in the several morphine-induced pharmacological actions. In the present study, morphine produced a dose-dependent place preference in control B0 mice (Fig. 1A), as reported previously [10,17]. Morphine at the low dosage that elicited neither place preference nor place aversion in control B0 mice produced a significant place preference in BPA-treated mice (Fig. 1A). As well as the rewarding effect, a significant increase in the morphine-induced hyperlocomotion was observed by chronic BPA treatment ($F_{1,340} = 6.617$, $P < 0.05$ vs. B0 group, Fig. 1B).

Recently, several investigations have provided evidence that the treatment of adult animals with BPA could not affect the reproductive function and social behaviors [2,3]. We have already confirmed that acute administration of BPA with adult mice could not affect the dopamine-related behaviors (data not shown). These findings indicate that prenatal and neonatal exposure to BPA may cause the neuronal toxicity specifically in the developmental process.

Many studies have pointed to the mesolimbic dopaminergic system, which originates from the VTA projecting mainly to the nucleus accumbens, as a critical site for the initiation of opioid addiction [7,13,14,16,20]. Morphine has been shown to indirectly activate dopamine neurons in the VTA as a consequence of inhibiting non-dopaminergic neurons, presumably γ-aminobutyric acid-containing neur-

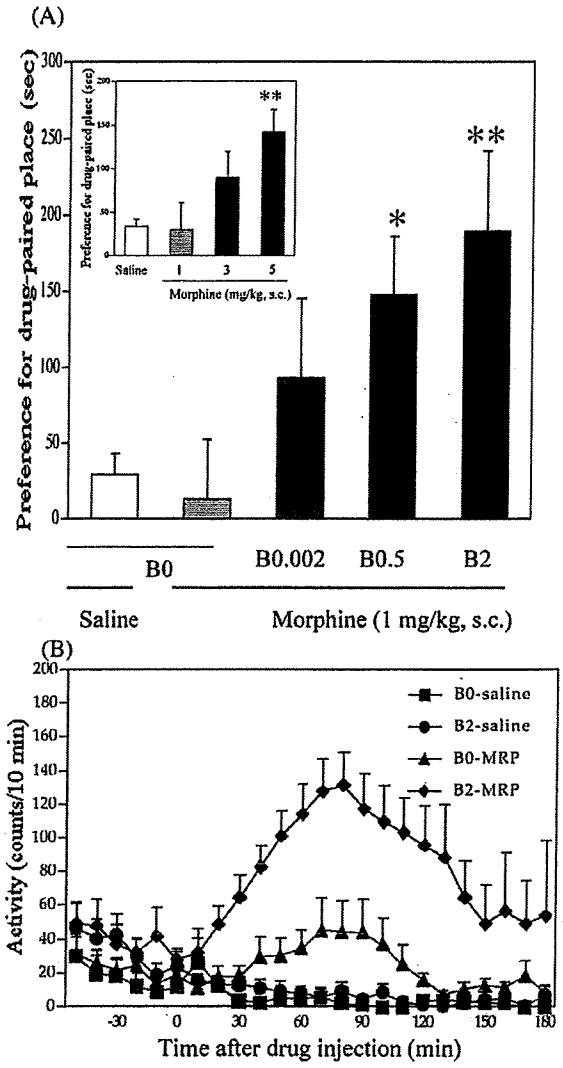

Fig. 1. (A) Effect of prenatal and neonatal exposure to BPA on the rewarding effect of morphine in mice. (Inner) Dose-response for the morphine-induced place preference in control B0 mice (*$P < 0.05$ vs. saline-treated mice). (Outer) Effect of BPA (0.002–2 mg/g of food: B0.002–B2) on the morphine (1 mg/kg, s.c.)-induced place preference. The BPA-non-treated group (hatched bar) did not show any place preference or place aversion with morphine at 1 mg/kg. The BPA-treated group (filled bar) showed a significant place preference induced by morphine at this dosage (*$P < 0.05$, **$P < 0.01$ vs. BPA-non-treated group). Each column represents the mean place preference score with SEM of six to ten mice. (B) Effect of BPA on morphine (10 mg/kg, s.c.)-induced hyperlocomotion. The BPA (2 mg/g of food)-treated group (diamond: B2 mice) revealed a significant potentiation of the morphine-induced locomotor-enhancing effect ($F_{1,340} = 6.617$, $P < 0.05$ vs. B0 group: triangle). Each point represents the mean activity counts for 10 min with SEM of nine to ten mice.

ons, leading to the increased dopamine release in the nucleus accumbens [10]. In order to investigate the mechanism of the enhancement of morphine's effect, we demonstrated whether prenatal and neonatal exposure to BPA could directly affect the opioidergic system associated with the mesolimbic dopaminergic system. In the [^{35}S]GTPγS binding assay, prenatal and neonatal BPA exposure failed to enhance μ-opioid receptor-mediated G-protein activation by morphine in the lower midbrain (Fig. 2A). As well as G-protein activation, the expression of μ-opioid receptor mRNA was not changed by chronic BPA treatment, suggesting that μ-

