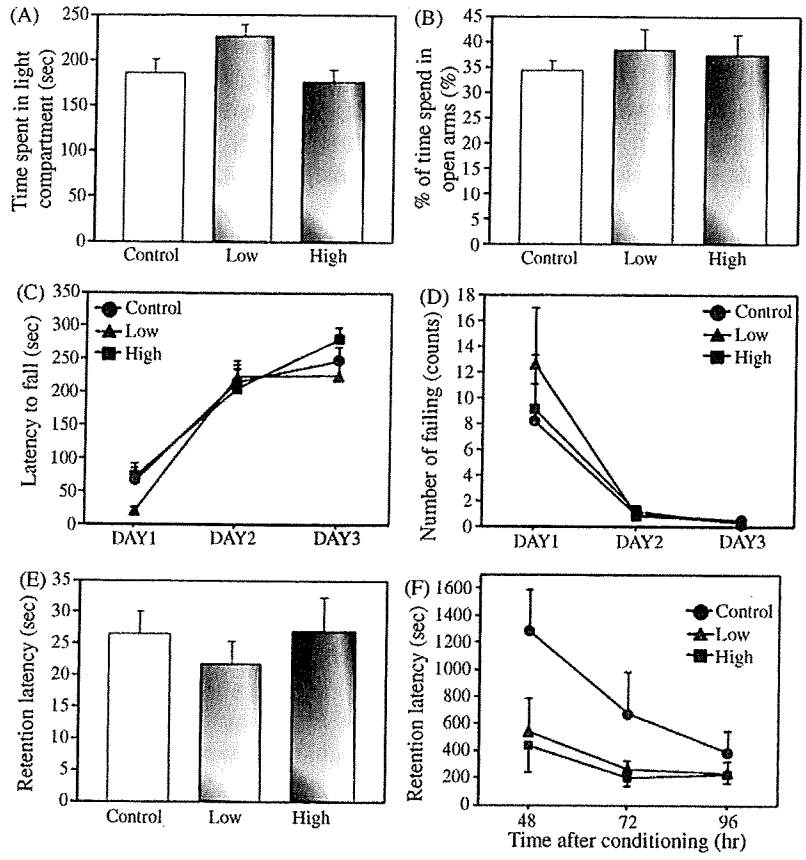

Fig. 1. Behavioral analysis in adult mice prenatally and neonatally exposed to bisphenol-A. (A) Lack of anxiogenic effects by prenatal and neonatal exposures to bisphenol-A using the light–dark test procedure. Prenatal and neonatal exposures to bisphenol-A failed to change the time spent in the lighted 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

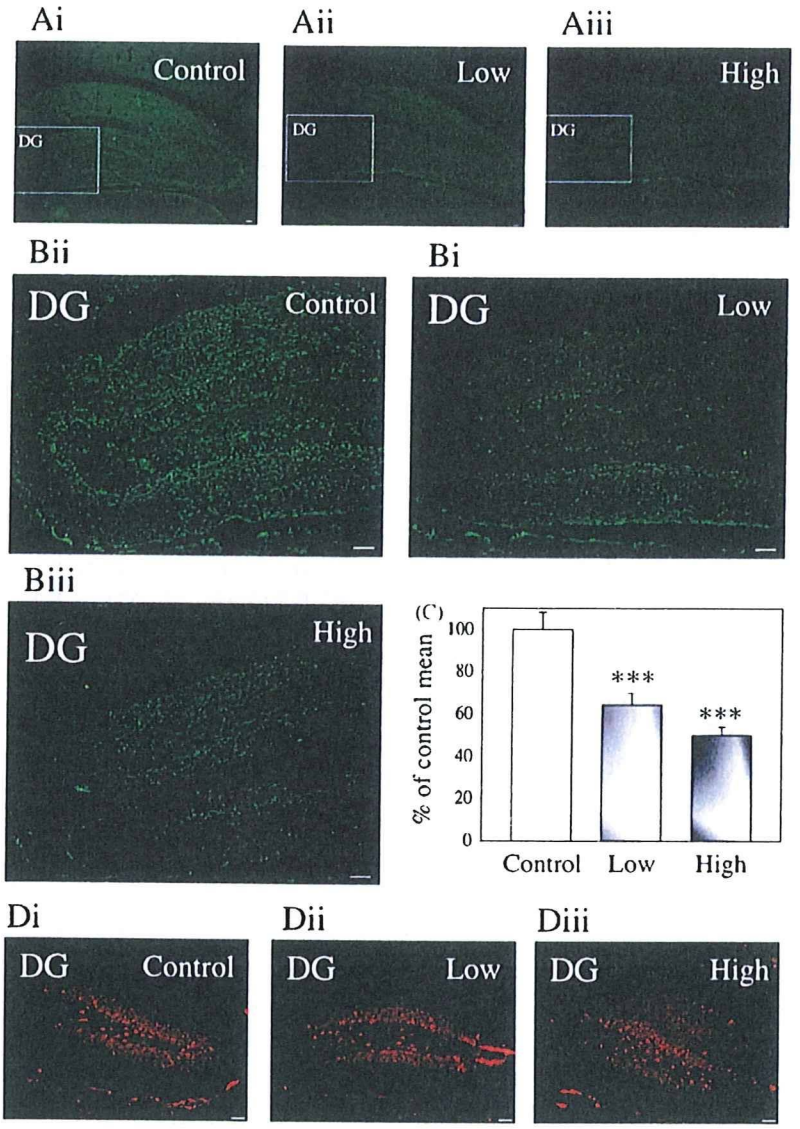

Fig. 2. Dramatic reduction in ChAT-like immunoreactivity in the hippocampus of the mice prenatally and neonatally exposed to low and high doses of bisphenol-A. (Ai–iii) Prenatal and neonatal exposures to low (Aii) and high doses (Aiii) of bisphenol-A dramatically decreased the level of ChAT-IR in the hippocampus compared to control (Ai). (Bi–iii) High magnification images showed that ChAT-IR 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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References

[1] R.T. Bartus, R.L. Dean 3rd, B. Beer, A.S. Lippa, The cholinergic hypothesis of geriatric memory dysfunction, Science 217 (1982) 408–414.
[2] A. Bilkei-Gorzo, I. Gyertyan, G. Levay, mCPP-induced anxiety in the light-dark box in rats–a new method for screening anxiolytic activity, Psychopharmacology (Berl) 136 (1998) 291–298.
[3] D.A. Donahue, E.J. Dougherty, L.A. Meserve, Influence of a combination of two tetrachlorobiphenyl congeners (PCB 47; PCB 77) on thyroid status, choline acetyltransferase (ChAT) activity, and short- and long-term memory in 30-day-old Sprague-Dawley rats, Toxicology 203 (2004) 99–107.

