malities such as anxiogenic behavior, motor learning behavior, and memory.

The low-dose endocrine disrupting actions of various environmental toxicants are serious problems. However, little is known about the effects on the central nervous system induced by low-dose bisphenol-A. We also investigated whether prenatal and neonatal exposures to low-dose bisphenol-A in mice could induce behavioral abnormalities.

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

All experiments were performed using 7–11-week-old male C57BL/6J mice (Japan SLC Inc., Shizuoka, Japan) that had been prenatally and neonatally exposed to bisphenol-A (Wako Pure Chemical Industries Ltd., Osaka, Japan). Adult female mice (10 weeks old) were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 30 ng/g diet (Low), and 2 mg/g diet (High) from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. During the treatment with bisphenol-A, the animals did not show any body weight loss. In the present study, at least 10 dams were used per group. We randomly selected a few pups per litter and housed to undergo the behavioral tests. To obtain unbiased results, we appropriately distributed mice for each behavioral study.

To investigate changes in anxiogenic-like effects, the mice were tested using the light–dark paradigm [2,19]. We used a box consisting of a small (18 cm × 13 cm × 18 cm) dimly lit compartment with black walls and a black floor, connected by a small opening (5 cm × 5 cm) to a large (18 cm × 18 cm × 18 cm) intensely lit (500 lux) compartment with white walls and a white floor. Each animal (n = 11–14/group, 7 weeks old) was placed in the dark compartment at the beginning of the observation session. The compartment entry and exit were defined as all four paws into and out of the chamber, respectively. The time spent in the lit compartment was recorded for 10 min. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

The elevated plus-maze consists of two opposing open arms (30 cm × 6 cm × 0.3 cm) and two opposing enclosed arms (30 cm × 6 cm × 15 cm) that are connected by a central platform (9 cm × 9 cm, 100 lux), thus forming the shape of a plus sign. Each animal (n = 5–7/group, 7 weeks old) was tested using the elevated plus-maze in each experiment. We recorded for 5 min the time spent in open or enclosed arms and the time spent entering into the arms. The results were calculated as mean ratios of the time spent in the open arms to the total time spent in both the open and enclosed arms. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

To investigate possible changes in motor learning impairment with prenatal and neonatal exposures to bisphenol-A, the mice were tested using the rota-rod performance procedure (rota-rod test). The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a nonslippery surface. The 30-cm-long rod was placed at a height of 15 cm from the base and was divided into five equal sections by six disks. Thus the mice were tested simultaneously on the apparatus with a rod-rotating speed of 10 rpm. Each animal (n = 6–7/group, 9 weeks old) was placed on the rotating rod, requiring forward locomotion. Each animal was tested for a total of 5 min. If/when a mouse fell from the rod it was immediately replaced. We measured the time until falling the first time and the numbers of falls during the 5 min as the indicators of motor impairment twice a day. The score was the mean of latencies or numbers of falls in two trials. The data are presented as the mean with S.E.M. Statistical analyses were performed using two-way ANOVA with Bonferroni/Dunnett's test.

The experimental apparatus for the step-through passive avoidance test is a shuttle box that is divided into an illuminated small compartment (12 cm × 5 cm × 14 cm) and a dark/large compartment (25 cm × 25 cm × 20 cm) by a wall with a guillotine door. On the first day (conditioning day), each animal (n = 5–7/group, 11 weeks old) was placed in the illuminated compartment. After 90 s, the door was opened and the mouse was freely moved into the dark compartment. The door was closed as soon as the mouse stepped into the dark compartment, and an inescapable foot shock (0.5 mA, 0.5 s) was delivered through the grid floor. After 48 h from conditioning day, a retention test was started. The retention test was performed in a similar manner but without the electric shock and the step-through latency to the dark compartment was recorded. The maximal cut-off time for step-through latency was 30 min. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way or two-way ANOVA with Bonferroni/Dunnett's test.

In the immunohistochemical approach, the mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfusion fixed with 4% paraformaldehyde (pH 7.4). The brains were then quickly removed after perfusion, and thick coronal sections of the midbrain, including the hippocampus, were initially dissected using Brain Blocker. The brain coronal sections were postfixed in 4% paraformaldehyde for 2 h. After the brains were permeated with 20% sucrose for 1 day and 30% sucrose for 2 days, they were frozen in embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at −30 °C until use. Frozen 8-μm-thick coronal sections were cut with a cryostat (CM1510; Leica, Heidelberg, Germany) and thaw-mounted on poly-L-lysine-coated glass slides. The brain sections were blocked in 3% normal horse serum with 0.2% triton (for choline acetyltransferase (ChAT); Chemicon International Inc., CA, USA) and 10% normal goat serum (NGS) (for NeuN; Chemicon) in 0.01 M PBS for 1 h at room temperature. The primary antibody of ChAT (1:100) or NeuN (1:250) was diluted in 0.01 M PBS containing 3% NHS with 0.2% triton or 10% NGS and incubated for 2 days at 4 °C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 or Alexa 546 for 2 h at room temperature. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA). Fluorescence immunolabeling was detected using

a light microscope (AX-70; Olympus Optical, Tokyo, Japan) and photographed with a digital camera (Polaroid PDMCII/OL; Olympus Optical). Digitized images of the dentate gyrus were captured at a resolution of 140–200 pixels with a digital camera (Polaroid PDMCII/OL; Olympus). The density of ChAT labeling was measured with a computer-assisted imaging analysis system (Image J program, developed at the National Institutes of Health available at http://rsb.info.nih.gov/ij). The upper and lower threshold density ranges were adjusted to encompass and match the immunoreactivity; this provided an image with immunoreactive material appearing in black pixels and non-immunoreactive material in white pixels. A standardized rectangle was positioned over the hippocampus of control mice. The area and density of pixels within the threshold value representing immunoreactivity were calculated and the integrated density was the product of the area and density. The same box was then "dragged" to the corresponding position on the hippocampus of bisphenol-A treated mice, and the integrated density of pixels within the same threshold was again calculated. The data are presented as the mean with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

In the present study, prenatal and neonatal exposures to bisphenol-A failed to induce anxiogenic-like effects using the light–dark paradigm (Fig. 1A). As another measurement of anxiety, the mice prenatally and neonatally exposed to bisphenol-A were evaluated by the elevated plus-maze paradigm. The percentage of time spent in the open arms in the mice prenatally and neonatally exposed to bisphenol-A are shown in Fig. 1B. The prenatal and neonatal exposures to bisphenol-A failed to affect the percentage of time spent in the open arms. These results suggest that prenatal and neonatal exposures to bisphenol-A induced no anxiogenic-like behaviors. In the light–dark paradigm, the time spent in the light compartment of the mice prenatally and neonatally exposed to low-dose of bisphenol-A was increased as compared with the mice prenatally and neonatally exposed to high-dose of bisphenol-A (Fig. 1A). Although it is very difficult to explain the difference between the effect of low and high doses of bisphenol-A, it is necessary to consider the effects of the difference of the dosage on the difference of the action.

To investigate possible changes in motor learning impairment with prenatal and neonatal exposures to bisphenol-A, the mice were tested using the rota-rod test. In the present study, the mice prenatally and neonatally exposed to low or high doses of bisphenol-A progressively improved their skill in the rota-rod test, as the control mice did (Fig. 1C and D). The improvement of latency to fall and the number of fallings in the rota-rod test were indistinguishable between control and bisphenol-A treated mice (Fig. 1C and D). These results suggest that prenatal and neonatal exposures to bisphenol-A have no direct effect on motor skill learning.

The influence of prenatal and neonatal exposures to bisphenol-A on memory processes in mice was evaluated by the step-through passive avoidance test. In the conditioning trial, the step-through latency of the mice prenatally and neonatally exposed to low and high doses of bisphenol-A was similar to that of control mice (Fig. 1E). Although the latency to step-through increased in all groups compared to the latency shown at conditioning, the latencies to step into the dark compartment dramatically decreased in mice prenatally and neonatally exposed to bisphenol-A as compared to control mice ($F_{(2,30)} = 5.766$, $p < 0.05$, Fig. 1F). These results strongly suggest that chronic treatment with low and high doses of bisphenol-A induced the memory impairment. The contextual fear conditioning is hippocampal-dependent memory. Therefore, we next investigated the morphological and/or functional changes in the hippocampus of mice prenatally and neonatally exposed to low and high doses of bisphenol-A.

Immunohistochemical study showed that prenatal and neonatal exposures to low and high doses of bisphenol-A dramatically decreased the level of choline acetyltransferase-like immunoreactivity (ChAT-IR) in the widespread regions of the hippocampus compared to control (Fig. 2Ai–iii). Especially as shown in high magnification images, cholinergic fiber was dramatically decreased in mice prenatally and neonatally exposed to low and high doses of bisphenol-A compared to control (Fig. 2Bi–iii). Furthermore, these phenomena were observed in several regions of the hippocampus, such as CA1, CA2, and CA3 (data not shown). Using semi-quantitative analysis, prenatal and neonatal low and high doses of bisphenol-A produced a significant decrease in the level of ChAT-IR in the hippocampus (Low: $64.3 \pm 5.6\%$ of control mean, (***) $p < 0.001$ versus control mice; High: $50.0 \pm 3.8\%$ of control mean, (***) $p < 0.001$ versus control mice, Fig. 2C). It is widely accepted that cholinergic function in the hippocampus is important for the learning and memory processes [1,4,12]. Among the cholinergic parameters described for the brains of Alzheimer's disease patients, the decrease in ChAT activity is the most prominent and provides an excellent biochemical correlation to the severity of dementia. These reports strongly support our findings that the memory impairment corresponded well to the dysfunction of cholinergic neuron in the hippocampus of mice prenatally and neonatally exposed to bisphenol-A. Prenatal and neonatal exposure to bisphenol-A failed to affect the NeuN-IR in the dentate gyrus (Fig. 2Di–iii) and other regions of hippocampus compared to control, indicating that prenatal and neonatal exposure to bisphenol-A failed to cause cell death or layer formation on the mature pyramidal or granular cells in the hippocampus. Taken together, we hypothesized that although the prenatal and neonatal exposure to bisphenol-A could induce the dynamic morphological changes in cholinergic fibers associated with the disruption of the neuron development, it did not modify the hippocampal content of all immunogens.