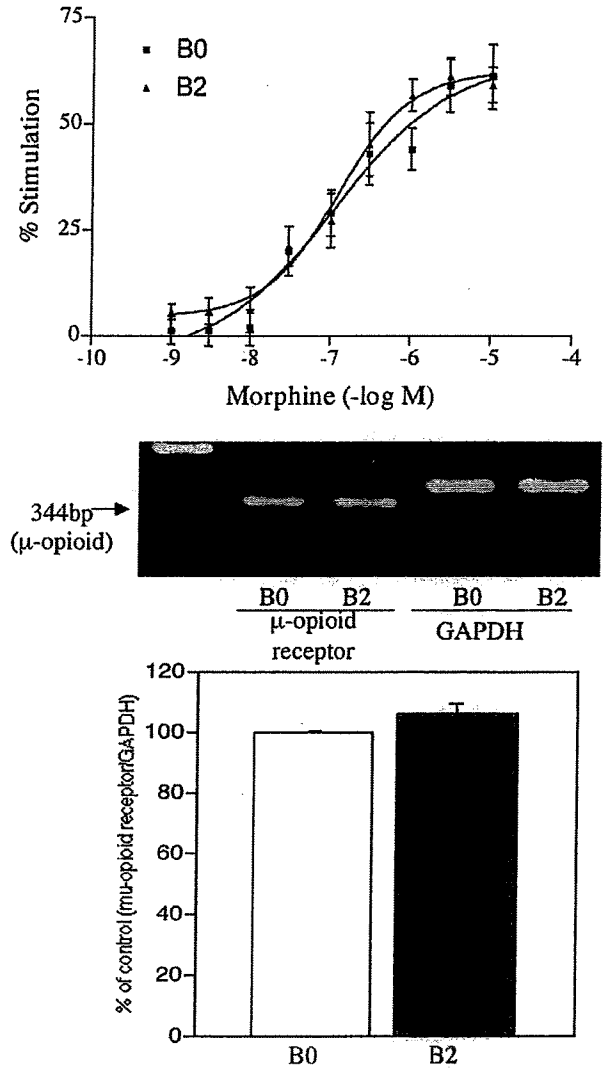

Fig. 2. No changes in the μ-opioid receptor in the mice prenatal and neonatal exposure to BPA. (A) Comparison of the stimulation of [^{35}S]GTPγS binding by morphine to membranes of the lower midbrain obtained from control (B0: square) and BPA-treated (B2: triangle) mice. Membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with morphine (10^{-9}–10^{-5} M). The values are expressed as % increase of the value in B0 mice. Data are expressed as the mean ± SEM of three independent samples. (B) Effect of prenatal and neonatal exposure to BPA on the expression of μ-opioid receptor mRNA in the mouse brain. (Upper) Representative RT-PCR for the μ-opioid receptor mRNAs in the whole brain minus cerebellum obtained from B0 and B2 mice. (Lower) There were no changes in the expression of μ-opioid receptor mRNAs in the whole brain obtained from B2 mice (filled bar) as compared to that from B0 mice (open bar). The values are expressed as a percentage of the value in B0 mice. Each column represents the mean ± SEM of three independent samples.

opioid receptor function is likely to be directly unaffected in this region. We have already reported that prenatal and neonatal exposure to BPA lead to an up-regulation of dopamine D_1 receptor in the limbic forebrain [19]. Taken together, these results indicate that the enhancement of the morphine-induced rewarding effect and hyperlocomotion may mainly result from the direct up-regulation of the dopaminergic transmission in the limbic forebrain.

BPA is considered to disrupt the endocrine systems via binding to estrogen receptor by mimicking estrogenic action. However, weak estrogenicity of BPA was confirmed with approximately 15,000 times less potency than 17β-estradiol [5]. Furthermore, BPA binds to estrogen receptor with low affinity and transactivates the estrogen responsive element-driven reporter gene in vitro [5]. Recently, it has been reported that BPA acts on thyroid receptor as an antagonist [9]. In addition, Song et al. have reported that BPA induces the expression and steroidogenesis of the orphan nuclear receptor Nur77 gene in mice Leydig cells [15]. Several subfamilies of Nur77, for example NGFI-B or Nurr1, have been shown to be highly expressed in the brain [21]. In particular, it has been accepted that NGFI-B is highly expressed in the basal ganglia, and is involved in the development of dopaminergic and opioidergic systems [1, 22]. These findings suggest that, although we cannot completely exclude the possibility of estrogenic action of BPA, the present action of BPA may mainly result from novel mechanisms associated with dopaminergic transmission.

In conclusion, the present data provide further evidence that prenatal and neonatal exposure to BPA can directly influence the development of the central dopaminergic system in the limbic area. These effects could lead to a change in sensitivity to morphine. Further investigation is necessary to fully understand the molecular mechanism of BPA in the CNS.