[4] P. Dutar, M.H. Bassant, M.C. Senut, Y. Lamour, The septohippocampal pathway: structure and function of a central cholinergic system, Physiol. Rev. 75 (1995) 393–427.
[5] E. Gould, C.S. Woolley, M. Frankfurt, B.S. McEwen, Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood, J. Neurosci. 10 (1990) 1286–1291.
[6] L.M. Juarez de Ku, M. Sharma-Stokkermans, L.A. Meserve, Thyroxine normalizes polychlorinated biphenyl (PCB) dose-related depression of choline acetyltransferase (ChAT) activity in hippocampus and basal forebrain of 15-day-old rats, Toxicology 94 (1994) 19–30.
[7] J.B. Knaak, L.J. Sullivan, Metabolism of bisphenol A in the rat, Toxicol. Appl. Pharmacol. 8 (1966) 175–184.
[8] Z. Li, R. Yang, Z. Chen, Effects of iodine and thyroid hormone deficiency during brain development on activities of cholinergic neurone-related enzymes in central nervous system of rats, Zhonghua Yu Fang Yi Xue Za Zhi 30 (1996) 337–339.
[9] R.H. Lustig, M. Sudol, D.W. Pfaff, H.J. Federoff, Estrogenic regulation and sex dimorphism of growth-associated protein 43 kDa (GAP-43) messenger RNA in the rat, Brain Res. Mol. Brain Res. 11 (1991) 125–132.
[10] J.B. Matthews, K. Twomey, T.R. Zacharewski, In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta, Chem. Res. Toxicol. 14 (2001) 149–157.
[11] M. McKinney, C. Kent, Differential expression of GAP-43 mRNA in adult central cholinergic neuronal populations, Brain Res. Mol. Brain Res. 23 (1994) 213–220.
[12] M. Miyamoto, J. Kato, S. Narumi, A. Nagaoka, Characteristics of memory impairment following lesioning of the basal forebrain and medial septal nucleus in rats, Brain Res. 419 (1987) 19–31.
[13] M. Miyatake, K. Miyagawa, K. Mizuo, M. Narita, T. Suzuki, Dynamic changes in dopaminergic neurotransmission induced by a low concentration of bisphenol-A in neurones and astrocytes, J. Neuroendocrinol. 18 (2006) 434–444.
[14] K. Mizuo, M. Narita, K. Miyagawa, E. Okuno, T. Suzuki, Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice, Neurosci. Lett. 356 (2004) 95–98.
[15] K. Mizuo, M. Narita, T. Yoshida, T. Suzuki, Functional changes in dopamine D3 receptors by prenatal and neonatal exposure to an endocrine disruptor bisphenol-A in mice, Addict. Biol. 9 (2004) 19–25.
[16] M. Narita, K. Miyagawa, K. Mizuo, T. Yoshida, T. Suzuki, Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect, Neurosci. Lett. 402 (2006) 249–252.
[17] L.H. Pottenger, J.Y. Domoradzki, D.A. Markham, S.C. Hansen, S.Z. Cagen, J.M. Waechter Jr., The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration, Toxicol. Sci. 54 (2000) 3–18.
[18] J. Prange-Kiel, G.M. Rune, Direct and indirect effects of estrogen on rat hippocampus, Neuroscience 138 (2006) 765–772.
[19] N.L. Schramm, M.P. McDonald, L.E. Limbird, The alpha(2a)-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety, J. Neurosci. 21 (2001) 4875–4882.
[20] R.W. Snyder, S.C. Maness, K.W. Gaido, F. Welsch, S.C. Sumner, T.R. Fennell, Metabolism and disposition of bisphenol A in female rats, Toxicol. Appl. Pharmacol. 168 (2000) 225–234.
[21] T. Suzuki, K. Mizuo, K. Miyagawa, M. Narita, Exposure to bisphenol-A affects the rewarding system in mice, Nihon Shinkei Seishin Yakurigaku Zasshi 25 (2005) 125–128.
[22] T. Suzuki, K. Mizuo, H. Nakazawa, Y. Funae, S. Fushiki, S. Fukushima, T. Shirai, M. Narita, Prenatal and neonatal exposure to bisphenol-A enhances the central dopamine D1 receptor-mediated action in mice: enhancement of the methamphetamine-induced abuse state, Neuroscience 117 (2003) 639–644.

PRECLINICAL STUDY

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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).

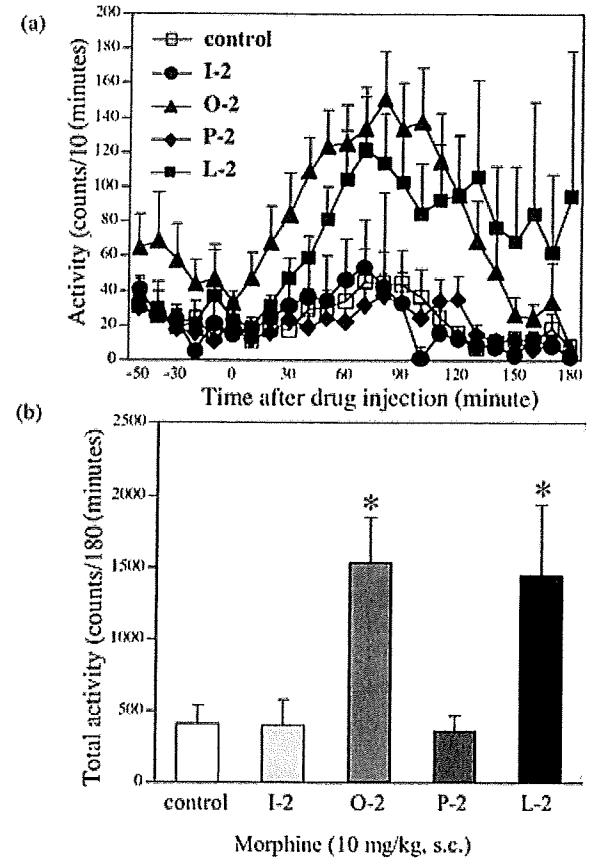

Figure 1 Enhancement of the morphine-induced hyperlocomotion in mice exposed to bisphenol-A during organogenesis or lactation. (a) Time-course changes in the morphine-induced hyperlocomotion in control and bisphenol-A-treated (I-2, O-2, P-2 and L-2) mice (I = implantation, O = organogenesis, P = parturition, 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

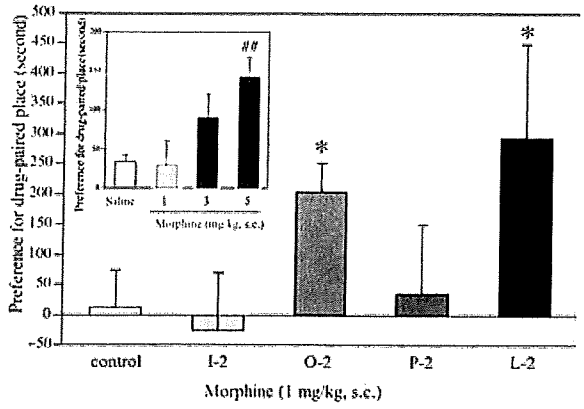

Figure 2 Enhancement of the morphine-induced rewarding effect in mice exposed to bisphenol-A during organogenesis or lactation. (Inner) Dose–response for the morphine-induced place preference in control mice (##$P < 0.01$ versus saline-treated mice). (Outer) Mice exposed to bisphenol-A in each period 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

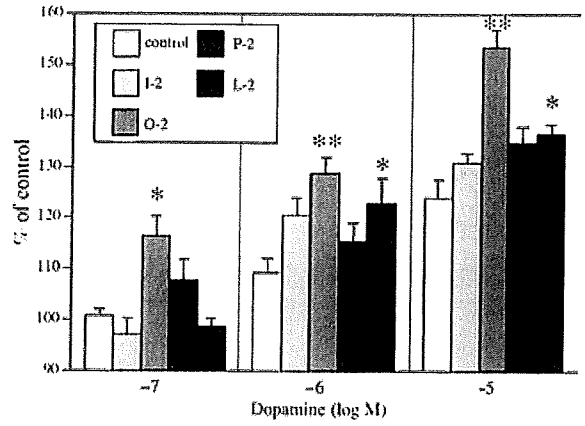

Figure 3 Comparison of the stimulation of [^{35}S]GTPγS binding to membranes from the limbic forebrain by dopamine between control and bisphenol-A-treated mice. Membranes were incubated with [^{35}S]GTPγS (50 pM) and guanosine-5′-diphosphate (GDP) (30 μM) with dopamine. The data are shown as the percentage 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

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References

Ashby J, Tinwell H (1998) Uterotrophic activity of bisphenol A in the immature rat. Environ Health Perspect 106:719–720.

BPA Safety Committee of Japanese Manufactures (2002) [www document] http://www.bisphenol-a.gr.jp (accessed on 31 March 2007).

Cagen SZ, Waechter JM Jr, Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, Harris LR (1999) Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. Toxicol Sci 50:36–44.

De Boer P, Enrico P, Wright J, Wise LD, Timmerman W, Moor E, Dijkstra D, Wikstrom HV, Westerink BH (1997) Characterization of the effect of dopamine D_3 receptor stimulation on locomotion and striatal dopamine levels. Brain Res 758:83–91.

Farabollini F, Porrini S, Della Seta D, Bianchi F, Dessi-Fulgheri F (2002) Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. Environ Health Perspect 110:409–414.

Funada M, Suzuki T, Misawa M (1994) The role of dopamine D_1-receptors in morphine-induced hyperlocomotion in mice. Neurosci Lett 169:1–4.

Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T, Safe S, McDonnell DP, Gaido KW (1998) Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. Mol Cell Endocrinol 142:203–214.

Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenbergh JG, vom Saal FS (1999) Exposure to bisphenol A advances puberty. Nature 401:763–764.

Koob GF (1992) Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends Pharmacol Sci 13:177–184.

Krishnan AV, Stathis P, Permuth SF, Tokes L, Feldman D (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132: 2279–2286.

Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252–4263.