It is widely accepted that one of the most common endocrine disruptors, bisphenol-A, has weak estrogenic activity. Moreover, endogenous estrogen, 17β-estradiol, plays the critical role in the neurotransmission of the hippocampus associated with spinogenesis or neuroprotection [5,18]. Furthermore, many cholinergic neurons also express the growth-associated protein GAP-43, may be taken as a marker of neurite outgrowth [11]. Estrogens, among other factors, up-regulate the expression of GAP-43 in the developing and adult brain [9]. On the other hand, bisphenol-A also disrupts thyroid hormone. Thyroid hormone deficiency during brain development disrupts on the activities

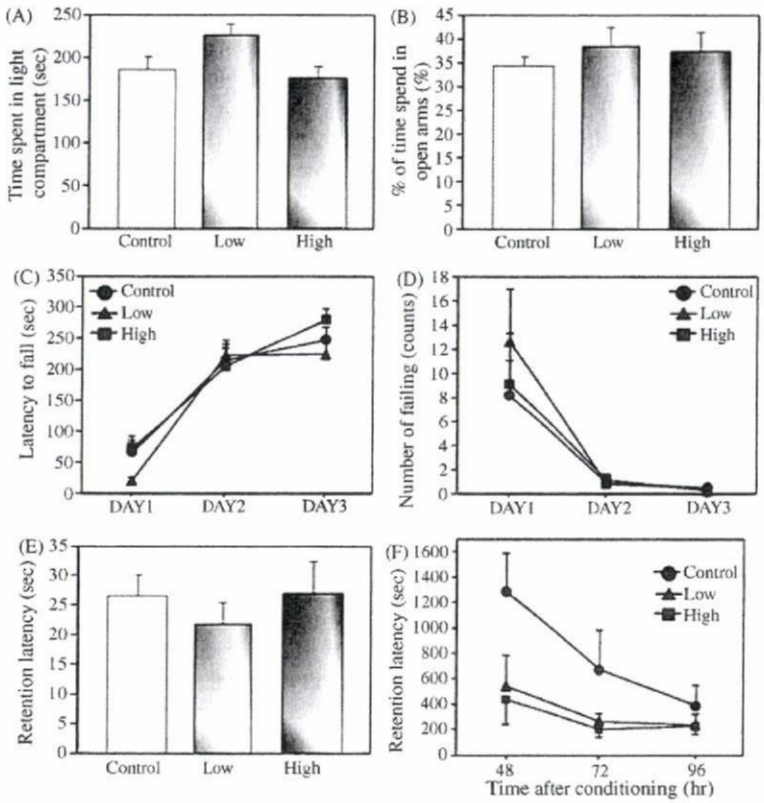

Fig. 1. Behavioral analysis in adult mice prenatally and neonatally exposed to bisphenol-A. (A) Lack of anxiogenic effects by prenatal and neonatal exposures to bisphenol-A using the light–dark test procedure. Prenatal and neonatal exposures to bisphenol-A failed to change the time spent in the lighted compartment as compared with control mice. On the other hand, time spent in the light compartment of the mice prenatally and neonatally exposed to low-dose of bisphenol-A was increased as compared with the mice prenatally and neonatally exposed to high-dose of bisphenol-A (All group: $F_{(2, 35)} = 3.467$, $p < 0.05$, Control vs. Low: $F_{(1, 22)} = 3.790$, $p > 0.05$, Control vs. High: $F_{(1, 25)} = 0.227$, $p > 0.05$, Low vs. High: $F_{(1, 23)} = 7.438$, $p < 0.05$). Each column represents the mean with S.E.M. of 11–14 mice/group. (B) Lack of anxiogenic effect by prenatal and neonatal exposures to bisphenol-A using the elevated-plus-maze procedure. Prenatal and neonatal exposures to bisphenol-A failed to change the percentage of time spent in the open arms (All group: $F_{(2, 15)} = 0.391$, $p > 0.05$, Control vs. Low: $F_{(1, 9)} = 1.128$, $p > 0.05$, Control vs. High: $F_{(1, 11)} = 0.429$, $p > 0.05$, Low vs. High: $F_{(1, 10)} = 0.044$, $p > 0.05$). Each column represents the mean with SEM of 5–7 mice/group. (C and D) Lack of motor learning impairment by prenatal and neonatal exposures to bisphenol-A using the rota-lod test. (C) The time that the animal remained on a rotating rod at 10 rpm was measured twice a day. A maximum of 300 s was allowed for each animal per trial. The score was the mean of latencies in two trials (All group: $F_{(2, 34)} = 0.283$, $p > 0.05$, Control vs. Low: $F_{(1, 22)} = 1.045$, $p > 0.05$, Control vs. High: $F_{(1, 24)} = 0.284$, $p > 0.05$, Low vs. High: $F_{(1, 22)} = 2.271$, $p > 0.05$). Each point represents the mean with S.E.M. of 6–7 mice/group. (D) The number of falls from a rotating rod to the ground during 300 s was counted twice a day. The score was the mean of numbers of fallings in two trials (All group: $F_{(2, 34)} = 0.318$, $p > 0.05$, Control vs. Low: $F_{(1, 22)} = 0.645$, $p > 0.05$, Control vs. High: $F_{(1,24)} = 0.027$, $p > 0.05$, Low vs. High: $F_{(1, 22)} = 0.259$, $p > 0.05$). Each point represents the mean with S.E.M. of 6–7 mice/group. (E and F) The effects of prenatal and neonatal exposures to bisphenol-A on performance in a step-through passive avoidance procedure. (E) At conditioning, the mice were placed in the lighted compartment of a two-compartment box and received a foot shock as soon as they stepped into the dark compartment. The step-through latency of the mice prenatally and neonatally exposed to low and high doses of bisphenol-A was similar to that of the control mice (All group: $F_{(2, 15)} = 0.401$, $p > 0.05$, Control vs. Low: $F_{(1, 9)} = 0.882$, $p > 0.05$, Control vs. High: $F_{(1, 10)} = 0.003$, $p > 0.05$, Low vs. High: $F_{(1, 11)} = 0.584$, $p > 0.05$). (F) Prenatal and neonatal exposures to low and high doses of bisphenol-A induced a significant memory impairment (All group: $F_{(2, 30)} = 5.766$, $p < 0.05$, Control vs. Low: $F_{(1, 18)} = 6.246$ $p < 0.05$, Control vs. High: $F_{(1, 20)} = 9.167$, $p < 0.05$, Low vs. High: $F_{(1, 22)} = 0.222$, $p > 0.05$). Each point represents the mean with S.E.M. of 5–7 mice/group.

of enzymes of central acetylcholine metabolism, the activities of ChAT and acetylcholinesterase in the hippocampus [8]. Actually, exposure to polychlorinated biphenyls, well known as one of the most common thyroid hormone disruptors, suppressed ChAT activity and spatial learning and memory deficits [3,6]. According to these reports, we hypothesized that the memory impairment induced by prenatal and neonatal exposures to bisphenol-A could be mediated by the disruption of endogenous hormones in the developing brain.

In the present study, we found that memory impairment associated with a drastic reduction of ChAT-IR in the hippocampus was induced by prenatal and neonatal exposures not only to high-dose, but also to low-dose bisphenol-A. Although it is very difficult to explain where the primary site of bisphenol-A is, we therefore must take into account its mechanism through nonhormonergic effects.

Knaak and Sullivan first reported the metabolic fate of bisphenol-A in rats, showing that the major metabolite in urine was the glucuronide of bisphenol-A; considerable amounts of free bisphenol-A and hydroxylated bisphenol-A were found in feces [7]. Many reports have shown that bisphenol-A is metabolized and excreted rapidly [10,17,20]. Taken together, it is almost impossible that the bisphenol-A remaining in the adult brain of mice directly affects CNS. Therefore, prenatal and neonatal

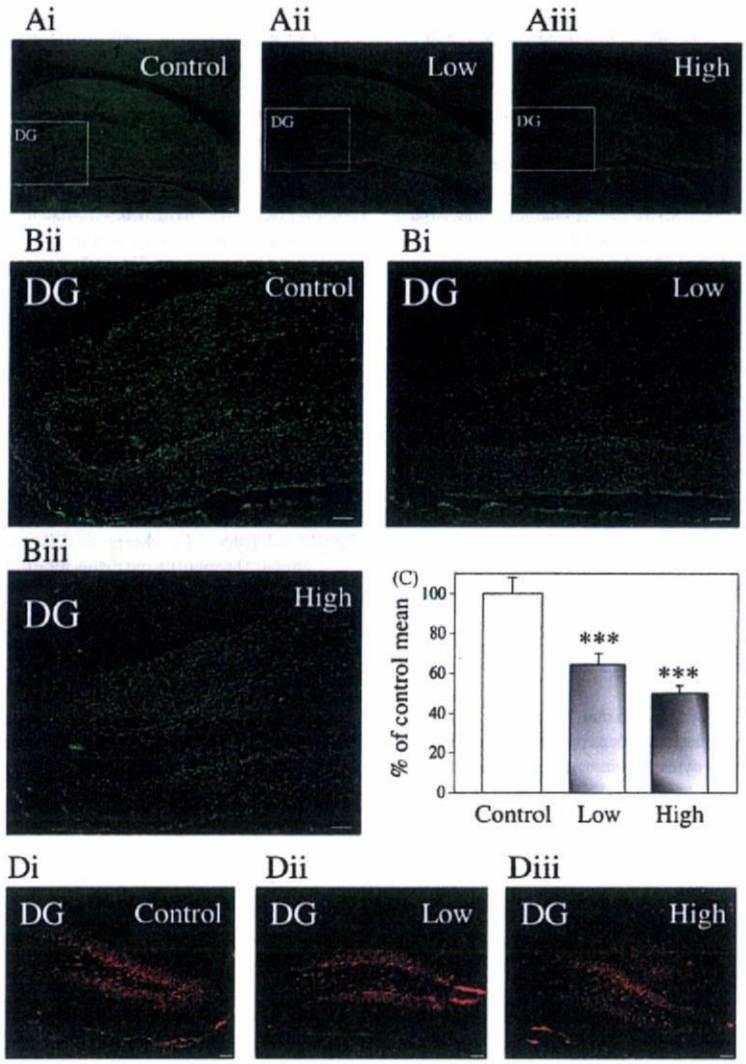

Fig. 2. Dramatic reduction in ChAT-like immunoreactivity in the hippocampus of the mice prenatally and neonatally exposed to low and high doses of bisphenol-A. (Ai–iii) Prenatal and neonatal exposures to low (Aii) and high doses (Aiii) of bisphenol-A dramatically decreased the level of ChAT-IR in the hippocampus compared to control (Ai). (Bi–iii) High magnification images showed that ChAT-IR in the DG. ChAT-IR was dramatically decreased in the DG by prenatal and neonatal exposures to low (Bii) and high doses (Biii) as compared to control (Bi). (C) A semi-quantitative analysis of ChAT-IR was performed using Image J (Low: 64.3 ± 5.6% of control mean, (***) $p < 0.001$ vs. control mice; High: 50.0 ± 3.8% of control mean, (***) $p < 0.001$ vs. Control mice). (Di–iii) On the other hand, prenatal and neonatal exposure to low (Dii) and high doses (Diii) of bisphenol-A faild to change NeuN-IR in the DG compared to control (Di). Each column represents the mean ± S.E.M. of three independent samples. DG: dentate gyrus. Scale bars: 50 μM.

exposures to bisphenol-A disrupt the neuron development, resulting in behavior abnormalities in the adult animals.