Acknowledgements

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Original Article

Effects of in Utero and Lactational Exposure to Di(2-ethylhexyl)phthalate on Somatic and Physical Development in Rat Offspring

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Abstract: Di(2-ethylhexyl)phthalate (DEHP) has been reported to act as an antiandrogen and to affect the reproductive organs and accessory genital glands. Thus, to assess the reproductive toxicity of DEHP it is important to examine both its adverse effects on the development of offspring following maternal exposure and its effects on sexual function and fertility. In the present study, we examined whether in utero and lactational exposure to DEHP affects postnatal somatic growth of offspring in the rat. Pregnant females were orally administered various doses of DEHP (0, 25, 100 or 400 mg/kg body weight/day) from gestational day (GD) 6 through postnatal day (PND) 20. There were no significant changes in body weight, body length, tail length, or the weight of individual organs between the control and DEHP-treated groups. Somatic hormonal parameters were the same for all DEHP doses. These findings suggest that in utero and lactational exposure to various concentrations of DEHP has very little effect on postnatal development or endocrine and physical status of male and female rat offspring under the experimental conditions of the present study.

Key words: Di(2-ethylhexyl)phthalate, Postnatal development, In utero and lactational exposure, Offspring, Rat

Introduction

To date, several compounds have been suspected of exerting endocrine-disturbing effects even at ultra-low concentrations. Phthalates have been produced and used in the manufacture of chemically derived materials and products. Di(2-ethylhexyl)phthalate (DEHP) has been most widely used in polyvinyl chloride to impart structural flexibility, and it is used as a plasticizer in products such as food packaging, children's products (toys and crib bumpers) and medical devices. Significantly, DEHP has been detected in plasma samples[1]. Mono(2-ethylhexyl)phthalate (MEHP), which is an active and the predominant DEHP metabolite, is also considered as a testicular toxicant[2]. It has been estimated that mean DEHP intake is 8.2 μg/kg body weight per day for adults[3]. During recent years, DEHP has been excluded from many products to avoid consumer exposure. However, recent heightened public concerns about environmental exposure to high concentrations of DEHP have raised new questions about its possible occupational and medical health hazards.

Developmental toxicity studies of DEHP have been conducted in laboratory mice[4–8] and rats[8–10]. These reports suggest that in utero exposure to high doses of DEHP induces embryotoxicity and/or teratogenicity. Animal reproductive toxicity studies of DEHP have also been reported. In a study of adult male rats, testicular defects such as atrophy of the seminiferous tubules, loss of spermatogenesis and vacuolation of Sertoli cells were observed after 90 days of dietary exposure to DEHP at 500 and 5,000 ppm (equivalent to 37.6 and 375.2 mg/kg/day, respectively)[11]. Perinatal exposure to DEHP in rats from gestational day (GD) 14 through postnatal day (PND) 3 reduced anogenital distance, testis weight or the weight of androgen-dependent tissues[12].

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Dietary exposure of adult male rats given 0, 320, 1,250, 5,000, and 20,000 ppm DEHP (equivalent to 0, 17.5, 69.2, 284.1 and 1156.4 mg/kg/day, respectively) for 60 days, when mated with untreated adult females, did not affect the rate of neonatal death, initial pup weight or growth (up to PND 7), whereas the average litter size decreased in rats fed 20,000 ppm DEHP[13]. Inhalation exposure of adult male Wistar rats to 25 mg/m^3 for 6 h/day for 8 wk increased plasma testosterone level and seminal vesicle weight in a dose-dependent manner[14]. In a study of adult female rats, DEHP induced prolonged estrous cycles and suppressed plasma concentrations of estradiol and subsequent ovulation[15].

Several studies have shown that in utero and lactational exposure to DEHP leads to abnormalities in the hypothalamus-pituitary-testicular axis. Sprague-Dawley rats were orally dosed with DEHP (0–1,500 mg/kg/day) from GD 3 through PND 21, and dose-related effects in the male offspring included several parameters involved in sexual development[16]. Oral exposure of pregnant female Long-Evans rats to 100 mg/kg/day DEHP from GD 12–21 induced significantly increased levels of testosterone and luteinizing hormone in male offspring on PND 21 and PND 35, but by PND 90 the levels were comparable between treated and untreated animals[17], indicating that the magnitude of DEHP toxicity on reproductive function is influenced by the stage of development.

Thus, DEHP toxicity studies in laboratory animals have focused on embryotoxicity, teratogenicity and reproductive toxicological effects in addition to some developmental effects in the early postnatal period, yet extensive toxicity information for long-term development after DEHP exposure is still lacking. The purpose of the present study was to evaluate postnatal growth and physical development following in utero and lactational exposure to DEHP in male and female rat offspring until the post-pubertal period. We examined the effects of DEHP on pubertal development, and doses of DEHP were chosen based on the levels that caused no overt maternal toxicity. Additionally, the exposure period was extended to examine the effects of lactational exposure in addition to the effects of in utero exposure, to complement previous studies[4–10]. Thus, we administered several doses of DEHP orally by gavage to pregnant rats using an experimental schedule identical to one used previously[18], and we examined the effects on postnatal somatic and organ growth, as assessed by body weight, body length, tail length and main organ weights, including reproductive organs, in male and female offspring. In addition, to better assess physical status following DEHP exposure, we evaluated the levels of several plasma hormonal landmarks with regard to postnatal somatic growth.