Maldonado R, Dauge V, Feger J, Roques BP (1990) Chronic blockade of D_2 but not D_1 dopamine receptors facilitates behavioural responses to endogenous enkephalins, protected by kelatorphan, administered in the accumbens in rats. Neuropharmacology 29:215–223.

Mizuo K, Narita M, Miyagawa K, Okuno E, Suzuki T (2004a) Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice. Neurosci Lett 356:95–98.

Mizuo K, Narita M, Yoshida T, Suzuki T (2004b) Functional changes in dopamine D_3 receptors by prenatal and neonatal exposure to an endocrine disruptor bisphenol-A in mice. Addict Biol 9:19–25.

Narita M, Funada M, Suzuki T (2001) Regulations of opioid dependence by opioid receptor types. Pharmacol Ther 89:1–15.

Narita M, Mizuo K, Mizoguchi H, Sakata M, Narita M, Tseng LF, Suzuki T (2003) Molecular evidence for the functional role of dopamine D_3 receptor in the morphine-induced rewarding effect and hyperlocomotion. J Neurosci 23:1006–1012.

Narita M, Takahashi Y, Takamori K, Funada M, Suzuki T, Misawa M, Nagase H (1993) Effects of kappa-agonist on the antinociception and locomotor enhancing action induced by morphine in mice. Jpn J Pharmacol 62:15–24.

Phillips AG, LePiane FG, Fibiger HG (1983) Dopaminergic mediation of reward produced by direct injection of enkephalin into the ventral tegmental area of rat. Life Sci 33:2505–2511.

Seiwa C, Nakahara J, Komiyama T, Katsu Y, Iguchi T, Asou H (2004) Bisphenol A exerts thyroid-hormone-like effects on mouse oligodendrocyte precursor cells. Neuroendocrinology 80:21–30.

Shippenberg TS, Bals-Kubik R, Herz A (1993) Examination of the neurochemical substrates mediating the motivational effects of opioids: role of the mesolimbic dopamine and D-1 versus D-2 dopamine receptors. J Pharmacol Exp Ther 265:53–59.

Sokoloff P, Giros B, Martres M-P, Bouthnet ML, Schwartz JC (1990) Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. Nature 347:146–151.

Staples CA, Dorn PB, Klecka GM, O'Block ST, Harris LR (1998) A review of the environmental fate, effects, and exposures of bisphenol A. Chemosphere 36:2149–2173.

Stinus L, Nadaud D, Jauregui J, Kelley AE (1986) Chronic treatment with five different neuroleptics elicits behavioral supersensitivity to opiate infusion into the nucleus accumbens. Biol Psychiatry 21:34–48.

Suzuki T (1996) Conditioned place preference in mice. Methods Find Exp Clin Pharmacol 18:75–83.

Suzuki T, Funada M, Narita M, Misawa M, Nagase H (1991) Pertussis toxin abolishes mu- and delta-opioid agonist-induced place preference. Eur J Pharmacol 205:85–88.

Suzuki T, Funada M, Narita M, Misawa M, Nagase H (1993) Morphine-induced place preference in the CXBK mouse: characteristics of m opioid receptor subtypes. Brain Res 602:45–52.

Suzuki T, Maeda J, Funada M, Misawa M (1995) The D_3-receptor agonist (+/–)-7-hydroxy-N,N-di-npropyl-2-aminotetralin (7-OH-DPAT) attenuates morphine-induced hyperlocomotion in mice. Neurosci Lett 187:45–48.

Suzuki T, Mizuo K, Nakazawa H, Funae Y, Fushiki S, Fukushima S, Shirai T, Narita M (2003) Prenatal and neonatal exposure to bisphenol-A enhances the central dopamine D_1 receptor-mediated action in mice: enhancement of the methamphetamine-induced abuse state. Neuroscience 117:639–644.

Temple S (2001) The development of neural stem cells. Nature 414:112–117.

Wise RA, Rompre PP (1989) Brain dopamine and reward. Annu Rev Psychol 40:191–225.

〔Original Article〕

Changes in Central Dopaminergic Systems with the Expression of Shh or GDNF in Mice Perinatally Exposed to Bisphenol-A

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Abstract: In the previous study, we reported that exposure to bisphenol-A induced the potentiation of dopamine receptor functions in the mouse limbic area, resulting in supersensitivity to methamphetamine-induced pharmacological actions. The present study was undertaken to investigate whether prenatal exposure to bisphenol-A could produce morphological change in dopaminergic neuron and the pattern of expression of genes regulating the dopaminergic neuron development. Here we found that prenatal and neonatal exposures to bisphenol-A increased the tyrosine hydroxylase- and dopamine transporter-like immunoreactivities in the adult mouse limbic area. The present molecular biological study shows that chronic bisphenol-A treatment produced a significant decrease in the dopaminergic neuron development factors, sonic hedgehog and glial cell line-derived neurotrophic factor, which were also decreased by prenatal exposure to bisphenol-A. These results suggest that chronic exposure to bisphenol-A could disrupt the dopaminergic neurotransmission in the process of dopaminergic neuron development.

Key words: Bisphenol-A, Dopamine, Sonic hedgehog, Glial cell line-derived neurotrophic factor, Endocrine disrupting chemicals

In recent years there has been increasing public concern that chemicals in the environment may affect the endocrine function of humans and wildlife (Colborn, 1995). Bisphenol-A is an environmental endocrine-disrupting chemical that affects reproduction in wildlife. Bisphenol-A is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins, which are used in 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 (Brotons et al, 1995). This chemical is also released from polycarbonate flasks during autoclaving (Krishnan et al, 1993). Moreover, it has been reported that significant amounts of bisphenol-A are detected in the saliva of dental patients treated with fissure sealants (Olea et al, 1996).

We previously demonstrated that prenatal and neonatal exposures to bisphenol-A markedly enhanced the rewarding effects or hyperlocomotion induced by methamphetamine (Suzuki et al, 2003) and morphine (Mizuo et al, 2004a; Narita et al, 2006). We also demonstrated that in adult mice, prenatal and neonatal exposures to bisphenol-A enhanced function mediated by central dopamine D_1 receptors, which plays a substantial role in the rewarding effect of methamphetamine (Suzuki et al, 2003). These treatments also attenuated the function mediated by the dopamine D_3 receptor subtype that contributes to the inhibitory modulation of postsynaptic dopamine D_1/D_2 receptor-mediated signaling (Mizuo et al, 2004b). These findings indicate that exposure to bisphenol-A during development alters postsynaptic regulation of dopaminergic neurotransmission in the central nervous system (CNS), which results in an enhancement of psychological dependence on drugs of abuse. Although bisphenol-A may affect dopaminergic signaling in the CNS, little is known about the direct role of bisphenol-A in the development of dopaminergic neurotransmission. The purpose of the present study was then to clarify the effect of bisphenol-A on dopaminergic neuron development in mice.

In addition, 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 CNS. Their activation may control the structural and functional plasticity of synapses in the CNS. On the other hand, long-term exposure to drugs of abuse can induce neuronal plasticity, and we have shown that treatment of mouse cortical neuron/glia cocultures with methamphetamine or morphine caused morphological changes in astrocytes (Narita et al, 2005). Moreover, treatment with methamphetamine increased the

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Abbreviations ac: anterior commissure, ANOVA: analysis of varience, BPA: bisphenol-A, CNS: central nervous system, D_1R: dopamine D_1 receptor, D_2R: dopamine D_2 receptor, D_3R: dopamine D_3 receptor, DAT: dopamine transporter, DAMGO: [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin, EDs: embryonic days, FGF: fibroblast growth factor, GABA: γ-aminobutyric acid, GAPDH: D-glyceraldehyde-3-phosphate dehydrogenase, GDNF: glial cell-line derived neurotrophic factor, GFAP: glial fibrillary acidic protein, LMX: LIM homeobox transcription factor, Pax: paired- and homeodomain-containing transcription factor, PBS: phosphate-buffered saline, RT-PCR: reverse transcription-polymerase chain reaction, RXR: retinoid receptor, Shh: sonic hedgehog, TGF: transforming growth factor, TH: tyrosine hydroxylase, VTA: ventral tegmental area

sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids (Narita et al, 2005). Furthermore, astrocytes play a critical role in dopaminergic neuron development. We recently reported that *in vitro* treatment of bisphenol-A in mouse-purified astrocytes and neuron/glia cocultures caused the activation of astrocytes, as detected by a stellate morphology and an increase in levels of glial fibrillary acidic protein (GFAP). Therefore, we also investigated here whether prenatal and neonatal exposures to bisphenol-A induces astrocytic activation associated with the alternation of the dopaminergic neuron development.