In conclusion, the present findings provide direct evidence that prenatal and neonatal exposures not only to high-dose, but also to low-dose of bisphenol-A dramatically decreases the cholinergic transmission in the adult brain, resulting in learning and memory deficits.

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Changes in central dopaminergic systems and morphine reward by prenatal and neonatal exposure to bisphenol-A in mice: evidence for the importance of exposure period

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ABSTRACT

Bisphenol-A has been extensively evaluated for toxicity in a variety of tests as the most common environmental endocrine disruptors. In a previous study, we reported that exposure to bisphenol-A affects the development of the central dopaminergic system in the mouse limbic area. The present study was undertaken to investigate the relationship between the developmental toxicity of bisphenol-A and its exposure period. The exposure to bisphenol-A during either organogenesis or lactation, but not implantation and parturition, significantly enhanced the morphine-induced hyperlocomotion and rewarding effects. Furthermore, exposure to bisphenol-A during either organogenesis or lactation also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These results indicate that both organogenesis and lactation are more sensitive to the bisphenol-A-induced developmental neuronal toxicology than any other periods. In conclusion, the present data suggest that the organogenesis and lactation are the most important period to cause the alternation of dopaminergic system by bisphenol-A exposure in the mouse.

Keywords Bisphenol-A, endocrine disruptor, exposure periods, hyperlocomotion, morphine, rewarding effect.

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INTRODUCTION

Many aquatic ecosystems might be polluted with bisphenol-A, a possible endocrine disruptor that is widely used as a monomer for the production of plastics, resins and coatings (Staples *et al.* 1998). The global mass production is 1.7 million tons in the world and 0.35 million tons in Japan (reported by the BPA Safety Committee of Japanese Manufactures 2002) and the chemical is widely used all over the world. Bisphenol-A is a global environmental contaminant, used in polycarbonate plastics, and is reported to display weak estrogenic activity both *in vitro* and *in vivo* (Krishnan *et al.* 1993; Ashby & Tinwell 1998; Gould *et al.* 1998; Kuiper *et al.* 1998). The bioactivity of bisphenol-A is 1000–15 000 times lower than 17β-estradiol.

It has been reported that administration of bisphenol-A 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 (Howdeshell *et al.* 1999). Bisphenol-A also inhibits the differentiation of oligodendrocyte precursor cells induced by exposure to thyroid hormone (Seiwa *et al.* 2004). These results support the idea that bisphenol-A may cause toxicity in the developmental process.

Recently, we have demonstrated that prenatal and neonatal exposure to bisphenol-A markedly enhances the rewarding effects induced by drugs of abuse, such as methamphetamine (Suzuki *et al.* 2003) and morphine (Mizuo *et al.* 2004a). In addition, prenatal and neonatal exposure to bisphenol-A enhances central dopamine D_1 receptor function (Suzuki *et al.* 2003) and attenuates dopamine D_3 receptor function (Mizuo *et al.* 2004b) in mice. These findings indicate that exposure to bisphenol-A may cause alterations in dopaminergic neurotransmission in the central nervous system resulting in the enhancement of drug reward. The aim of the present

study was to further investigate the relationship between the effects of bisphenol-A on dopamine-related behaviors and the bisphenol-A exposure period, and to determine the most sensitive period in prenatal and neonatal exposure to bisphenol-A in mice.

MATERIALS AND METHODS

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 that had been prenatally and neonatally exposed to bisphenol-A (Wako Pure Chemical Industries Ltd, Osaka, Japan). Adult female mice were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 2 mg of bisphenol-A/g of food during the period of implantation [I-2; embryonic day (ED) 0–7], organogenesis (O-2; ED 7–14), parturition (P-2; ED 14–20) and lactation (L-2; postnatal day 0–20). Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. During the treatment with bisphenol-A, animals did not show weight loss or disrupted maternal behaviors. In addition, the pups did not show weight loss or decrease of birth rate. All experiments used mice aged 7–9 weeks.

Place conditioning

Place conditioning was conducted as previously described (Suzuki *et al.* 1991; Narita, Funada & Suzuki 2001). The apparatus was a shuttle box (15 × 30 × 15 cm: width × length × height), which was made of an acrylic resin board and divided into two equal-sized compartments. One compartment is white with a textured floor, and the other is 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 injection. The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. Conditioning sessions (3 days for morphine, 3 days for saline) were conducted once daily for 6 days. Immediately after subcutaneous injection (s.c.) injection of morphine (1 mg/kg), animals were placed in one compartment for 1 hour. On alternate days, animals receiving vehicle were placed in the other compartment for 1 hour. On day seven, tests of conditioning were performed as follows. The partition separating the two compartments was raised to 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. The mice were not treated with either morphine or saline, and then placed on the platform. The time spent in each compartment during a 900-second session was then recorded automatically using an infrared beam sensor (KN-80, Natsume Seisakusyo Co., Tokyo, Japan). The preference for drug-paired place was shown as a mean difference between the time spent during the drug-conditioning compartment and saline-conditioning compartment. All sessions were conducted under conditions of dim illumination (28-lux lamp) and white masking noise. In this study, we used 6–16 mice.

Measurement of locomotor activity

The locomotor activity of mice was measured by an ambulometer as described previously (Narita *et al.* 1993). 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 hours following the injection of saline (10 ml/kg, s.c.) or morphine (10 mg/kg, s.c.). In this study, we used 9–10 mice.

[^{35}S]GTPγS binding assay

In the membrane preparation, mice were killed by decapitation and the limbic forebrain was then dissected as described previously (Narita *et al.* 2001). 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), $MgCl_2$ and 1 mM ethylene glycol-bis-(beta-aminoethyl ether)-*N,N'*-tetra-acetic acid (EGTA) for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 4°C for 10 minutes at 48 000 × g. The pellet was resuspended in [^{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 minutes at 48 000 × g. The resultant pellet was resuspended in [^{35}S]GTPγS binding assay buffer and stored at −70°C until used. The membrane homogenate (3–8 μg of protein/assay) was incubated at 25°C for 2 hours in 1 ml of assay buffer with various concentrations of dopamine, 30 μM guanosine-5′-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL, USA). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters pre-soaked in 50 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$ at 4°C for 2 hours. Filters were then washed three times with 5 ml of 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 hours, 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.

Statistical analysis

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

RESULTS

Enhancement of morphine-induced hyperlocomotion in mice exposed to bisphenol-A during the organogenesis or lactation period

Treatment with 10 mg/kg (s.c.) morphine produced a locomotor-enhancing effect in all groups. In both O-2 and L-2 mice, but not I-2 and P-2, the hyperlocomotion induced by morphine was dramatically potentiated as compared with that in control ($P < 0.05$ versus control, Fig. 1).

Enhancement of morphine-induced rewarding effect in mice exposed to bisphenol-A during the organogenesis or lactation period

At the dose of 1 mg/kg, morphine produced neither place preference nor place aversion in control, I-2 and P-2 mice. However, treatment with 1 mg/kg morphine produced a significant place preference in both O-2 and L-2 ($P < 0.05$ versus control, Fig. 2).

The dopamine-induced G-protein activation in the limbic forebrain of mice exposed to bisphenol-A during the organogenesis or lactation period

Dopamine (0.1–10 μM) produced a concentration-dependent increase in [^{35}S]GTPγS binding to membranes from the limbic forebrain including the nucleus accumbens of control, I-2, O-2, P-2 and L-2 mice. It should be noted that the stimulation of [^{35}S]GTPγS binding induced by dopamine was markedly potentiated in O-2 and L-2 mice (Fig. 3).

DISCUSSION

Drug addiction in a pathological behavior characterized by compulsive drug seeking and drug ingestion despite severe adverse consequences. The place-conditioning paradigm has become the most frequently used method to evaluate the motivational properties and its use has been reported more frequently than the self-administration paradigm (Suzuki 1996).

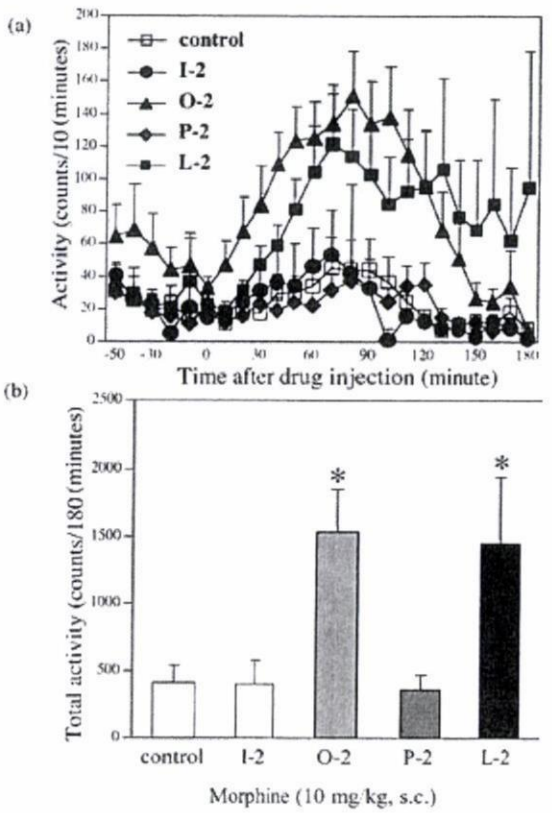

Figure 1 Enhancement of the morphine-induced hyperlocomotion in mice exposed to bisphenol-A during organogenesis or lactation. (a) Time-course changes in the morphine-induced hyperlocomotion in control and bisphenol-A-treated (I-2, O-2, P-2 and L-2) mice (I = implantation, O = organogenesis, P = parturition, L = lactation). Each point represents the mean activity counts for 10 minutes with SEM of 9–10 mice. O-2 (triangle), $F_{(1,299)} = 11.4$, $P < 0.01$ versus control (open square); L-2 (filled square), $F_{(1,299)} = 4.1$, not significant. (b) Total activity in the morphine-induced hyperlocomotion in control and bisphenol-A-treated (I-2, O-2, P-2 and L-2) mice. Mice exposed to bisphenol-A in each period are shown as I-2, O-2, P-2 and L-2 groups. Each column represents the mean total activity counts for 180 minutes with SEM of 9–10 mice. *$P < 0.05$ versus control. s.c. = subcutaneous injection