Materials and Methods

Chemicals and experimental animals

DEHP (purity >99.9%, Cat# 289-10442) and corn oil were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. A total of 52 pregnant (GD 3) female rats (Crj: CD (SD) IGS strain, 9 wk of age) were purchased from Charles River Japan, Inc. (Tsukuba, Japan). The presence of a copulatory plug defined GD 0. They were acclimated on GD 3–6 and housed individually in plastic cages with sterilized wood chips (Soft chip, Japan Slc Inc., Shizuoka, Japan) for bedding and were maintained under controlled temperature (23 ± 1°C) and humidity (55 ± 5%) and with a 12-h light-dark cycle (08:00–20:00) throughout the study. A standard laboratory diet (CE-2, Clea Japan, Inc., Tokyo, Japan) and drinking water were available ad libitum.

Dose range-finding evaluation

Dams were randomly divided into five groups (four pregnant females per group). The DEHP-exposed groups were orally administered 500, 1,000, 1,500, or 2,000 mg DEHP/kg/day in corn oil vehicle (10 ml/kg of body weight); DEHP was given between 08:30 and 09:30 for five consecutive days each week (Monday–Friday) from GD 6 through GD 20, and the control group was given the same amount of corn oil during the same period. During the exposure period, we recorded maternal body weights and noted any clinical signs or abnormal behavior that may have resulted from toxic effects. These results were used to determine the range of the DEHP dose for the main study.

Main study

Dams were randomly divided into four groups (eight pregnant females per group) and weighed once daily from GD 3 through PND 20 (except for GD 4 and 5). The DEHP-exposed groups were orally administered 25, 100 or 400 mg DEHP/kg/day in corn oil vehicle (10 ml/kg of body weight); DEHP was given between 08:30 and 09:30 from GD 6 through PND 20, and the control group was given the same amount of corn oil during the same period. Maternal data were recorded as described above. For each dam, the gestational duration was recorded, and weight gain during gestation and lactation was measured. Dams were checked for birth until 10:00 on each day; the day on which pups were first observed was designated PND 0. The number of

live births and the weight of each live pup on PND 1 were recorded. The litter size was standardized to 10 (five males and five females when possible) between 10:00 and 11:00 on PND 7 (1 wk of age). Litters with a total of nine or fewer pups were not culled regardless of the sex ratio. Culled pups were used for the analysis at 1 wk of age. On PND 21, the remaining offspring were weaned, and thereafter males and females were housed in separate stainless steel cages by litter. Body weights were recorded with an electric balance (Shimadzu, Kyoto, Japan). Body length and tail length (millimeters) were measured with a digital caliper (Mitutoyo, Kanagawa, Japan). The nose-anus length was considered the body length. One male and one female offspring from each dam were dissected at 3 and 9 wk of age when possible. While the rat was under ether anesthesia, liver, kidneys and testes, prostate and seminal vesicles or ovaries and uterus were carefully removed and weighed.

Hormone determinations

For hormone determinations, blood samples were collected from the postcaval vein following euthanasia by ether inhalation at 9 wk of age. Plasma samples were obtained by centrifugation at 4°C and stored at –20°C until the analysis. Concentrations of the plasma thyroid hormones thyroxine (T_4) and tri-iodothyronine (T_3) were determined by a time-resolved fluoroimmunoassay (DELFIA T_4 Reagents and DELFIA T_3 Reagents, respectively, PerkinElmer Life and Analytical Sciences, Inc., MA, USA). Plasma growth hormone (GH) concentrations were determined by enzyme immunoassay (EIA) (Rat GH EIA Biotrak system, GE Healthcare Bio-Sciences Corp., NJ, USA). Plasma insulin-like growth factor-I (IGF-I) concentrations were also measured by EIA (ACTIVE mouse/rat IGF-I EIA kit, Diagnostic Systems Laboratories, Inc., TX, USA). Time-resolved fluorescence and absorbance were measured by a multilabel counter (VICTOR^2, PerkinElmer Life and Analytical Sciences, Inc.). All hormones were assayed according to the manufacturer's instructions.

Statistical analysis

The differences from the corresponding control group were statistically analyzed by an analysis of variance followed by Dunnett's test (significance at $p<0.05$).

Results

Dose range-finding evaluation

In the 1,000 mg/kg/day and higher DEHP groups, maternal toxicity was clearly manifested as greatly suppressed weight gain during gestation, which led us to discontinue subsequent dosing by GD 17 of this preliminary study. In the 500 mg/kg/day group, mean body weights decreased slightly at later stages of gestation compared with the control group (data not shown). Based on these observations, we set the highest dose at 400 mg/kg/day to exclude the influence of maternal toxicity and observe the effect of DEHP on the offspring. The lowest dose and the middle dose were set at 25 mg/kg/day and 100 mg/kg/day, respectively.