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.

Animals

All experiments were performed using 10–14-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 powdered food containing 0 (control) or 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. In addition, RT-PCR was also performed using embryonic C57BL/6J mice that had been prenatally exposed to bisphenol-A from mating to embryo 14 days (same concentration as mentioned above).

It should be mentioned that the blood level of bisphenol-A in the present study (approximately 10 ng/ml, data not shown) is considered to be more than 30 times higher than the healthy human-exposure level (Inoue et al, 2000). However, our previous study clearly indicate that even much lower concentrations of bisphenol-A exposure (3×10^{-2} μg bisphenol-A/g of food) produced the enhancement of the pharmacological actions induced by morphine (Narita et al, 2006).

RT-PCR

In the RNA preparation and semiquantitative analysis by reverse transcription-PCR, total RNA in the whole brain (adult mice: excluding cerebellum, embryonic mice: including cerebellum) was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the instructions of the manufacturer. First-strand cDNA was prepared as described previously (Narita et al, 2001), and the targeted genes were amplified in 50 μl of a PCR solution containing $MgCl_2$, dNTP mix and DNA polymerase (Invitrogen, Carlsbad, CA) with synthesized primers of dopamine D_1 receptor (103 bp) (sense, 5-CTC ATA AGC TTT TAC ATC CCC G-3; antisense, 5-CCC TCT CCA AAG CTG AGA TG-3), dopamine D_2 receptor (202 bp) (sense, 5-CTC TAC CCT CCA ATC CAC TCC-3; antisense, 5-TAA GGC AGA GGC ACT GGC-3), dopamine D_3 receptor (136 bp) (sense, 5-GCA GTG GTC ATG CCA GTT CAC TAT CAG-3; antisense, 5-CCT GTT GTG TTG AAA CCA AAG AGG AGA GG-3), DAT (540 bp) (sense, 5-AAG ATC TGC CCT GTC CTG AAA G-3; antisense, 5-CAT CGA TCC ACA CAG ATG CCT C-3), Shh (243 bp) (sense, 5-CTG GCC AGA TGT TTT CTG GT-3; antisense, 5-GAT GTC GGG GTT GTA ATT GG-3) or GDNF (403 bp) (sense, 5-ACC AGA TAA ACA AGC GGC AG-3; antisense, 5-TCA GAT ACA TCC ACA CCG TTT AG-3). Samples were heated to 94°C for 5 min, 55°C for 1 min, and 72°C for 1 min, and cycled 35 times through 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. The final incubation was at 72°C for 7 min. The mixture was subjected to 2% agarose gel electrophoresis with the indicated markers and primers for the internal standard glyceraldehyde-3-phosphate dehydrogenase. Each sample was applied to more than two lanes in the same gel. The agarose gel was stained with ethidium bromide and photographed with ultraviolet transillumination. The intensity of the bands was analyzed and quantified by computer-assisted densitometry using NIH Image software.

Immunohistochemistry

In the immunohistochemical approach, mice were deeply anaesthetized with sodium pentobarbital (70 mg/kg, ip) 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 ventral tegmental area (VTA) or the limbic forebrain including the nucleus accumbens were initially dissected using Brain Blocker. The brain coronal sections were postfixed in 4% paraformaldehyde for 2 hr. 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 10% normal horse serum (NHS) in 0.01 M PBS for 1 hr at room temperature. Each primary antibody was diluted in 0.01 M PBS containing 10% NGS [1:1000 tyrosine hydroxylase (TH) (Chemicon, Temecula, CA), 1:10 GFAP (NICHIREI, Tokyo, Japan) and 1:2500 DAT (Chemicon)] and incubated for 2 days at 4°C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 and Alexa 546 for 2 hr 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).

Drugs

The drug used in the present study was bisphenol-A (Wako Pure Chemical Industries Ltd.).

Statistical analysis

All data are expressed as mean ±SEM. Statistical analyses were performed using Student's *t*-test.

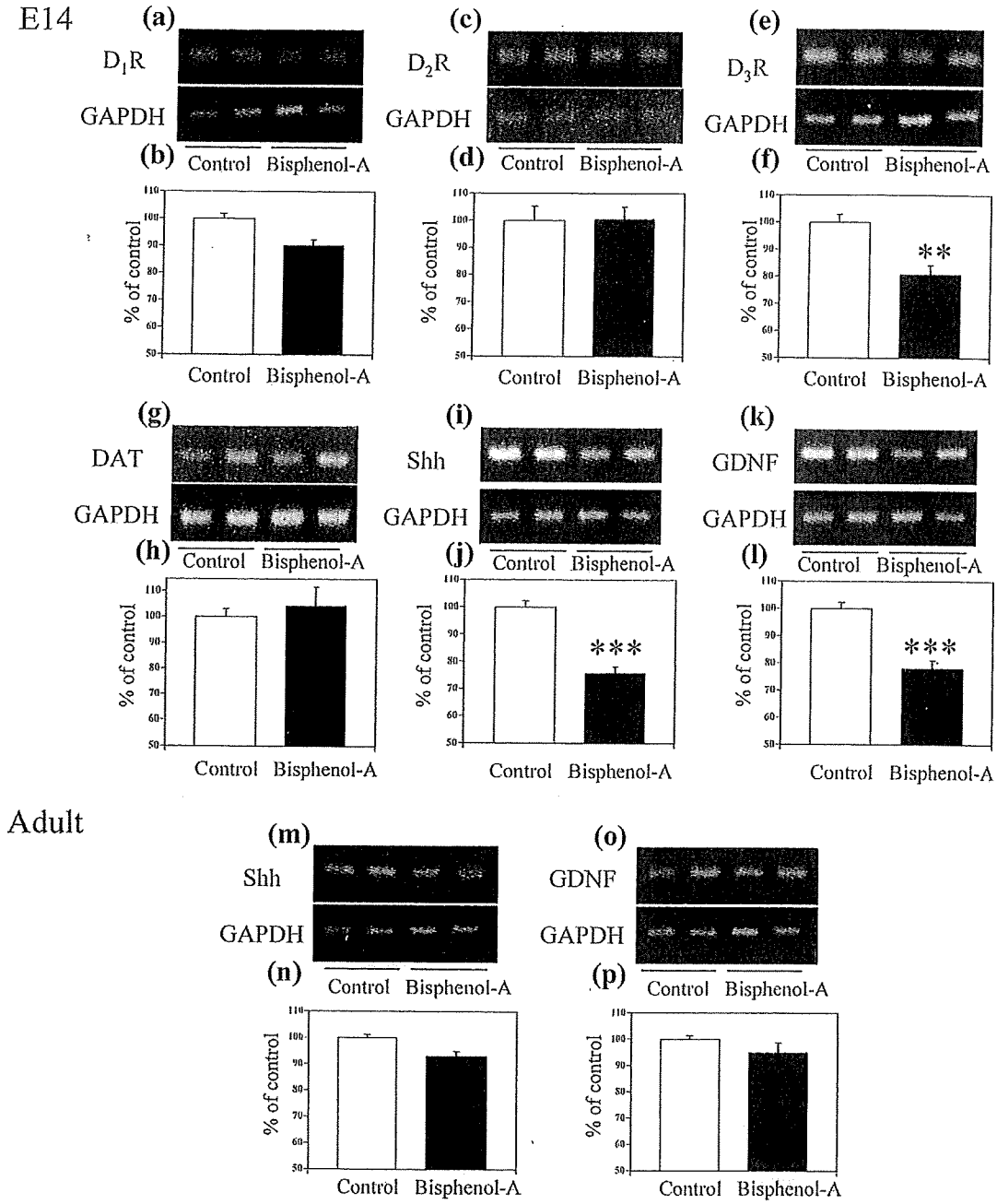

Fig. 1 Down-regulation of the expression of dopamine D_3 receptor, Shh and GDNF mRNAs in the whole brain obtained from embryonic mice prenatally exposed to bisphenol-A. (a, c, e, g, i, k) Representative RT-PCR for the dopamine D_1 receptor (D_1R; a), dopamine D_2 receptor (D_2R; c), dopamine D_3 receptor (D_3R; e), DAT (g), Shh (i) and GDNF (k) mRNAs in the whole brain obtained from embryonic mice. (f, j, l) Significant decrease in the expression of dopamine D_3 receptor (f), Shh (j) and GDNF (l) mRNAs in the whole brain obtained from embryonic mice prenatally exposed to bisphenol-A (filled bar) compared to that from control mice (open bar). (b, d, h) Under these conditions, no changes in mRNA levels of dopamine D_1 receptor (a), dopamine D_2 receptor (d) or DAT (h) were noted. (m, o) Representative RT-PCR for the Shh (m) and GDNF (o) mRNAs in the whole brain minus cerebellum obtained from adult mice. (n, p) No changes in the expression of Shh (n) or GDNF (p) mRNAs in the whole brain minus cerebellum obtained from mice prenatally and neonatally exposed to bisphenol-A (filled bar) as compared to control mice (open bar). The values are expressed as a percentage of the value in the control mice. Each column represents the mean ±SEM of 3 independent samples. **: $P<0.01$, ***: $P<0.001$ vs. control mice.