Many studies have suggested that the mesolimbic dopaminergic system that projects from the ventral tegmental area (VTA) to the nucleus accumbens is critical for the initiation of opioid reinforcement and hyperlocomotion (Stinus *et al.* 1986; Wise & Rompre 1989; Koob 1992). Either [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO)- or morphine-induced place preference can be blocked by dopamine receptor antagonists (Phillips, LePiane & Fibiger 1983; Shippenberg, Bals-Kubik & Herz 1993). In addition, hyperlocomotion induced by morphine can be blocked by treatment with dopamine receptor antagonists in the nucleus accumbens (Maldonado *et al.* 1990; Funada, Suzuki & Misawa 1994). These

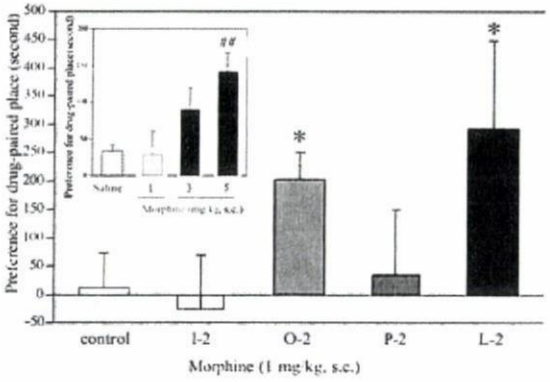

Figure 2 Enhancement of the morphine-induced rewarding effect in mice exposed to bisphenol-A during organogenesis or lactation. (Inner) Dose–response for the morphine-induced place preference in control mice (##$P < 0.01$ versus saline-treated mice). (Outer) Mice exposed to bisphenol-A in each period are shown as I-2, O-2, P-2 and L-2 groups (I = implantation, O = organogenesis, P = parturition, L = lactation). Each column represents the mean conditioning score with SEM of 6–16 mice. *$P < 0.05$ versus control. s.c. = subcutaneous injection

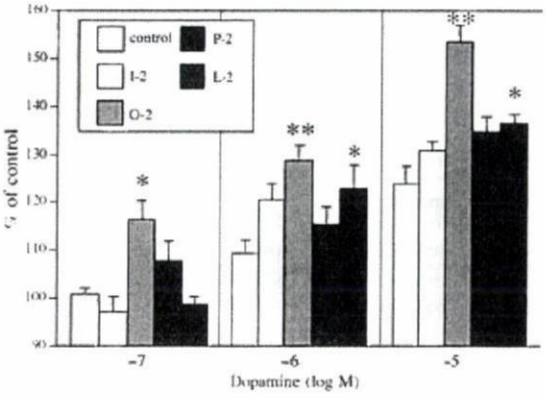

Figure 3 Comparison of the stimulation of [^{35}S]GTPγS binding to membranes from the limbic forebrain by dopamine between control and bisphenol-A-treated mice. Membranes were incubated with [^{35}S]GTPγS (50 pM) and guanosine-5′-diphosphate (GDP) (30 μM) with dopamine. The data are shown as the percentage of basal [^{35}S]GTPγS binding measured in the presence of GDP and absence of dopamine. Mice exposed to bisphenol-A in each period are shown as I-2, O-2, P-2 and L-2 groups (I = implantation, O = organogenesis, P = parturition, L = lactation). Each column represents the mean with SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$ versus control

findings indicate that the dopamine-containing neuron of the midbrain VTA, which has a high density of μ-opioid receptors, plays a critical role in the rewarding effects and hyperlocomotion by μ-opioid receptor agonists. In terms of dopamine receptor involvement at the terminal site of the mesolimbic dopamine system, the rewarding effects of abused drug have been shown to be mediated by dopamine D_1 receptors (Shippenberg *et al.* 1993; Suzuki *et al.* 1993). In addition, the dopamine D_3 receptor cloned by Sokoloff *et al.* (1990) has been characterized extensively. The dopamine D_3 receptor shows a distinct distribution in limbic areas of the brain, including the nucleus accumbens and olfactory tubercle (Sokoloff *et al.* 1990). Several pharmacological studies with dopamine D_3 receptor-preferring agonists such as 7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OH-DPAT) suggest that the dopamine D_3 receptor regulates the inhibitory effect to produce hyperlocomotion in rodents (Suzuki *et al.* 1995; De Boer *et al.* 1997). In addition, we found that the morphine-induced rewarding effect and hyperlocomotion were markedly enhanced in mice lacking the dopamine D_3 receptor gene (Narita *et al.* 2003). These findings suggest that the dopamine D_3 receptor plays a critical role in mediating drug-induced effects on dopamine neurotransmission.

In the previous study, we reported that prenatal and neonatal exposure to bisphenol-A enhances the rewarding effect of drugs of abuse, which is associated with the up-regulation of central dopamine D_1 receptor function and down-regulation of the functional dopamine D_3 receptors in mice (Suzuki *et al.* 2003; Mizuo *et al.* 2004a,b). In the present study, we investigated the relationship between these neuronal changes and the exposure period for bisphenol-A. The exposure to bisphenol-A during either organogenesis or lactation significantly enhanced the morphine-induced hyperlocomotion and rewarding effect. These findings suggest that prenatal and neonatal, especially organogenetical and lactational, exposure to bisphenol-A leads to the supersensitivity of drugs of abuse-induced pharmacological actions.

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 neurons, leading to the increased dopamine release in the nucleus accumbens. In a previous study, we reported that prenatal and neonatal exposure to bisphenol-A failed to enhance μ-opioid receptor-mediated G-protein activation by morphine in the lower midbrain (Mizuo *et al.* 2004a). We further showed that the expression of μ-opioid receptor mRNA was not changed by chronic bisphenol-A treatment, suggesting that μ-opioid receptor function is unaffected in this region (Mizuo *et al.* 2004a). Therefore, we next investigated the influence of prenatal and neonatal exposure to bisphenol-A in mesolimbic dopaminergic function using [^{35}S]GTPγS binding assay. The exposure to bisphenol-A during either organogenesis or lactation also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These

results indicate that either organogenesis or lactation is more sensitive to the bisphenol-A-induced neuronal toxicity than any other periods.

Recently, several investigations have provided evidence that the treatment of adult animals with bisphenol-A could not affect the reproductive function and social behaviors (Cagen *et al.* 1999; Farabollini *et al.* 2002). We have already confirmed that acute administration of bisphenol-A with adult mice could not affect the dopamine-related behaviors (data not shown). On the other hand, several investigations clarified the behavioral abnormalities by prenatal and neonatal exposure to bisphenol-A. These findings indicate that prenatal and neonatal exposure to bisphenol-A may cause the neuronal toxicity specifically in the developmental process. In the present study, we focused on the relationship between these developmental changes and the exposure period for bisphenol-A. The exposure to bisphenol-A during either organogenesis or lactation significantly enhanced the morphine-induced hyperlocomotion and rewarding effect. Furthermore, the exposure to bisphenol-A during either organogenesis or lactation also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These results indicate that either organogenesis or lactation is more sensitive to the bisphenol-A-induced developmental toxicity than any other periods.

Generally, in cerebral development, it is well known that the proliferation, differentiation or migration of nerve cells and glia cells are carried out most briskly at organogenesis (Temple 2001). Additionally, the functional development of the central nerves system, synaptogenesis and the construction of the nerve network are carried out most briskly at lactation (Temple 2001). Therefore, these reports strongly support our present results that these are most sensitive periods for the influence of bisphenol-A exposure in the development of the central nervous system. Our findings suggest the idea that exposure during organogenesis to bisphenol-A could affect the differentiation or migration of neuronal stem cells. Additionally, lactation exposure to bisphenol-A affects the functional development of the central nervous system including synaptogenesis and the construction of the neuronal network.

Taken together, the present data may explain that although the treatment of adult animals with bisphenol-A could not affect the reproductive function and social behaviors, the prenatal and neonatal exposure, especially either organogenesis or lactation, to bisphenol-A induced developmental neuronal toxicity in the midbrain of rodents. Our findings warn that exposure to bisphenol-A during either organogenesis or lactation may predispose their children to the development of dopamine-related disorders.

Acknowledgements

This work was supported in part by grants from the Ministry of Health, Labour and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect

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Abstract

Bisphenol-A has been extensively evaluated for toxicity in a variety of tests as the most common environmental endocrine disruptors. In the previous study, we reported that prenatal and neonatal exposure to high-dose of bisphenol-A affects the development of central dopaminergic system in the mouse limbic area. The present study was then undertaken to investigate whether prenatal and neonatal exposure to lower dose of bisphenol-A could change the morphine-induced several pharmacological actions such as rewarding effect and hyperlocomotion in mice. Prenatal and neonatal exposure to low-dose of bisphenol-A enhanced the morphine-induced hyperlocomotion and rewarding effect. Additionally, the treatment with bisphenol-A produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain, which is thought to play a critical role for hyperlocomotion and rewarding effects by drugs of abuse. These findings suggest that prenatal and neonatal exposure to low-dose of bisphenol-A can potentiate the central dopamine receptor-dependent neurotransmission, resulting in the supersensitivity of the morphine-induced hyperlocomotion and rewarding effects in the mouse.

Keywords: Bisphenol-A; Dopamine; Morphine; Rewarding effect; Hyperlocomotion; Endocrine disruptor

Bisphenol-A is an environmental endocrine-disrupting chemical that affects reproduction in wildlife [4,5]. Bisphenol-A is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins, which are used in the food cans and found as a contaminant not only in the liquid of the preserved foods, but also in the water autoclaved in the cans [1,7]. This chemical is also released from polycarbonate flasks during autoclaving [9]. Moreover, it has been reported that significant amounts of bisphenol-A are detected in the saliva of dental patients treated with fissure sealants [15].