Main study

Dams

Table 1 shows the number of dams and their offspring used for examinations in each group. Weight gain did not differ between dams from the control group and the DEHP groups from GD 6 through GD 21. In the 400 mg/kg/day group, one dam was found dead on GD 23, and thus the dam was excluded from the analysis. No significant differences were observed between the control group and the DEHP groups with regard to gestational duration or the number of live births per litter on PND 1.

Figure 1 shows maternal body changes during gestation (left panel) and lactation (right panel). There were no statistically significant differences among groups with regard to maternal body weight during the gestation and lactation periods, although the 25 mg/kg/day group showed a transient but not significant weight reduction during early lactation.

Offspring

The number of offspring examined is shown in Table 2. In male and female offspring, there were no statistically significant differences in body weight, body length or tail length between the control and DEHP-exposed groups at 1, 3 or 9 wk of age (Figs. 2, 3 and 4). There were no statistically significant effects on liver or kidney weights in males or females at 1, 3 or 9 wk of age (Table 3, 4). In male offspring, testis weights did not differ among the control group and DEHP groups at 3 or 9 wk of age (Table 3). Prostate and seminal vesicle weights did not differ among the control group and DEHP groups at 9 wk of age (Table 3). In female offspring, ovary and uterus weights did not differ among the groups at 3 or 9 wk of age (Table 4).

Physical status of offspring

In male offspring, no statistically significant differences in plasma concentrations of T_4, T_3, GH or IGF-I were observed among the control group and the DEHP groups at 9 wk of age (Table 5). In female offspring, no statistically significant differences in plasma concentrations of T_4, T_3,

Table 1. Dams and litter data

	DEHP dose (mg/kg/day)			
	0	25	100	400
Females (n)	8	8	8	8
Pregnant females (n)	8	8	8	8
Dam weight gain (GD 6-21)	130 ± 7[a]	127 ± 5	135 ± 4	133 ± 5
Gestational period (days)	21.1 ± 0.1	21.4 ± 0.2	21.3 ± 0.2	21.3 ± 0.2
Live births/litter on PND 1	11.8 ± 0.7	13.6 ± 0.6	13.5 ± 0.5	11.7 ± 0.5 (7)[b]

[a]Values are mean ± SEM.

[b]The number in parentheses represents dams per dose group. One dam was found dead on GD 23, and thus the dam was excluded from the analysis.

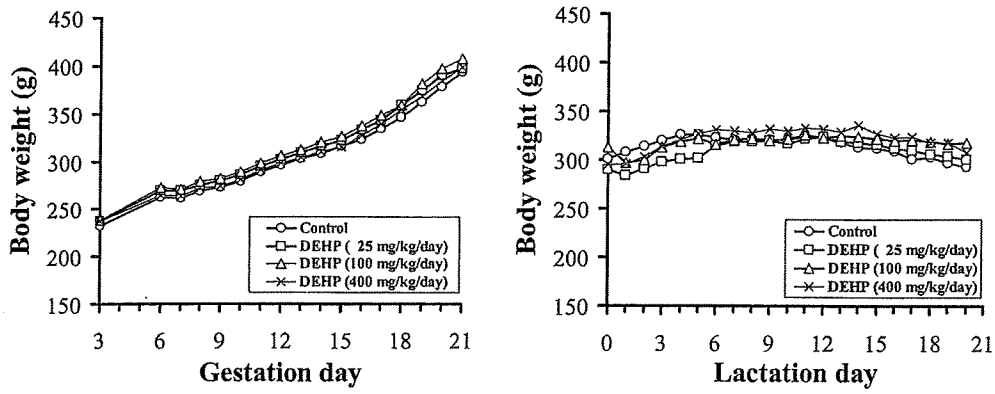

Fig. 1. Effects of exposure to di(2-ethylhexyl)phthalate (DEHP) on maternal body weight during gestation (left panel) and lactation (right panel).
Each point represents the mean.

GH or IGF-I were observed between the control group and the DEHP groups at 9 wk of age (Table 6).

Discussion

In recent years, the issue of endocrine-disrupting chemicals has been the topic of much discussion. Nagel *et al.*[19] and vom Saal *et al.*[20] reported that *in utero* exposure to low doses of bisphenol A (2 and/or 20 μg/kg/day) affects prostate and preputial gland weight and decreases daily sperm production efficiency in mouse offspring; moreover, their results indicated that exposure to low doses of xenoestrogens during a critical period can affect the reproductive organ systems of male offspring. On the other hand, other investigators have failed to find such effects in mouse offspring when using an identical experimental design[21,22]. Thus, the issue of low-dose exposure to these potential endocrine-disrupting chemicals remains a matter of debate among investigators. Hence, as more refined analytical methods become available, risk assessment for previously characterized chemical substances should be repeated.