RESULT

Down-regulation of the expression of dopamine D_3 receptor, Shh and GDNF mRNAs in the whole brain obtained from embryonic mice prenatally exposed to bisphenol-A

In the RT-PCR assay, chronic bisphenol-A treatment produced a significant decrease in the dopamine D_3 receptor ($P<0.01$, Fig. 1e, f), Shh ($P<0.001$, Fig. 1i, j) and GDNF ($P<0.01$, Fig. 1k, l) production in the whole brain obtained from embryonic mice. On the other hand, no changes in mRNA levels of Shh (Fig. 1m, n) or GDNF (Fig. 1o, p) were noted in the whole brain obtained from the adult mice prenatally and neonatally exposed to bisphenol-A. Under these conditions, no changes in mRNA levels of dopamine D_1 receptor (Fig. 1a, b), dopamine D_2 receptor (Fig. 1c, d) and DAT (Fig. 1g, h) were noted in the whole brain obtained from embryonic mice.

Increases in DAT, TH and GFAP-like immunoreactivities in the nucleus accumbens and ventral pallidum of mice prenatally and neonatally exposed to bisphenol-A

We first investigated the possible morphological changes in dopaminergic neurons. The DAT or TH-like immunoreactivity (DAT-IR or TH-IR) in the VTA was observed by immunohistochemical analysis (Fig. 2a–d). Prenatal and neonatal exposures to bisphenol-A failed to induce morphological changes in dopamine cell bodies or the number of dopaminergic neurons. On the other hand, prenatal and neonatal exposures to bisphenol-A produced a dramatic increase in the levels of DAT-IR and TH-IR in the nucleus accumbens (Fig. 2e–h). In addition, GFAP-like immunoreactivity (GFAP-IR) was increased in the mouse ventral pallidum by prenatal and neonatal exposures to bisphenol-A (Fig. 2i, j). Double-labelling experiments showed that the increased DAT-IR was expressed in nonglial cells of the nucleus accumbens obtained from bisphenol-A treated mice, as shown by no apparent colocalization with GFAP-IR (Fig. 2k, l).

DISCUSSION

Recently, several investigations have provided evidence that the treatment of adult animals with bisphenol-A could not affect the reproductive function or social behaviors (Cagen et al, 1999). In the previous study, we found that acute administration of bisphenol-A to adult mice failed to affect the dopamine-related behaviors (Narita et al, 2006). On the other hand, several investigations clarified the behavioral abnormalities from prenatal and neonatal exposures to bisphenol-A (Mizuo et al, 2004a, 2004b; Narita et al, 2006; Suzuki et al, 2003). These findings indicate that prenatal and neonatal exposures to bisphenol-A may cause the neuronal toxicity, specifically in the developmental process. In the previous study, we reported that prenatal and neonatal exposures to bisphenol-A induced the abnormality of the dopamine receptor functions in the mouse limbic area, resulting in supersensitivity of methamphetamine-induced pharmacological actions (Suzuki et al, 2003). These findings indicate that exposure to bisphenol-A during development alters postsynaptic regulation of dopamine neurons. In the present study, we therefore focused on the change in the dopaminergic neuron during development.

Here, we found that chronic bisphenol-A treatment produced a significant decrease in sonic hedgehog (Shh) and glial cell line-derived neurotrophic factor (GDNF) production in the whole brain obtained from embryonic mice. On the other hand, no changes in mRNA levels of Shh and GDNF were noted in the whole brain obtained from the adult mice prenatally and neonatally exposed to bisphenol-A. Progenitor cells develop into dopaminergic neurons through the actions of Shh and fibroblast growth factor 8 (FGF8) (Lee et al, 2000). Maturation is orchestrated by several transcription factors, including the orphan nuclear receptor (Nurr-1), which is widely expressed in both the adult and developing CNS (Zetterstrom et al, 1996). Furthermore, it was reported that Shh, FGF8 and Nurr-1 collaborate to induce dopaminergic phenotypes (Kim et al, 2003). GDNF is also one of the most potent trophic factors for dopaminergic neurons, playing a role in development and survival (Lin et al, 1993). Therefore, the present data support the idea that prenatal and neonatal exposures to bisphenol-A may disrupt dopaminergic neuron development associated with the expression of Shh and GDNF.

We previously reported that prenatal and neonatal exposures to bisphenol-A induced the functional reduction in dopamine D_3 receptors in mice (Mizuo et al, 2004b). Du et al. reported that the pharmacological action of GDNF was regulated by activation of dopamine D_3 receptor (Du et al, 2005). In our previous study, exposure to bisphenol-A during organogenesis (Embryonic days (EDs) 7–14), but not implantation (EDs 0–7) or parturition (EDs 14–20), significantly enhanced the morphine-induced hyperlocomotion and rewarding effect. Furthermore, exposure to bisphenol-A during organogenesis also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These findings strongly support our hypothesis that bisphenol-A disrupts dopaminergic neuron development.

Next, we further investigated whether prenatal and neonatal exposures to bisphenol-A could affect the dopaminergic neuron in the adult brain. Immunohistochemical study showed that prenatal and neonatal exposures to bisphenol-A failed to change DAT-IR and TH-IR in the VTA. These results suggest that prenatal and neonatal exposures to bisphenol-A failed to induce cell death, overexpression of or morphological changes in dopaminergic neuron in the VTA. On the other hand, we found that prenatal and neonatal exposures to bisphneol-A dramatically increased DAT-IR and TH-IR in the nucleus accumbens. These results suggest that prenatal and neonatal exposures to bisphenol-A induce the abnormalities at axon terminals of dopaminergic neurons.

Another key finding of the present study was that prenatal and neonatal exposures to bisphenol-A induced astroglial proliferation as characterized by the increase in GFAP-IR levels, and astroglial hypertrophy as detected by a stellate morphology of GFAP-IR in the ventral pallidum. Many toxic stimuli activate astrocytes. The activation of astrocytes may control the structural and functional plasticity of synapses in the