On the endocrine-disrupting chemical problems, the low-dose actions of the endocrine-disrupting chemicals are serious problems. However, little is known about its action on the central nervous system induced by low-dose of bisphenol-A. The aim of the present study was then undertaken to investigate whether prenatal and neonatal exposure to low-dose of bisphenol-A in mice could affect the rewarding effect and locomotor-enhancing effects induced by morphine.

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

All experiments were performed using 7 weeks old male ddY mice (Tokyo Animal Science Co., Tokyo, Japan) that had been prenatally and neonatally exposed to bisphenol-A (Wako Pure Chemical Industries Ltd., Osaka, Japan). Adult female mice (10 weeks old) were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 3×10^{-2}, 3×10^{-1}, 3, 5×10^{2}, 2×10^{3} μg bisphenol-A/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. During the treatment with bisphenol-A, animals did not show weight loss and disruption of maternal behaviors.

The place conditioning paradigm has been known to the method to evaluate the motivational properties as the self-administration paradigm [13,18]. The apparatus was a shuttle box ($15 \times 30 \times 15$ cm: $w \times l \times h$) which was made of acrylic resin board and divided into two equal-sized compartments. One compartment is white with a textured floor, and the other is

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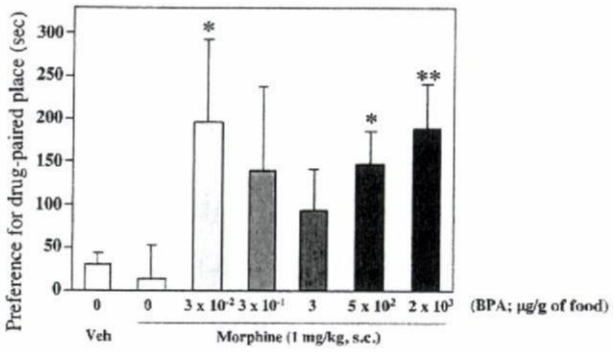

Fig. 1. Effect of prenatal and neonatal exposure to a wide range of concentrations of bisphenol-A on the morphine (1 mg/kg, s.c.) -induced rewarding effect in mice. Each column represents the mean conditioning score with S.E.M. of 6–14 mice. $^{*}p<0.05$, $^{**}p<0.01$ vs. morphine-treated control group.

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 injection. 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 is placed in the test apparatus without any confinements. At the dose of 1 mg/kg (s.c.), morphine produced neither place preference nor place aversion in control mice (Fig. 1). However, treatment with 1 mg/kg (s.c.) of morphine produced a significant place preference in the mice chronically treated with low- and high-dose of bisphenol-A, but not middle-dose of bisphenol-A, from mating to weaning (Fig. 1). The locomotor activity of mice was measured by an ambulometer as described previously [14]. 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. Treatment with 10 mg/kg (s.c.) of morphine produced a locomotor-enhancing effect in all groups (Fig. 2). In mice chronically treated with low- and high-dose of bisphenol-A, but not middle-dose of bisphenol-A, from mating to weaning, the hyperlocomotion induced by morphine was dramatically potentiated as compared to that in control (Fig. 2). These findings suggest that prenatal and neonatal exposure to low- and high-dose of bisphenol-A lead to the supersensitivity of morphine-induced pharmacological actions. It should be mentioned that prenatal and neonatal exposure to bisphenol-A shows the biphasic effect on the supersensitivity of morphine-induced pharmacological actions. Recently, several investigations have provided evidence that the treatment of adult animals with bisphenol-A could not affect the reproductive function and social behaviors [2,3]. We have already confirmed that acute administration of bisphenol-A with adult mice could not affect the dopamine-related behaviors (data not shown). On the other hand, the behavioral abnormalities are induced by prenatal and neonatal exposure to bisphenol-A [11,12,19,20]. These findings indicate that prenatal and neonatal exposure to bisphenol-A may cause the neuronal toxicity specifically in the developmental process. We next investigated the influence of prenatal and neonatal exposure to bisphenol-A in the development of central dopaminergic function using [^{35}S]GTPγS binding assay as described previously [20]. In the membrane preparation, mice were killed by decapitation and the limbic forebrain including the nucleus accumbens was then dissected as described previously [17]. 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), $MgCl_2$ 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 of [^{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. 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 dopamine, 30 μM guanosine-5′-diphosphate

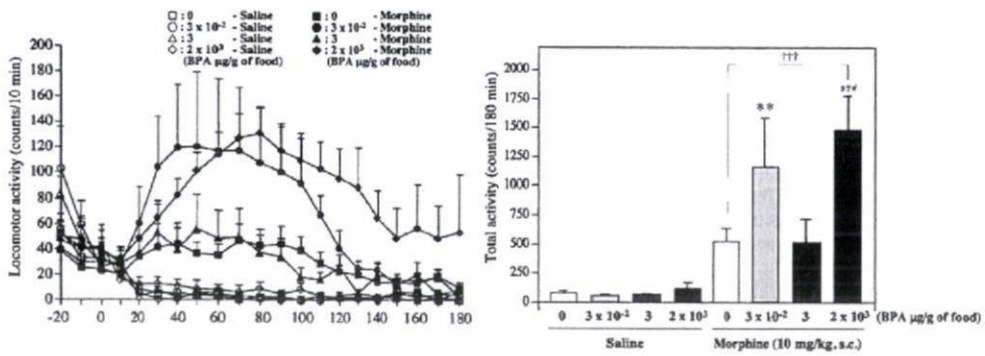

Fig. 2. Effect of prenatal and neonatal exposure to a wide range of concentrations of bisphenol-A on the morphine-induced hyperlocomotion in mice. (A) Time course changes in the morphine-induced hyperlocomotion in mice. Each point represents the mean activity counts for 10 min with S.E.M. of 5–15 mice. (B) Total locomotor activity of morphine-induced locomotor enhancing effect in mice. Each column represent the mean activity for 180 min with S.E.M. of 5–15 mice. $^{**}p<.0.01$ vs. saline-treated 3×10^{-2} BPA μg/g of food-treated group. $^{\#\#\#}p<0.001$ vs. saline-treated 2×10^{3} BPA μg/g of food-treated group. $^{\dagger\dagger\dagger}p<0.001$ vs. morphine-treated control group.

(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 (10 μM) produced an increase in [^{35}S]GTPγS binding to membranes from the limbic forebrain including the nucleus accumbens of control mice. Under these conditions, the stimulation of [^{35}S]GTPγS binding induced by dopamine was potentiated in mice chronically treated with wide range of concentrations of bisphenol-A from mating to weaning. Especially, the enhancement of the stimulation of [^{35}S]GTPγS binding induced by dopamine in mice chronically treated with low- and high dose of bisphenol-A was noted, witch suggests that bisphenol-A shows the biphasic effect on the regulation of dopamine receptor function in the limbic forebrain (Fig. 3). Taken together, these findings suggest that the supersensitivity of morphine-induced pharmacological actions following prenatal and neonatal exposure to especially low- and high dose of bisphenol-A may result from a drastic up-regulation of dopamine receptor function in the limbic forebrain (see Fig. 4).

All of data represent the mean counts with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

As mentioned above, humans might be orally exposed to bisphenol-A in daily life. In the previous study, we chronically treated female mice with bisphenol-A-admixed powder food containing 2×10^3 μg of bisphenol-A/g of food, and this

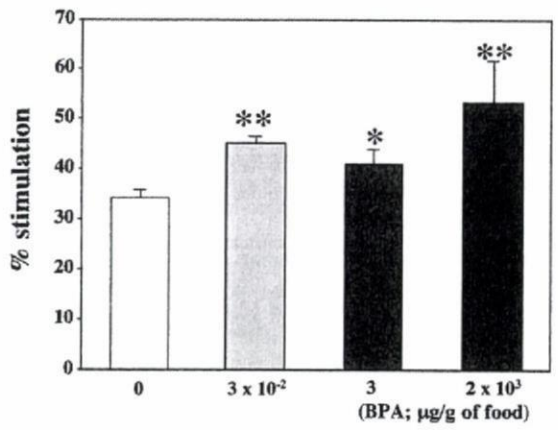

Fig. 3. Comparison of the stimulation of [^{35}S]GTPγS binding to membranes from the limbic forebrain by dopamine between control and a wide range of concentrations of bisphenol-A-treated mice. Membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with dopamine. The data are shown as the percentage of basal [^{35}S]GTPγS binding measured in the presence of GDP and absence of dopamine. Each column represents the mean with S.E.M. of three samples. $^{**}p < 0.01$ vs. control group.

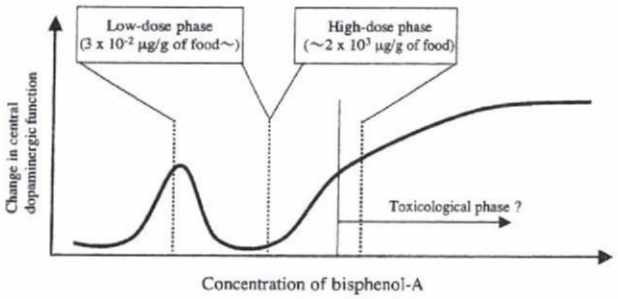

Fig. 4. A schematic drawing of the biphasic effect on the central dopaminergic function by prenatal and neonatal exposure to bisphenol-A. In the previous study, we reported that prenatal and neonatal exposure to high dose ($2–2 \times 10^3$ μg/g of food) of bisphenol-A change the central dopaminergic function. Here, our findings suggest that supersensitivity of morphine-induced pharmcological actions following prenatal and neonatal exposure to low dose (3×10^{-2}–3 μg/g of food) of bisphenol-A may result from an up-regulation of dopamine receptor function in the limbic forebrain.

enhanced the rewarding effect induced by drugs of abuse in their pups [11,12,20]. Under these conditions, the blood level of bisphenol-A in their pups was approximately 10 ng/ml, which is considered to be more than 30 times higher than the level for healthy human exposure [20]. On the other hand, vom Saal et al. estimated that humans are exposed to bisphenol-A at a dose of 2–20 μg/kg/day [21]. Based on these reports, we here ascertained the effects of low dose of exposure to bisphenol-A. Adult female mice were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 3×10^{-2}, 3×10^{-1}, 3×10^2, 5×10^2, 2×10^3 μg bisphenol-A/g of food from mating to weaning.