Table 2. Number of subjects examined

Group	DEHP dose (mg/kg/day)	No. of offspring examined			
		Age (wk)	1	3	9
Control	0	Male	8	8	8
		Female	6	8	8
DEHP	25	Male	10	7	7
		Female	11	7	7
DEHP	100	Male	13	8	7
		Female	9	8	8
DEHP	400	Male	9	7	6
		Female	7	7	7

Embryo-fetotoxicity and teratotoxicity of DEHP have been studied in mice[4-8] and rats[8-10]. These studies were conducted to elucidate whether in utero exposure to high doses of DEHP induces embryotoxicity and/or teratogenicity. The doses used in these previous studies were far in excess of human

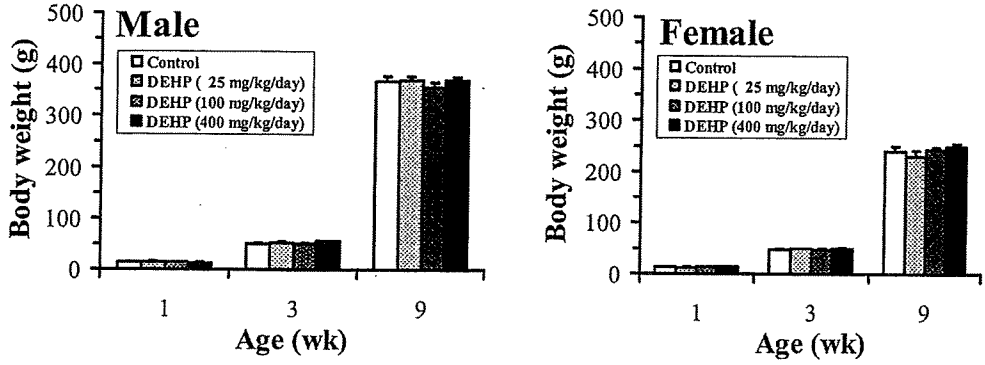

Fig. 2. Effects of maternal exposure to DEHP on postnatal body weight of offspring.
Body weights of male (left panel) and female (right panel) offspring are shown at 1, 3 and 9 wk of age. Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

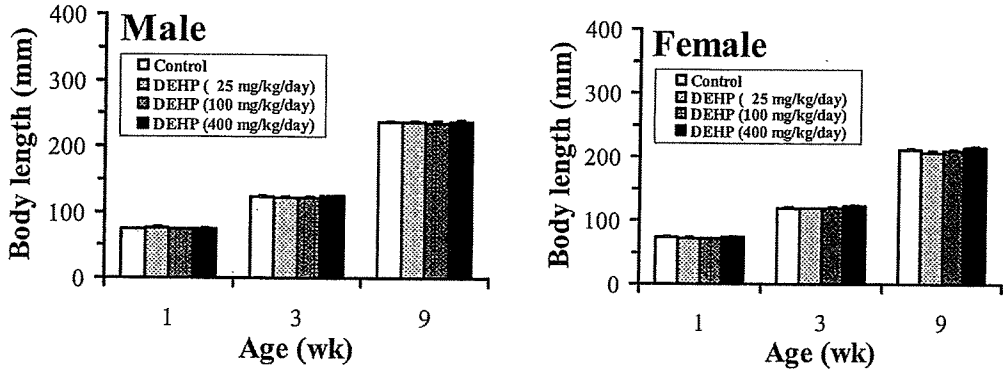

Fig. 3. Effects of maternal exposure to DEHP on postnatal body length of offspring.
Body lengths (nose to anus) of males (left panel) and females (right panel) are shown at 1, 3 and 9 wk of age. Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

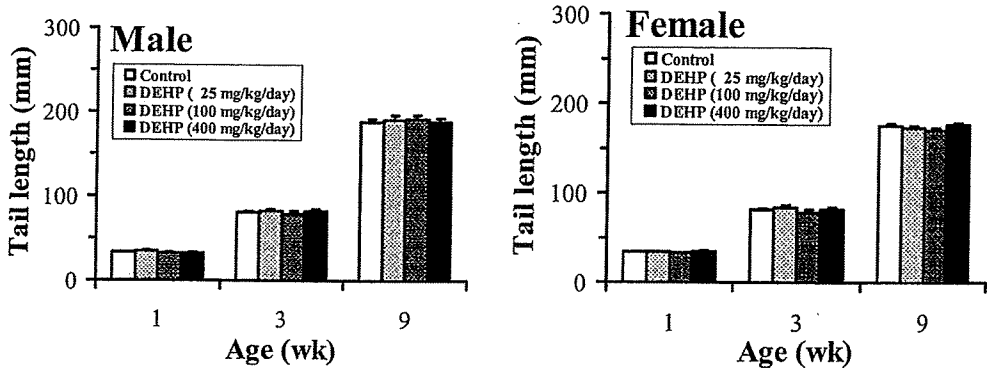

Fig. 4. Effects of maternal exposure to DEHP on postnatal tail length of offspring.
Tail lengths of males (left panel) and females (right panel) are shown at 1, 3 and 9 wk of age. Each column and vertical bar represent the mean and SEM, respectively. There were no significant differences among groups.