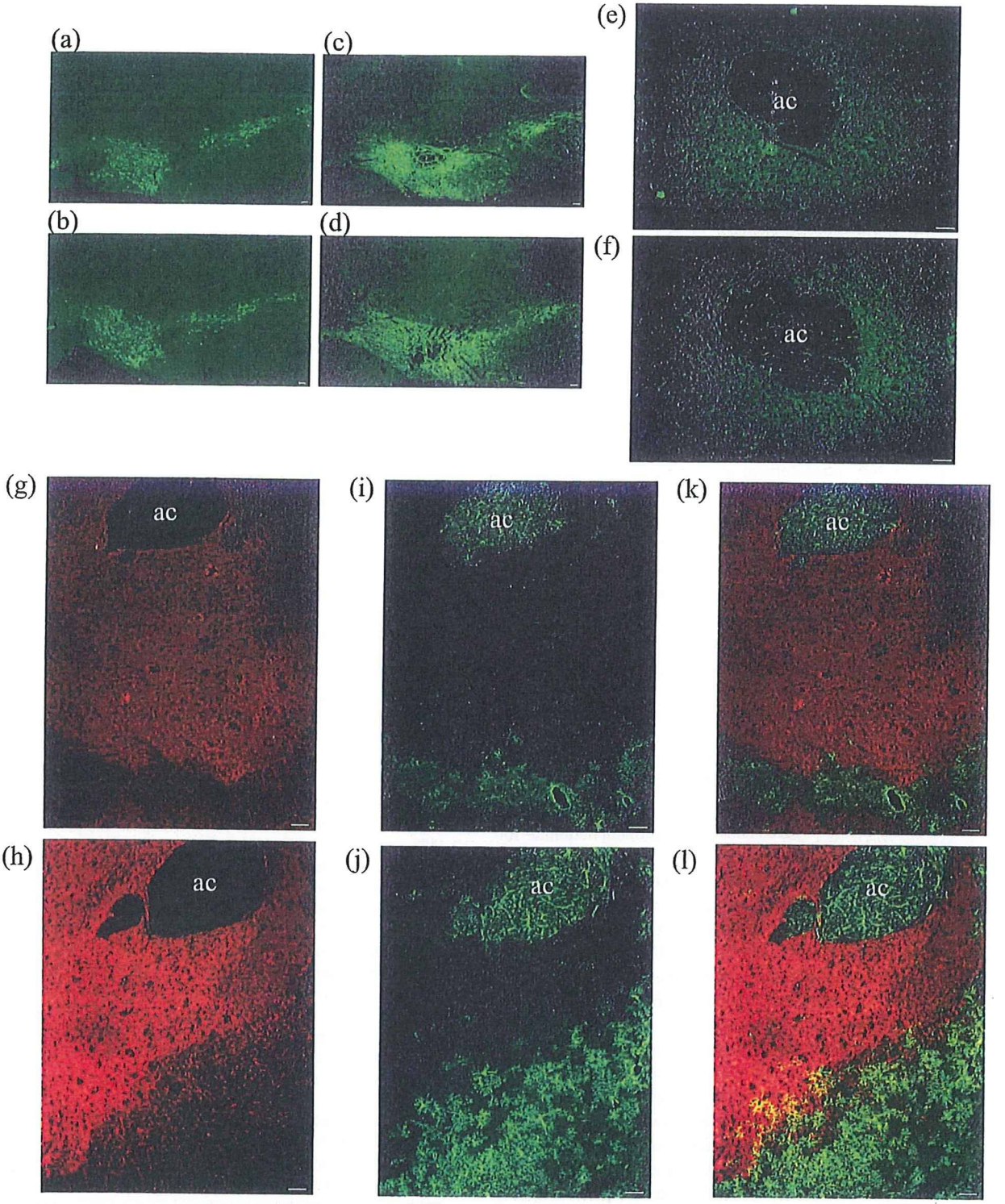

Fig. 2 Increase in DAT-, TH- and GFAP-IRs in the nucleus accumbens or ventral pallidum of mice prenatally and neonatally exposed to bisphenol-A. (a, b) The TH-IR in the ventral tegmental area (VTA) did not change in mice prenatally and neonatally exposed to bisphenol-A (b) compared to control mice (a). (c, d) Similarly, no change of the DAT-IR in the VTA was noted in mice prenatally and neonatally exposed to bisphenol-A (d) compared to control mice (c). (e, f) On the other hand, the increased TH-IR in the nucleus accumbens was noted in mice prenatally and neonatally exposed to bisphenol-A (f) compared to control mice (e). (g, h) The increased DAT-IR in the nucleus accumbens was also observed in mice prenatally and neonatally exposed to bisphenol-A (h) compared to control mouse (g). The GFAP-IR in the ventral pallidum was dramatically increased with morphological changes in mice prenatally and neonatally exposed to bisphenol-A (j) compared to control mice (i). The green labeling for DAT and the red labeling for GFAP show no apparent colocalization in the limbic area (k, l). ac: anterior commissure. Scale bars: 50 μm.

CNS. Recent accumulating evidence suggests that astrocytes express a repertoire of neurotransmitter receptors mirroring that of neighboring synapses. Such receptors are stimulated during synaptic activity and spread information by calcium signaling into the astrocyte network via gap-junction channels (Pasti et al, 1997). It has been widely accepted that long-term exposure to drugs of abuse can induce neuronal plasticity. We have shown that treatment of mouse cortical neuron/glia cocultures with methamphetamine or morphine causes morphological changes in astrocytes (Narita et al, 2005). Moreover, treatment with methamphetamine increases the sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids (Narita et al, 2005). Interestingly, treatment of mouse purified astrocytes and neuron/glia cocultures with bisphenol-A caused the activation of astrocytes, as detected by stellate morphology and an increase in levels of GFAP (Miyatake et al, 2006). It has been reported that the projection from the nucleus accumbens to the ventral pallidum regulates the reinstatement of cocaine seeking behavior in rats extinguished from cocaine self-administration (Tang et al, 2005). The nucleus accumbens and ventral pallidum have a pivotal role in regulating exploratory motor behaviors. Pharmacological manipulation of dopamine or enkephalin transmission in the nucleus accumbens induces motor activity. The nucleus accumbens has a prominent GABAergic projection to the ventral pallidum. The motor response elicited by microinjecting the μ-opioid agonist D-Ala-Tyr-Gly-NMePhe-Gly-OH (DAMGO) or dopamine into the accumbens is blocked by stimulating $GABA_A$ receptors in the ventral pallidum with the agonist muscimol (Austin and Kalivas, 1989). Collectively, these reports strongly support the idea that the astrocytic activation in the ventral pallidum of mice prenatally and neonatally exposed to bisphenol-A plays a critical role in the supersensitivity to methamphetamine following bisphenol-A treatment.

As mentioned above, prenatal and neonatal exposures to bisphenol-A may dramatically change the dopaminergic transmission. Knaak and Sullivan first reported the metabolic fate of bisphenol-A in rats (Knaak and Sullivan, 1966), 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. Many reports have showed that bisphenol-A is metabolized and excreted rapidly (Volkel et al, 2002). In addition, we reported that acute administration of bisphenol-A to adult mice did not affect the dopamine-related behaviors (Narita et al, 2006). In our preliminary biochemical studies, bisphenol-A did not increase or decrease [^{35}S]GTPγS bindings to brain membranes. In addition, we could not make the Scatchard plot using [^{3}H] bisphenol-A in brain membranes. Taken together, it is almost impossible that bisphenol-A that remains in the adult brain of mice directly affects dopaminergic neurotransmission.

At the present time, it is very hard to anticipate where the primary site of bisphenol-A is. Although bisphenol-A has weak estrogenic activity, prenatal and neonatal exposures to 17β-estradiol failed to induce supersensitivity to morphine (Miyatake et al, 2006). In addition, treatment with 17β-estradiol failed to induce astrocytic activation (Miyatake et al, 2006). Furthermore, although it is well known that bisphenol-A disrupts thyroid hormone, prenatal and neonatal exposures to propylthiouracil, a thyroid hormone inhibitor, reduced the activation of dopaminergic neurons (unpublished observation). These findings indicate that disruption of dopaminergic neuron development induced by prenatal and neonatal exposures to bisphenol-A can be mediated by non-hormonergic actions of bisphenol-A.

In conclusion, the present data suggest that bisphenol-A induces dopaminergic amplification following the disruption of the dopaminergic neuron development. This phenomenon could explain the aggravation of the development of dependence on drugs of abuse.

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REFERENCES

Austin, M. C. and Kalivas, P. W. (1989) Blockade of enkephalinergic and GABAergic mediated locomotion in the nucleus accumbens by muscimol in the ventral pallidum. Jpn J Pharmacol, 50: 487–490.

Brotons, J. A., Olea-Serrano, M. F., Villalobos, M., Pedraza, V. and Olea, N. (1995) Xenoestrogens released from lacquer coatings in food cans. Environ Health Perspect, 103: 608–612.

Cagen, S. Z., Waechter, J. M. Jr., Dimond, S. S., Breslin, W. J., Butala, J. H., Jekat, F. W., Joiner, R. L., Shiotsuka, R. N., Veenstra, G. E. and Harris, L. R. (1999) Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. Toxicol Sci, 50: 36–44.

Colborn, T. (1995) Environmental estrogens: Health implications for humans and wildlife. Environ Health Perspect, 103 [Suppl 7]: 135–136.

Du, F., Li, R., Huang, Y., Li, X. and Le, W. (2005) Dopamine D3 receptor-preferring agonists induce neurotrophic effects on mesencephalic dopamine neurons. Eur J Neurosci, 22: 2422–2430.

Inoue, K., Kato, K., Yoshimura, Y., Makino, T. and Nakazawa, H. (2000) Determination of bisphenol A in human serum by high-performance liquid chromatography with multi-electrode electrochemical detection. J Chromatogr B Biomed Sci Appl, 749: 17–23.

Kim, T. E., Lee, H. S., Lee, Y. B., Hong, S. H., Lee, Y. S., Ichinose, H., Kim, S. U. and Lee, M. A. (2003) Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem cell. Biochem Biophys Res Commun, 305: 1040–1048.