Bisphenol-A and alkylphenols have been reported to have estrogenic activity [8]. Recent molecular studies have suggested the transcriptional activation of the human dopamine D_1 receptor gene by estrogen [10]. Since bisphenol-A has only very weak estrogenic effects [23], it does not seem likely that low dose of bisphenol-A is accompanied by a classical estrogenic activity. It was reported that the binding of bisphenol-A to the non-classical membrane-bound estrogen receptor activates a guanylyl cyclase, protein kinase G and closing K_{ATP} channels [16]. Additionally, low dose of bisphenol-A can activate the transcription factor, cAMP-responsive element binding protein (CREB). Phosphorylated CREB has been shown to be increased after only a 5 min application of bisphenol-A in a calcium-dependent manner [16]. Therefore, the supersensitivity of morphine-induced pharmacological actions caused by prenatal and neonatal exposure to low dose of bisphenol-A may be mediated by non-classical membrane-bound estrogen receptors.

On the other hand, the animal model for hyperactivity was produced by Shaywitz et al., who demonstrated that rat pups treated with 6-hydroxydopamine (6-OHDA) via intracisternal administration at 5 days of age developed increased motor activity caused by the reduction of tyrosine hydroxylase (TH)-sensitive dopamine, leading to cognitive difficulties in shuttle-box learning between 2 and 4 weeks of age [17]. Additionally, Ishido et al. have reported that high dose of bisphenol-A affects the central dopaminergic system, resulting in hyperactivity due most likely to a large reduction TH activity in the midbrain

[6]. Based on these reports, we hypothesize that prenatal and neonatal exposure to high dose of bisphenol-A may cause the dopamine depletion for the limited time period and in turn induce the long-lasting supersensitivity of dopamine receptor-related action following chronic treatment with morphine.

It is very difficult to explain the fact that the prenatal and neonatal exposure to middle-dose of bisphenol-A have only weak effect on the disruption of functional changes in the dopaminergic transmission. Although the mechanisms of the weak effect by the prenatal and neonatal exposure to middle-dose of bisphenol-A remain unclear, one possibility is that the potentiation of the central dopaminergic transmission caused by the prenatal and neonatal exposure to low dose of bisphenol-A can be offset by the middle-dose of bisphenol-A through the negative feedback regulation. It is also likely that prenatal and neonatal exposure to high dose of bisphenol-A may potentiate the dopamine receptor function following a dramatic deletion of TH-sensitive dopamine and/or dysfunction of negative feedback mechanism against dopamine receptor function following the overshooting of its negative feedback.

On the endocrine-disrupting chemical problems, the low dose actions are serious problems. As well as described in the present study, it was recently reported that there were effects caused by exposure to low doses of bisphenol-A on rate of growth and sexual maturation, hormone levels in blood, reproductive organ function, fertility, immune function, enzyme activity, and brain structure, brain chemistry and behavior [22]. Therefore, our findings warn that prenatal and postnatal exposure to low- and high doses of bisphenol-A may dramatically change the neuronal transmission including dopaminergic transmission in the adult brain. This phenomenon could explain the aggravation of the development of dependence on drugs of abuse.

Acknowledgments

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Dynamic Changes in Dopaminergic Neurotransmission Induced by a Low Concentration of Bisphenol-A in Neurones and Astrocytes

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Key words: bisphenol-A, neurone, astrocyte, dopamine, 17β-oestradiol, drug abuse.

Abstract

One of the most common chemicals that behaves as an endocrine disruptor is the compound 4,4′-isopronylidenediphenol, called bisphenol-A (BPA). We previously reported that prenatal and postnatal exposure to BPA potentiated central dopaminergic neurotransmission, resulting in supersensitivity to psychostimulant-induced pharmacological actions. Many recent findings have supported the idea that astrocytes, which are a subpopulation of glial cells, play a critical role in neuronal transmission in the central nervous system. The present study aimed to investigate the role of neurone–astrocyte communication in the enhancement of dopaminergic neurotransmission induced by BPA. We found that treatment of mouse purified astrocytes and neurone/glia cocultures with BPA *in vitro* caused the activation of astrocytes, as detected by a stellate morphology and an increase in levels of glial fibrillary acidic protein. A low concentration of BPA significantly enhanced the Ca^{2+} responses to dopamine in both neurones and astrocytes. Furthermore, a high concentration of BPA markedly induced the activation of caspase-3, which is a marker of neuronal apoptotic cell death in mouse midbrain neurone/glia cocultures. By contrast, treatment with 17β-oestradiol (E_2) had no such effects. Prenatal and neonatal exposure to BPA led to an enhancement of the dopamine-dependent rewarding effect induced by morphine. These findings provide evidence that BPA alters dopamine responsiveness in neurones and astrocytes and that, at least in part, it may contribute to potentiate the development of psychological dependence on drugs of abuse.

The foetus uses natural hormonal messages that originate in its own hormone system and that of its mother for developmental guidance. Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. One of the most common endocrine disruptors is the compound 4,4′-isopronylidenediphenol, called bisphenol-A (BPA), which is used in the manufacture of many types of products. These include polycarbonate plastic food storage containers (i.e. baby bottles and water carboys), the lining of food or beverage cans (1, 2), dental composites and sealants and a bioactive bone cement, indicating the potential for human exposure to BPA in daily life.

Our recent studies suggest that exposure to BPA during prenatal and postnatal development has long-lasting effects on central dopaminergic systems linked with behavioural rewarding effects, as well as drug addiction and the reward induced by drugs of abuse (3, 4). The dopamine projection most often linked with a behavioural-rewarding effect is the mesolimbic dopamine system, which originates from the ventral tegmental area (VTA) and terminates at the nucleus accumbens.

We previously demonstrated that prenatal and neonatal exposure to BPA markedly enhanced the rewarding effects induced by drugs of abuse, such as methamphetamine (5) and morphine (6). We also demonstrated that, in adult mice, prenatal and neonatal exposure to BPA enhanced function mediated by central D_1 receptors, which play a substantial role in the rewarding effect of drugs of abuse (5). These treatments also attenuated dopamine function mediated by the D_3 receptor subtype that contributes to the inhibitory modulation of D_1/D_2 receptor-mediated signalling (7, 8). These findings indicate that exposure to BPA during development alters dopaminergic neurotransmission in the central nervous system (CNS), which results in enhancement of the psychological dependence on drugs of abuse. However, the mechanisms underlying these enduring effects of BPA are unknown.

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Many toxic stimuli activate astrocytes, as determined by morphological changes and by an increase in the levels of glial fibrillary acidic protein (GFAP), which is a marker of astrocytes (9, 10). The activation of astrocytes may control the structural and functional plasticity of synapses in the CNS. However, long-term exposure to drugs of abuse can induce neuronal plasticity (11, 12), and we have shown that treatment of mouse cortical neurone/glia cocultures with drugs of abuse, such as methamphetamine and morphine, caused morphological changes in astrocytes (13). Moreover, treatment with methamphetamine increased the sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids (13). Together, these findings indicate that astrocytes may play an important role in the development of dependence on drugs of abuse.

Although BPA may affect dopaminergic signalling in the CNS, little is known about the role of BPA in neurone-astrocyte communication. The present study aimed to clarify the effect of BPA in neurone-glia communication. We used mouse midbrain neurone/glia cocultures and purified astrocytes to determine the effects of BPA in the mesolimbic dopamine system.

Because the sex steroid hormones (oestrogens and androgens) have been shown to exert profound effects on brain differentiation, neural plasticity and central neurotransmission (14, 15) and BPA has an affinity for oestrogen receptors, albeit 1 : 2000 that of 17β-oestradiol (E_2) (1), we also investigated the effect of E_2 on astrocytic and neuronal responses and the rewarding effect induced by morphine.

Materials and methods

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Tissue processing

Purified midbrain astrocytes

Midbrains were dissected from ICR mice at postnatal 1 day (Tokyo Laboratory Animals Science, Tokyo, Japan), minced, and treated with trypsin (0.025%; Invitrogen, Grand Island, NY, USA) dissolved in phosphate-buffered saline (PBS) solution containing 0.02% L-cysteine monohydrate (Sigma-Aldrich, St Louis, MO, USA), 0.5% glucose (Wako Pure Chemicals, Osaka, Japan) and 0.02% bovine serum albumin (Wako Pure Chemicals). After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (20 min, 1000 *g*). After centrifugation, cells were plated in a flask (75 cm^2 culture flask; Corning Inc., Corning, NY, USA). Seven days after seeding in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% precolostrum newborn calf serum (FBS, Invitrogen), 5% heat-inactivated (56 °C, 30 min) horse serum (HS, Invitrogen), 10 U/ml penicillin and 10 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C, the flask was shaken for 12 h at 37 °C to remove nonastrocytic cells. Seven days after seeding, the cells were seeded at a density of 1×10^5 cells/cm^3, and maintained for 7 days in DMEM supplemented with 5% FBS, 5% HS, 10 U/ml penicillin and 10^{-5} g/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Midbrain neurone/glia cocultures

Midbrains were obtained from newborn ICR mice at postnatal 1 day, minced, and treated with papain (9 U/ml; Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% bovine serum albumin. After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (10 min, 1000 *g*). The cells were then seeded at a density of 2×10^6 cells/cm^3. The cells were maintained for 7 days in DMEM supplemented with 10% FBS, 10 U/ml penicillin and 10 μg/ml streptomycin. Eight days after seeding, the cells were treated with drugs. In this culture condition, NeuN-positive neurones are surrounded by astrocytes (16).