Table 3. Organ weights in male offspring

Organ	Group	DEHP dose (mg/kg/day)	Age (wk)		
			1	3	9
Liver (g)	Control	0	0.372 ± 0.011[a]	1.974 ± 0.090	15.55 ± 0.439
	DEHP	25	0.367 ± 0.024	1.984 ± 0.156	16.73 ± 0.560
	DEHP	100	0.334 ± 0.016	1.936 ± 0.138	14.78 ± 0.735
	DEHP	400	0.372 ± 0.037	2.276 ± 0.122	15.83 ± 0.691
Kidneys (g)	Control	0	0.191 ± 0.004	0.618 ± 0.018	2.951 ± 0.093
	DEHP	25	0.188 ± 0.008	0.585 ± 0.037	3.049 ± 0.124
	DEHP	100	0.164 ± 0.007	0.582 ± 0.042	2.842 ± 0.078
	DEHP	400	0.163 ± 0.015	0.632 ± 0.024	3.071 ± 0.092
Testes (g)	Control	0	-[b]	0.222 ± 0.009	3.065 ± 0.095
	DEHP	25	-	0.225 ± 0.014	2.999 ± 0.102
	DEHP	100	-	0.213 ± 0.011	2.834 ± 0.050
	DEHP	400	-	0.241 ± 0.012	3.070 ± 0.092
Prostate (g)	Control	0	-	-	0.443 ± 0.026
	DEHP	25	-	-	0.428 ± 0.033
	DEHP	100	-	-	0.372 ± 0.032
	DEHP	400	-	-	0.358 ± 0.026
Seminal vesicles (g)	Control	0	-	-	1.109 ± 0.057
	DEHP	25	-	-	1.064 ± 0.060
	DEHP	100	-	-	0.979 ± 0.034
	DEHP	400	-	-	1.014 ± 0.096

[a]Values are mean ± SEM. [b]-, not examined.

environmental exposure, and the duration of dosing was limited to the period of gestation. The present study was thus designed to investigate whether in utero and lactational exposure to DEHP affects the development of the next generation. For the main study, we set the highest dose at 400 mg/kg/day to avoid the influence of maternal toxicity and observe the effect of DEHP on the offspring. The exposure period was prolonged to examine the effects of lactational exposure in addition to the effects of gestational exposure. The offspring of dams in which no overt toxicity was observed (0, 25, 100 and 400 mg/kg/day), as determined by body weight and general behavior during gestation and lactation, were used in our study.

In recent years, certain studies have focused on the effects of DEHP and its antiandrogenic action on the hypothalamus-pituitary-gonadal axis[16, 17, 29]; very few studies, however, have reported the effect of DEHP on longer term postnatal development. Hence, it is important to examine the developmental toxicity of DEHP from birth until puberty. In this regard, our study was performed to evaluate the effects of in utero and lactational exposure to DEHP in rat offspring with a special focus on postnatal growth and physical status. We found that somatic and tissue growth and related endocrine landmarks were not affected by DEHP exposure.

Liver weights were slightly increased in the 400 mg/kg/day group for both male and female offspring at 3 wk of age, but no significant differences were observed among treatment groups. DEHP and other phthalates, such as di(2-ethylhexyl) adipate (DEHA) and butylbenzyl phthalate, are peroxisome proliferators that activate peroxisome proliferator–activated receptors and cause liver enlargement[23]. Induction of peroxisome proliferator–activated receptors could result in liver enlargement following DEHP exposure (Table 3, 4). This phenomenon could be an adaptive response following consecutive exposures to DEHP. However, this trend was no longer apparent at 9 wk of age. Since the DEHP groups were not exposed to the compound after 3 wk of age, body burden might be decreased because of metabolic clearance.

In a study of reproductive and accessory organ development following DEHP exposure, dose-dependent reductions in ventral, dorsolateral and/or anterior prostate weight were reported in rat offspring on PND 21 and PND 63 in response to oral administration of DEHP (0, 375, 750 and 1,500 mg/kg/day, GD3–PND21)[16]. This study also showed that DEHP significantly reduced testis weight on PND 21 and PND 63 in a dose-dependent manner. In the present study, on the other hand, testis weights were not