Knaak, J. B. and Sullivan, L. J. (1966) Metabolism of bisphenol A in the rat. Toxicol Appl Pharmacol, 8: 175–184.

Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L. and Feldman, D. (1993) Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology, 132: 2279–2286.

Lee, S. H., Lumelsky, N., Studer, L., Auerbach, J. M. and McKay, R. D. (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol, 18: 675–679.

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S. and Collins, F. (1993) GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science, 260: 1130–1132.

Miyatake, M., Miyagawa, K., Mizuo, K., Narita, M. and Suzuki, T.

(2006) Dynamic changes in dopaminergic neurotransmission induced by a low concentration of bisphenol-A in neurones and astrocytes. J Neuroendocrinol, 18: 434–444.

Mizuo, K., Narita, M., Miyagawa, K., Okuno, E. and Suzuki, T. (2004a) Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice. Neurosci Lett, 356: 95–98.

Mizuo, K., Narita, M., Yoshida, T. and Suzuki, T. (2004b) Functional changes in dopamine D3 receptors by prenatal and neonatal exposure to an endocrine disruptor bisphenol-A in mice. Addict Biol, 9: 19–25.

Narita, M., Mizoguchi, H., Suzuki, T., Dun, N. J., Imai, S., Yajima, Y., Nagase, H. and Tseng, L. F. (2001) Enhanced μ-opioid responses in the spinal cord of mice lacking protein kinase Cγ isoform. J Biol Chem, 276: 15409–15414.

Narita, M., Miyatake, M., Shibasaki, M., Tsuda, M., Koizumi, S., Yajima, Y., Inoue, K. and Suzuki, T. (2005) Long-lasting change in brain dynamics induced by methamphetamine: Enhancement of protein kinase C-dependent astrocytic response and behavioral sensitization. J Neurochem, 93: 1383–1392.

Narita, M., Miyagawa, K., Mizuo, K., Yoshida, T. and Suzuki, T. (2006) Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect. Neurosci Lett, 402: 249–252.

Olea, N., Pulgar, R., Perez, P., Olea-Serrano, F., Rivas, A., Novillo-Fertrell, A., Pedraza, V., Soto, A. M. and Sonnenschein, C. (1996) Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Perspect, 104: 298–305.

Pasti, L., Volterra, A., Pozzan, T. and Carmignoto, G. (1997) Intracellular calcium oscillations in astrocytes: A highly plastic, bidirectional form of communication between neurons and astrocytes in situ. J Neurosci, 17: 7817–7830.

Suzuki, T., Mizuo, K., Nakazawa, H., Funae, Y., Fushiki, S., Fukushima, S., Shirai, T. and Narita, M. (2003) Prenatal and neonatal exposure to bisphenol-A enhances the central dopamine D1 receptor-mediated action in mice: Enhancement of the methamphetamine-induced abuse state. Neuroscience, 117: 639–644.

Tang, X. C., McFarland, K., Cagle, S. and Kalivas, P. W. (2005) Cocaine-induced reinstatement requires endogenous stimulation of μ-opioid receptors in the ventral pallidum. J Neurosci, 25: 4512–4520.

Volkel, W., Colnot, T., Csanady, G. A., Filser, J. G. and Dekant, W. (2002) Metabolism and kinetics of bisphenol a in humans at low doses following oral administration. Chem Res Toxicol, 15: 1281–1287.

Zetterstrom, R. H., Solomin, L., Mitsiadis, T., Olson, L. and Perlmann, T. (1996) Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1. Mol Endocrinol, 10: 1656–1666.

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Congenit Anom (Kyoto). 2010 Apr 30. [Epub ahead of print]

Dietary exposure to low doses of bisphenol A: effects on reproduction and development in two generations of C57BL/6J mice.

Kobayashi K, Ohtani K, Kubota H, Miyagawa M.

National Institute of Occupational Safety and Health, Kawasaki, Japan.

Abstract

ABSTRACT This study was conducted to examine the effects of low-dose exposure to bisphenol A on reproduction and development in two generations of mice. Pregnant female C57BL/6J mice (F(0)) were fed a diet containing low doses of bisphenol A (0, 0.33, 3.3, or 33 ppm) from gestational day 6 through postnatal day 22, and the weanlings (F(1) and F(2)) from each F(0) and F(1) dam group, respectively, were also fed these same concentrations of bisphenol A ad libitum until sacrifice. There were no treatment-related changes in body weight, body weight gain, food consumption, gestation length, or the number of live births on postnatal day 1 in F(0) dams between the control group and bisphenol A groups. Sex ratio and viability were similar in all F(1) pups. No treatment-related changes were observed in body weight, food consumption, developmental parameters, anogenital distance, or weight of any of the organs (liver, kidney, heart, spleen, thymus, testis, ovary, or uterus) in F(1) and F(2) adults in either sex. The epididymis weight was slightly higher with 0.33 and 3.3 ppm in F(1) males, but this slight increase was neither dose dependent nor seen across generations. There were no treatment-related effects of bisphenol A on cauda epididymal sperm count or sperm motility in F(1) or F(2) males. These findings indicate that dietary exposure to bisphenol A between 0.33 and 33 ppm does not adversely affect reproduction or development as assessed in two generations of mice.

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臨床免疫・アレルギー科，51(1)：60-65，2009

総　説

Review

ダイオキシンによる免疫異常*

石丸直澄**　林　良夫**

Key Words：2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD), aryl hydrocarbon receptor(AhR), autoimmune disease, T cell

はじめに

環境ホルモンの一つとして知られるダイオキシン(図1)は免疫系，生殖系，神経系などの生物反応に重大な影響を及ぼすことが報告されてきた[1)~3)]．その中で免疫系への影響に関しては動物モデルを用いた研究が中心に行われ，免疫細胞の中で，T細胞やB細胞にダイオキシンの直接的な作用と間接的な影響に関して報告されてきた[4)~7)]．その中で，ダイオキシンのレセプターであるaryl hydrocarbon receptor(AhR)を介した分子シグナルの詳細が明らかにされようとしている．最近，ヘルパーT(Th)細胞の中でTh17細胞への分化をダイオキシンが調節することが判明した[8)]．さらに，Th17細胞が原因とされる自己免疫疾患の一つである多発性硬化症のモデルを用いた病態発症機序にダイオキシンが大きく影響を及ぼすことが明らかとされている[9)]．本稿ではこれまでのダイオキシンと免疫異常に関係する文献的知見を踏まえ，筆者らが明らかにしている自己免疫疾患に対するダイオキシンの影響に関する新知見を解説する．

免疫細胞へのダイオキシンの影響

正常マウスにダイオキシンを投与すると，胸腺が萎縮することが知られている[10)]．胸腺細胞の正負の選択に関連したアポトーシスにダイオキシンが影響を及ぼしている可能性や，胸腺間質細胞のFasLの発現にダイオキシンが調節因子として働きFasを発現した胸腺細胞のアポトーシスを制御しうるといったことが報告されているものの，明確な分子機序は不明である[11)]．さらに，ダイオキシン投与により，末梢のT細胞の機能低下が観察され，遅延型接触過敏反応やT細胞の細胞障害性活性の低下がみられる一方で，ダイオキシンによって各種刺激に対するT細胞の増殖反応やIL-2などのサイトカインの分泌は上昇することも知られている[12)13)]．また，卵白抗原(OVA)特異的なT細胞の反応性は初期の活性化には大きな影響は認められないかわりに，OVAに対するT細胞の増殖反応はダイオキシンによって亢進する[14)]．つまり，ダイオキシンの作用はT細胞の活性化ではなく生存に関係する分子群に影響を及ぼしている可能性がある．さらに，ダイオキシン投与により末梢でのCD25$^+$CD4$^+$調節性T細胞(regulatory T cell；Treg cell)を誘導可能であるというユニークな報告もある[8)]．

一方で，ダイオキシンのB細胞への影響として，ヒツジ赤血球抗原の免疫に対する抗体産生はダイオキシンの投与により抑制され，さらに，lipopolysaccaride(LPS)あるいはIgM抗体などによる刺激でB細胞の増殖反応がダイオキシン添加により阻害されることも報告されている[15)]．B細胞の最終分化段階である形質細胞への分化をダイオキシンが阻害する結果も知られている[16)]．加えて，ダイオキシン投与マウスへのインフル

* Immune disorder by dioxin.