Drug treatment and immunohistochemistry

Eight days after seeding, *in vitro*, the cells were treated with either normal medium or medium containing bisphenol A (BPA; 10 fM to 1 μM; Wako Pure Chemicals) or 1,3,5[10]-estratriene-3,17β-diol (E_2, 10 fM to 1 μM; Sigma-Aldrich) for 24 h. To explore role for steroid hormone receptors in mediating the effects of BPA (1 pM or 1 μM, 24 h), cells were pretreated with either an oestrogen receptor antagonist 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]-nonyl]estra-1,3,5(10)-triene-3.17-diol (ICI182,780; 100 nM, 1 μM or 2 μM; Tocris-Cookson, Ellisville, MO, USA), an oestrogen receptor agonist/antagonist tamoxifen (100 nM, 1 μM or 10 μM; Sigma-Aldrich), a progesterone receptor antagonist mifepristone (100 nM, 1 μM or 10 μM; Sigma-Aldrich) or an androgen receptor antagonist flutamide (100 nM, 1 μM or 10 μM; Sigma-Aldrich) for 24 h. Cells were then treated with normal medium or BPA (1 pM, 1 mM) with or without these steroid hormone receptor ligands for an additional 24 h. Glial cells were then identified by immunofluorescence using mouse anti-glial fibrillary acidic protein antibody (GFAP, dilution 1 : 1000; Chemicon Inc., Temecula, CA, U.S.A), rabbit anti-GFAP (dilution 1 : 1000, Chemicon) or mouse anti-neuronal nuclei (Neu-N) antibody (dilution 1 : 1000, Chemicon) followed by incubation with Alexa 488-conjugated goat anti-mouse IgG (dilution 1 : 4000), Alexa 488-conjugated goat anti-rabbit IgG (dilution 1 : 4000) or Alexa 546-conjugated goat anti-mouse IgG (dilution 1 : 4000). Images were collected using a Radiance 2000 laser-scanning microscope (Radiance 2000; Bio-Rad, Carlsbad, CA, USA).

The intensity of GFAP-like immunoreacivity was measured with a computer-assisted system (NIH Image, Bethesda MD, USA). The upper and lower threshold intensity ranges were adjusted to encompass and match the immunoreactivity to provide an image with immunoreactive material appearing in black pixels, and nonimmunoreactive material as white pixels. The area and intensity of pixels within the threshold value representing immunoreactivity were calculated. We randomly chose 10 areas (80 × 80 pixels) for calculation of GFAP-like immunoreactivity in each image (512 × 512 pixels). The experiments were repeatedly performed by at least three independent culture preparations. The intensity of GFAP-like immunoreactivity was expressed as a percent increase (mean ± SEM) with respect to that in control cells, which were seeded on the same plate.

To evaluate the apoptotic neuronal cell death, mouse midbrain neurone/glia cocultures were treated with normal medium, BPA (1 pM, 1 nM or 1 μM) or E_2 (1 pM, 1 nm or 1 μM) for 24 h. The cells were then identified by immunofluorescence, using rabbit-anticleaved caspase-3 antibody (dilution 1 : 100; Cell Signaling Technology Inc., Beverly, MA, USA), followed by incubation with Alexa 488 conjugated goat anti-rabbit IgG (dilution 1 : 10000). Images were collected using a Radiance 2000 laser-scanning microscope.

Confocal Ca^{2+} imaging

Confocal Ca^{2+} imaging was conducted as previously described (13, 16). Mouse midbrain neurone/glia cocultures or purified astrocytes were incubated for 24 h with normal medium or medium containing BPA (1 pM, 1 nM or 1 μM). Cells were then loaded with 10 μM fluo-3 acetoxymethyl ester (Dojindo Molecular Technologies, Kumamoto, Japan) during a 90-min incubation at room temperature. After a further 20–30 min of de-esterification with the acetoxymethyl ester, the cells which seeded on coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance 2000). Fluo-3 was excited with the 488-nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths > 515 nm, and average baseline fluorescence (F_0) of each cell was calculated. To compensate for the uneven distribution of fluo-3, self-ratios were calculated (ratio: $R_s = F/F_0$). The amplitude was determined by subtracting the average of baseline fluorescence ratio (F_{basal}/F_0) from the maximum of fluorescence ratio after a drug treatment (F_{max}/F_0). Dopamine (1, 10 or 100 μM; Sigma-Aldrich) was perfused via a plastic tube for 30 s at 5 ml/min at room temperature in cultured cortical neurones or astrocytes followed by superfusion of basal salt solution (BSS,

pH 7.4) containing 150 mM of NaCl, 5 mM of KCL, 1.8 mM of $CaCl_2$, 1.2 mM of $MgCl_2$, 25 mM of N-2-hydroxyethylpiperazine-N'-2-ethaneslufonic acid and 10 mM of D-glucose.

Prenatal and neonatal exposure to BPA

All experiments were performed using male ddY mice (7 weeks old) (Tokyo Laboratory Animals Science) that had been indirectly exposed prenatally and neonatally to BPA, administered to their mothers.

Mice were orally administered either olive oil (control; 0.1 mg/kg), BPA (3 μg/kg/day or 200 mg/kg/day) dissolved in olive oil (Wako Pure Chemicals) or E_2 (3 μg/kg/day) through the stomach sonde. Female mice (10 weeks old) were orally treated with these chemicals three times a day (08.00, 14.00 and 20.00 h): from mating to weaning. Therefore, these chemicals were administered during pregnancy (20 days) and lactation (21 days, total 41 days).

Place conditioning

The place-conditioning procedure has been used to evaluate the motivation properties, such as rewarding or aversive effects, of drugs in adult rodents (17). Place conditioning was conducted as previously described (5, 6). 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 is white with a textured floor, and the other is black with a smooth floor to create equally preferred compartments. For conditioning, groups of mice (seven mice in a group) were confined to one compartment after morphine injections (morphine-paired side) and to the other compartment after saline injection (saline-paired side). The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. Conditioning sessions (3 days for morphine, 3 days for saline) were conducted once daily for 6 days. Immediately after s.c. injection of morphine (1 mg/kg), animals were placed in one compartment for 1 h. On alternate days, animals receiving the vehicle were placed in the other compartment for 1 h. On day 7, tests of conditioning were performed. The partition separating the two compartments was raised to 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. The mice were not treated with either morphine or saline, and then placed on the platform. The time spent in each compartment during a 900-s session was then recorded automatically using an infrared beam sensor (KN-80, Natsume Seisakusyo Co., Tokyo, Japan). All sessions were conducted under conditions of dim illumination (28 lux lamp) and white masking noise.

Statistical analysis

Data for GFAP-like immunoreactivity and confocal Ca^{2+} imaging are presented as the mean ± SEM. The statistical significance of differences between the groups were assessed by one-way analysis of variance (ANOVA) followed by Student's t-test.

Conditioning scores for each mouse were obtained by subtracting the cumulative time spent in the saline-paired side from that in the morphine-paired side, and are expressed as means ± SEM. Statistical analysis for the place conditioning study was conducted using one-way ANOVA followed by Bonferroni/Dunnett's test.

Results

BPA, but not E_2, causes the activation of astrocytes

To ascertain the effect of BPA in mouse purified midbrain astrocytes, we performed immunohistochemical staining with a polyclonal antibody for GFAP. The results showed a biphasic response. Mouse midbrain purified astrocytes were treated with either normal medium or BPA (BPA: 10 fM, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM) for 24 h. Treatment with BPA (100 fM, 1 pM, 10 pM, 10 nM, 100 nM or 1 μM) for 24 h caused a robust activation of mouse purified midbrain astrocytes, as detected by a stellate morphology and an increase in the levels of GFAP-like immunoreactivity ($P < 0.001$ versus control cells) (Fig. 1A,B). On the other hand, treatment with the mid-range dosed of BPA (10 fM, 100 pM, 1 nM) for 24 h failed to produce morphological changes in mouse purified midbrain astrocytes (Fig. 1A,B).

Unlike BPA, treatment with E_2 (10 fM to 1 μM, 24 h) failed to produce morphological changes in midbrain astrocytes at all concentrations tested (Fig. 1C,D).

We next explored the effect of BPA on mouse midbrain neurone/glia cocultures. In this culture system, numerous glial cells, especially astrocytes, surround neurones. Mouse midbrain neurone/glia cocultures were treated with either normal medium or BPA (BPA: 10 fM to 1 μM) for 24 h. In neurone/glia cocultures, BPA caused biphasic activations of astrocytes. Treatment with BPA (100 fM, 1 pM, 10 pM, 100 nM or 1 μM, 24 h) caused a robust activation of astrocytes in midbrain neurone/glia cocultures (*$P < 0.05$, **$P < 0.01$, *** $P < 0.001$ versus control cells) (Fig. 2A,B), whereas treatment with BPA (10 fM, 100 pM, 1 nM or 10 nM, 24 h) failed to produce an increase in GFAP-like immunoreactivity in mouse midbrain neurone/glia cocultures. E_2 (10 fM to 1 μM) failed to produce an increase in GFAP-like immunoreactivity in mouse midbrain neurone/glia cocultures at any doses tested (Fig. 2C,D).

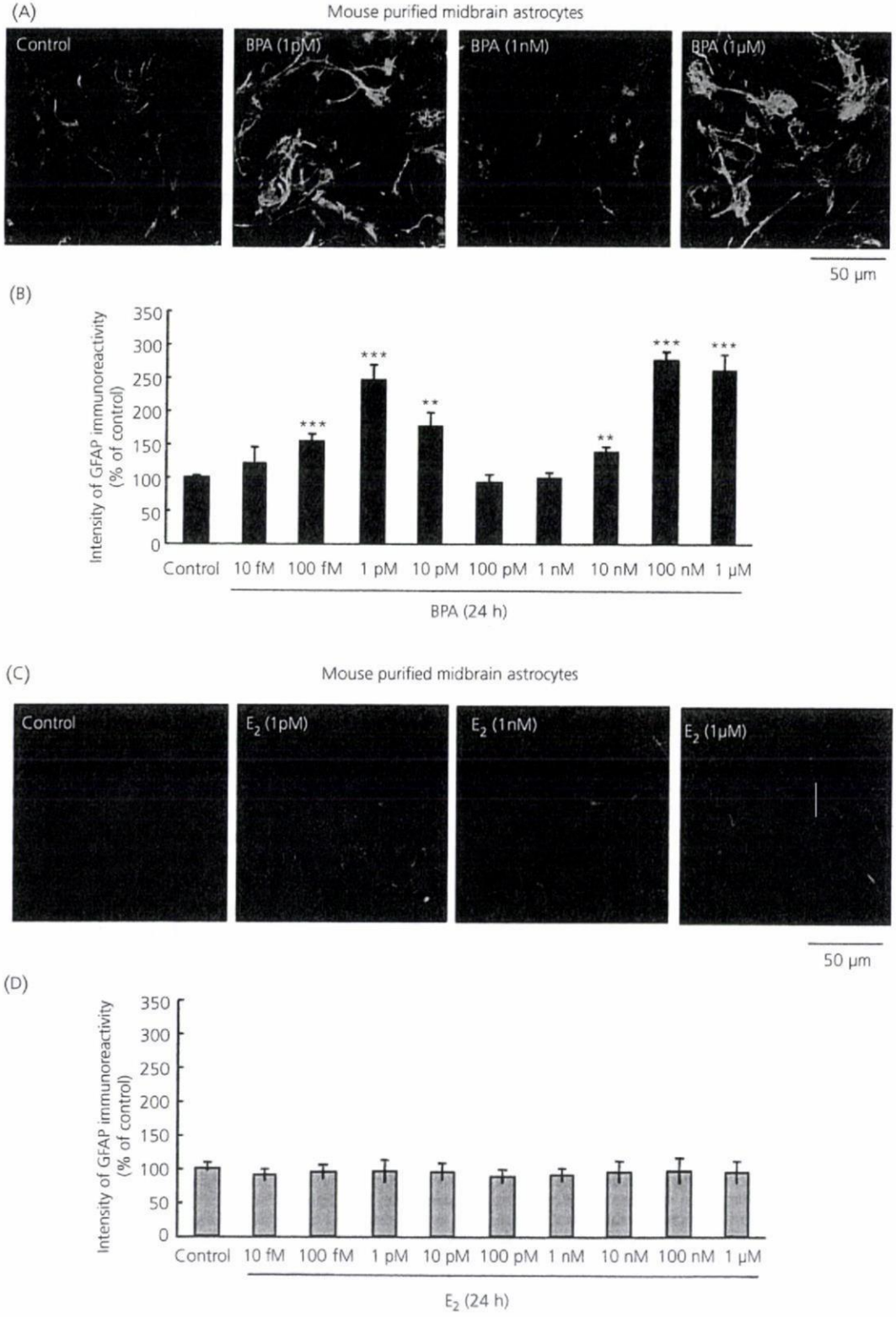
Enhancement of dopamine-induced Ca^{2+} responses by BPA