Table 4. Organ weights in female offspring

Organ	Group	DEHP dose (mg/kg/day)	Age (wk)		
			1	3	9
Liver (g)	Control	0	0.338 ± 0.007[a]	1.899 ± 0.117	9.665 ± 0.573
	DEHP	25	0.322 ± 0.015	1.886 ± 0.103	9.279 ± 0.511
	DEHP	100	0.349 ± 0.014	1.808 ± 0.105	9.760 ± 0.505
	DEHP	400	0.367 ± 0.030	2.046 ± 0.092	9.643 ± 0.441
Kidneys (g)	Control	0	0.176 ± 0.006	0.605 ± 0.026	2.039 ± 0.078
	DEHP	25	0.177 ± 0.006	0.593 ± 0.025	1.849 ± 0.091
	DEHP	100	0.179 ± 0.007	0.583 ± 0.023	1.983 ± 0.055
	DEHP	400	0.171 ± 0.007	0.583 ± 0.020	1.959 ± 0.039
Ovaries (mg)	Control	0	-[b]	18.95 ± 0.76	79.57 ± 4.08
	DEHP	25	-	17.80 ± 1.98	74.28 ± 8.14
	DEHP	100	-	14.83 ± 1.83	71.00 ± 4.26
	DEHP	400	-	16.67 ± 0.82	73.42 ± 3.29
Uterus (mg)	Control	0	-	26.03 ± 1.91	327.4 ± 25.3
	DEHP	25	-	30.72 ± 3.95	300.7 ± 14.2
	DEHP	100	-	31.96 ± 2.37	376.3 ± 30.9
	DEHP	400	-	27.82 ± 2.15	340.5 ± 16.1

[a]Values are mean ± SEM. [b] -, not examined.

Table 5. Hormone determinations in male offspring at 9 wk of age

Parameter		DEHP dose (mg/kg/day)			
		0	25	100	400
T_4	(ng/ml)	83.1 ± 6.9[a]	74.1 ± 3.7	73.2 ± 4.7	81.2 ± 7.5
T_3	(ng/ml)	1.74 ± 0.05	1.70 ± 0.06	1.63 ± 0.07	1.81 ± 0.09
GH	(ng/ml)	140.0 ± 35.3	137.3 ± 30.2	130.5 ± 16.3	96.5 ± 19.5
IGF-I	(ng/ml)	669.6 ± 49.0	641.7 ± 57.8	758.6 ± 49.6	743.5 ± 23.8

[a]Values are mean ± SEM.

Table 6. Hormone determinations in female offspring at 9 wk of age

Parameter		DEHP dose (mg/kg/day)			
		0	25	100	400
T_4	(ng/ml)	70.0 ± 7.4[a]	70.7 ± 5.4	67.7 ± 4.8	69.1 ± 6.4
T_3	(ng/ml)	1.88 ± 0.11	1.91 ± 0.06	1.76 ± 0.06	1.79 ± 0.10
GH	(ng/ml)	98.4 ± 9.6	99.5 ± 19.6	121.3 ± 22.4	109.4 ± 19.4
IGF-I	(ng/ml)	499.0 ± 34.4	574.0 ± 34.6	528.6 ± 42.5	632.6 ± 66.0

[a]Values are mean ± SEM.

significantly different between the control and DEHP groups. No significant differences in prostate weights were observed among the groups, although they were reduced in a dose-dependent manner (Table 3). The outcomes of the present study at the highest dose (400 mg/kg/day) were in accordance with those of Moore *et al.*, who conducted a study that used 375 mg/kg/day as the lowest dose[16]. The magnitude of DEHP effects in the present study was much smaller than that found in the study by Moore *et al.*[16]; this discrepancy could be explained by the large difference in dosage range.

Thyroid hormones play pivotal roles in normal growth, neuronal development and metabolism in animals. Endocrine disturbance following chemical exposure is suspected to occur at the embryonic and/or neonatal stage rather than at

the adult stage. An epidemiological study has suggested that toxicants such as polychlorinated biphenyls and dioxins, which are persistent and cumulative compounds in the environment, may affect growth and development through thyroid impairment[24]. Animal studies have reported that 2,3,7,8-tetracholorodibenzo-*p*-dioxin disrupts thyroid homeostasis[25] and causes developmental defects[26] and bone growth deficits[27]. Thyroid hormones are hormonal regulators of bone growth. The principal hormonal regulators during postnatal development are GH and IGF-I, and these hormones, which are regulated by thyroid hormones, are considered biomarkers for longitudinal somatic growth[28]. In the present study, hormonal parameters regarding developmental somatic growth were determined in the offspring to better assess the physical status following DEHP exposure. There were no significant differences in any parameters in male and female rat offspring (Table 5, 6). The fact that normal hormonal parameters were observed in rat offspring following exposure of dams to DEHP (even at high doses) leads us to conclude that postnatal development remains intact in the offspring.

The level of DEHP exposure used in the present study was much greater (~1,000-fold higher) than the estimated intake due to either medical exposure or consumer exposure in adult humans[3]. It was recently suggested that the magnitude of testicular toxicity after DEHP exposure is associated with the duration and/or the route of exposure[14, 29]. Inhalation of DEHP caused an elevation of plasma testosterone without affecting gonadotropin and several steroid enzymes that are involved in testosterone synthesis in male prepubertal rats[14]. These findings suggest that levels of DEHP that cause hormonal disturbance when inhaled may not have the same effect if consumed orally.

In conclusion, our results suggest that prenatal and postnatal exposure to DEHP does not affect postnatal somatic growth or endocrine and physical status of either males or females under the experimental conditions we used. The effects of DEHP exposure, however, remain uncertain and must be clarified using a wider dosage range, an extended exposure period, a side-by-side comparison of different exposure routes and a larger number of animals.

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