Dopamine (1–100 μM) produced a transient increase in the intracellular Ca^{2+} concentration in mouse purified midbrain astrocytes (Fig. 3). The Ca^{2+} responses to dopamine (100 μM) in astrocytes were significantly enhanced by pretreatment with a low concentration of BPA (1 pM, 24 h, **$P < 0.01$ versus control cells) (Fig. 3). By contrast, treatment with a high concentration of BPA (1 nM or 1 μM, 24 h) had no effect on the Ca^{2+} responses to dopamine in mouse purified midbrain astrocytes (Fig. 3).

Using immunocytochemical methods shown in Fig. 4(A) according to the study described previously (16), Neu-N-positive neurones are surrounded by GFAP-positive astrocytes in mixed neurone/glia cocultures. On the basis of morphological appearance, neurone-like cells were selected for the Ca^{2+} imaging studies. Under these criteria, dopamine (1–100 μM) produced a transient increase in the intracellular

FIG. 1. Treatment with bisphenol-A (BPA) for 24 h caused biphasic astrocytic activation in mouse purified midbrain astrocytes. (A) Mouse purified midbrain astrocytes were treated with normal medium or BPA (1 pM to 1 μM). The cells were stained with a polyclonal antibody to glial fibrillary acidic protein (GFAP). (B) Mouse purified midbrain astrocytes were treated with normal medium or BPA (10 fM to 1 μM) for 24 h and stained with a polyclonal antibody to GFAP. The intensity of GFAP-immunoreactivity from ten areas in each image was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells. The experiments were repeatedly performed by at least three independent culture preparations. **$P < 0.001$, ***$P < 0.001$ versus control cells. (C) Mouse purified midbrain astrocytes were treated with normal medium or 17β-oestradiol (E_2, 1 pM to 1 μM). The cells were stained with a polyclonal antibody to GFAP. (D) Mouse purified midbrain astrocytes were treated with normal medium or E_2 (10 fM to 1 μM) for 24 h and stained with a polyclonal antibody to GFAP. The intensity of GFAP-immunoreactivity from ten areas in each image was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells. The experiments were repeatedly performed by at least three independent culture preparations.

Ca^{2+} concentration in cultured midbrain neurone-like cells (Fig. 4B,C). These Ca^{2+} responses were significantly enhanced by treatment with a low concentration of BPA (1 pM, 24 h, *P < 0.05, **P < 0.01, ***P < 0.001 versus control cells) (Fig. 4B,C). Treatment with a high concentration of BPA (1 nM, 24 h) had no effect on the Ca^{2+} response to any



concentrations of dopamine, whereas the highest concentration of BPA (1 μM) suppressed the Ca^{2+} response to 100 μM dopamine (***P < 0.001 versus control cells) (Fig. 4B,C).

Effects of steroid hormone antagonists on the activation of astrocytes induced by BPA

To explore the involvement of steroid hormone receptor-dependent signalling in the activation of astrocytes, we next investigated whether steroid hormone antagonists could affect the BPA-induced increase in GFAP expression in midbrain astrocyte or neuronal/glial cultures. The highly selective oestrogen receptor antagonist ICI182,780 (100 nM, 1 μM, 2 μM) was administered as pretreatment (24 h) and cotreatment (24 h) with BPA (1 pM, 1 μM) in both mouse purified midbrain astrocyte and neurone/glia cocultures. ICI182,780 failed to attenuate the activation of astrocytes induced by BPA (1 pM or 1 μM) (Fig. 5). Pretreatment (24 h) and cotreatment (24 h) with either the oestradiol receptor agonist/antagonist tamoxifen (100 nM, 1 μM or 10 μM), the progesterone receptor antagonist mifepristone (100 nM, 1 μM or 10 μM) or the androgen receptor antagonist flutamide (100 nM, 1 μM or 10 μM) failed to affect the activation of astrocytes induced by BPA (1 pM, 1 μM) in both mouse purified midbrain astrocytes (Fig. 6A–C) and neurone/glia cocultures (Fig. 6D–F). These results suggest that activation of astrocytes by BSA was not mediated via oestrogen receptors, progesterone receptors or androgen.

BPA-induced neuronal cell death

We next investigated whether *in vitro* treatment with either BPA or E_2 could induce neuronal cell death. Treatment with a high concentration of BPA (1 μM, 24 h) in mouse midbrain neurone/glia cocultures caused the robust activation of caspase-3, which is a marker of neuronal cell death (Fig. 7). Unlike BPA, a high concentration of E_2 failed to produce caspase-3 activation (Fig. 7).

Enhancement of morphine-induced rewarding effect in mice prenatally and neonatally exposed to BPA

Morphine modulates several physiological processes including a rewarding effect by stimulating opioid receptors. We previously reported that chronic treatment with morphine (3–5 mg/kg, s.c.) produced a robust place preference in mice (6, 8). However, chronic treatment with a low dose of morphine (1 mg/kg, s.c) produced neither place preference nor place aversion in control mice (Fig. 8). On the other hand, treatment with 1 mg/kg of morphine produced a significant place preference in mice whose mothers had been exposed to BPA at a dose of 200 mg/kg/day (*P < 0.05 versus control group) (Fig. 8). Treatment with morphine at 1 mg/kg also produced a significant place preference in offspring of mothers chronically treated with BPA at a dose of 3 μg/kg/day (*P < 0.05 versus control group) (Fig. 8). By contrast, treatment with morphine at 1 mg/kg failed to produce a place preference in offspring of mothers that had been chronically treated with E_2 (3 μg/kg/day) (Fig. 8).

Discussion

A growing body of evidence suggests that astrocytes are important modulators of synaptic transmission. Astrocytes can respond to neurotransmitters released within the synapse by generating elevations in intracellular Ca^{2+} concentration and release glutamate and/or ATP that signal back to neurones (18, 19). Therefore, it is worthwhile to determine the effects of BPA on astrocytes. In the present study, we investigated the dopaminergic changes in neurones and astrocytes induced by BPA.

We show here for the first time that *in vitro* treatment with BPA caused morphological changes in GFAP-positive astrocytes. In addition, this effect of BPA was biphasic: treatment with 1 pM or 1 μM of BPA caused the robust activation of astrocytes, whereas treatment with 1 nM of BPA had no detectable effect on the morphology of astrocytes.

Inoue *et al.* (20) previously reported that the concentration of BPA was 0.32 ng/ml (approximately 1.4 pM) in normal human serum. Accordingly, it seems likely that 1 μM of BPA is higher than is commonly found in the environment. On the other hand, the amount of BPA that humans are exposed to results in the exposure of astrocytes to concentrations greater than 1 pM.

Neurones and astrocytes respond to various and chemical stimuli, including neurotransmitters, neuromodulators and hormones, with an increase in the intracellular Ca^{2+} concentration. These Ca^{2+} responses result from the co-ordinated activity of several molecular cascades responsible for Ca^{2+} movement into or out of the cytoplasm by way of either the extracellular space or intracellular stores. We have demonstrated that the dopamine-induced Ca^{2+} responses in mixed cultures of neurones and astrocytes were significantly enhanced by treatment with BPA (1 pM, 24 h). These findings strongly support the idea that the enhancement of Ca^{2+} responses to dopamine induced by BPA could lead to an increase in the excitability of central dopaminergic neurotransmission.

It has been reported that the stimulation of dopamine D_1 receptor increased the intracellular Ca^{2+} concentration via

FIG. 2. Treatment with bisphenol-A (BPA) for 24 h caused biphasic astrocytic activation in mouse midbrain neurone/glia cocultures. (A) Mouse midbrain neurone/glia cocultures were treated with normal medium or BPA (1 pM to 1 μM). The cells were stained with a polyclonal antibody to glial fibrillary acidic protein (GFAP). (B) Mouse midbrain neurone/glia cocultures were treated with normal medium or BPA (10 fM to 1 μM) for 24 h and stained with a polyclonal antibody to GFAP. The intensity of GFAP-immunoreactivity was measured using NIH Image. The level of GFAP-like immunoreactivity from ten areas in each image is expressed as a percent increase (mean ± SEM) with respect to that in control cells. **P < 0.001, ***P < 0.001 versus control cells. The experiments were repeatedly performed by at least three independent culture preparations. (C) Mouse midbrain neurone/glia cocultures were treated with normal medium or 17β-oestradiol (E_2, 1 pM to 1 μM). The cells were stained with a polyclonal antibody to GFAP. (D) Mouse midbrain neurone/glia cocultures were treated with normal medium or E_2 (10 fM to 1 μM) for 24 h and stained with a polyclonal antibody to GFAP. The intensity of GFAP-immunoreactivity was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells.