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エンザウイルスの感染実験では，T細胞，B細胞の機能低下とともにインフルエンザウイルスに対する抗体の産生も劇的に抑制されることが判明した[17]．

また，LPSの腹腔内誘導によるマクロファージの活性化をダイオキシンがTNF-αの産生上昇を介して亢進させる働きがある[18]．さらに，樹状細胞へのダイオキシンの影響については，抗原の取り込みや活性化に関してはダイオキシンが阻害的効果を有しているものの，T細胞への抗原提示能を上昇させる作用があることも報告されている[19]．

ダイオキシンは免疫細胞の種類やそれらの細胞の種々の機能に対して幅広い影響が認められるが，免疫細胞の機能に対して抑制的な効果が目立つ．表1にそれぞれの免疫細胞におけるダイオキシンの影響についてまとめる．

ダイオキシンによる細胞内分子シグナル

細胞内に入ったダイオキシンは細胞質に存在するそのレセプターであるAhRと結合する(図2)．AhRはヘリックス-ループ-ヘリックス(helix-loop-helix；HLH)ファミリーに属する転写因子として知られている．ダイオキシンと結合して活性化したAhRはAhR nuclear translocator(ARNT)とヘテロダイマーを形成し，核内に移行した後，さまざまな遺伝子上に存在するdioxin responsive element(DRE)として知られるxenobiotic response element(XRE)に結合することによりその遺伝子の転写が調節される[1)20)21)]．AhR複合体の標的遺伝子として，もっとも知られているのがcytochrome P-450 1A1(CYP1A1)である．CYP1A1は増殖・アポトーシスなどの細胞の生死を中心

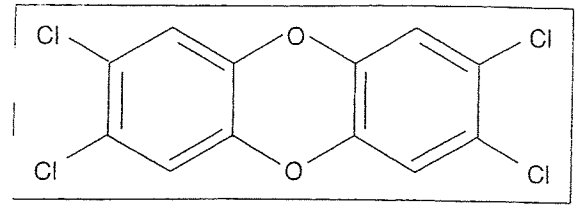

図1　ダイオキシン〔2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD)〕の化学式

に重要な役割を果たしており，AhRを介したダイオキシの細胞内における分子機序を解析するのに有効なメルクマールとして広く知られている[22)~24)]．また，AhRはnuclear factor-κB(NF-κB)のサブユニットの一つであるRelBとも結合することにより，免疫反応に重要な転写因子であるNF-κBの制御に影響を及ぼしていることも報告されている[25)]．AhRを中心としたダイオキシンの分子シグナルの解析にはAhRノックアウトマウスを用いることで明確な現象を観察することが可能となる．また，AhRノックアウトマウスを用いた免疫細胞への実験に関しても機能解析を中心に多くの報告がなされてきた[1)10)26)]．

ダイオキシンは，AhRを起点として，CYP1A1やNF-κBを介した多彩な免疫細胞機能に対して複雑に影響を及ぼしている．しかし，AhRのリガンドはダイオキシンだけでなくさまざまな生体物質あるいは非生体物質があげられることに加えて，ダイオキシン自体が内分泌かく乱物質としてエストロジェンレセプターと相互作用することにより，本来性ホルモンで制御されている生体機能のホメオスターシスの維持を破綻させる複雑な分子機序を有していることから，実際の生体内で起こっているダイオキシンの詳細な動態，正確な分子シグナルに関しては多くの謎が残されている．

表1　免疫細胞におけるダイオキシンの影響

免疫細胞	TCDDによる影響	文献番号
胸腺細胞	アポトーシス亢進	10)11)
T細胞	細胞障害性低下，増殖反応低下，Th17分化	8)9)12)~14)
調節性T細胞	誘導	8)
B細胞	抗体産生低下，増殖反応低下	15)~17)
マクロファージ	活性化亢進	18)
樹状細胞	活性化低下，抗原提示能亢進	19)

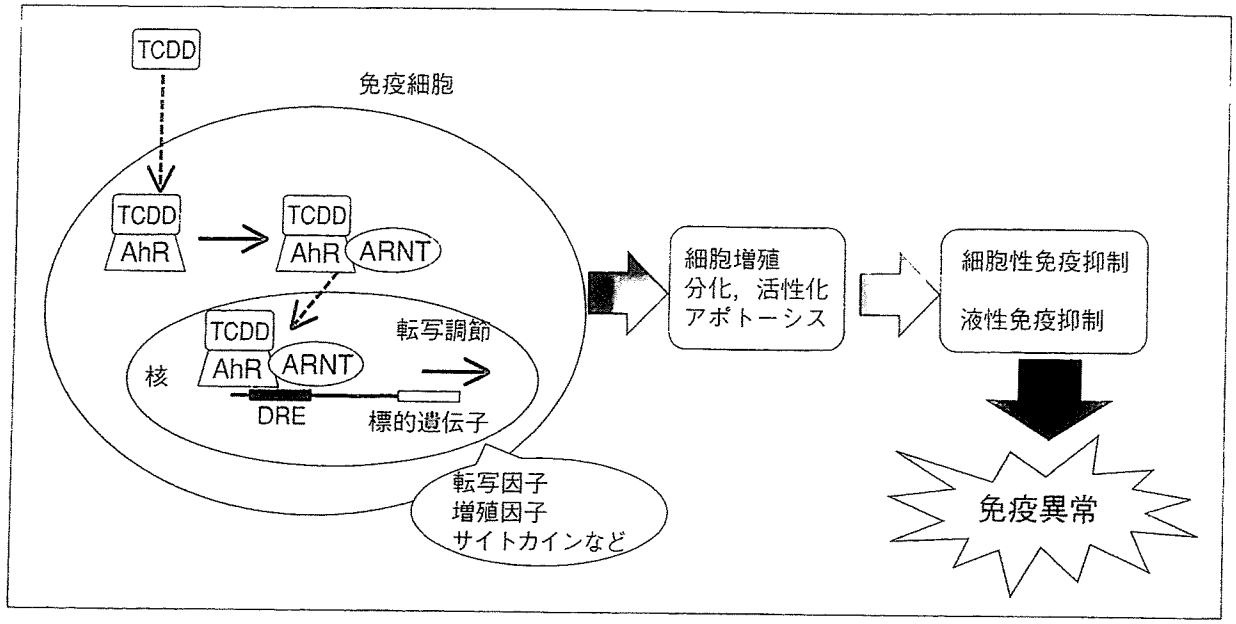

図2　免疫細胞におけるAhRを中心とした分子シグナル

ダイオキシンとTh17細胞

最近，Nature誌の同号にAhRとT細胞分化あるいは自己免疫疾患との関係を決定づける2本の論文が発表された[8)9)]．ダイオキシン(TCDD)の刺激で活性化されたAhRを介してTreg細胞においてもっとも重要とされる転写因子Foxp3の発現が亢進されることによって，Treg細胞の細胞数が増加することが判明した．さらに，多発性硬化症のモデルマウス〔実験的自己免疫性脳脊髄炎(experimental autoimmune encephalomyelitis；EAE)〕にダイオキシンを投与することによりTreg細胞の増加を介する病態の抑制効果があることを見出した．しかしながら，AhRの内因性リガンドの一つである6-formylindolo[3,2-b]carbazole(FICZ)をEAEモデルマウスに投与すると病態は増悪した．FICZはT細胞のIL-22およびIL-17の産生を上昇させることにより，EAEの病態形成にきわめて重要なTh17細胞の分化を促進させていることが明らかとなった．TCDDの投与で増加していたTreg細胞に関してはFICZ投与では影響がなかった．さらに，AhRの別のリガンドであるβ-naphthoflavoneを用いた実験においても，FICZと同様の効果が認められた．AhRは複数のリガンドと結合するため，リガンド依存性の転写制御機構が存在するものと考えられている．Th17細胞は従来知られていたIFN-γやIL-2などのサイトカインを分泌するTh1細胞とIL-4やIL-10などを分泌するTh2細胞とは異なるT細胞セブセットとして同定され，多発性硬化症などの自己免疫疾患の病態発症に重要な役割を果たしているという多くの報告がなされている．Nature誌に報告された2本の論文では共通してFICZは健常人の皮膚に存在し，紫外線によって活性型となりAhRと結合することが知られている．ダイオキシンや他のリガンドとAhRとの結合様式や親和性などいくつかの相違点があるものの，AhRの活性化機構に関しては不明な点が多い．また，Treg細胞におけるダイオキシンによるFoxp3の発現亢進の分子機序に関しても議論の余地を残している．

ダイオキシンと自己免疫疾患

上述のEAEの発症にFICZの投与によってAhRを介したT細胞異常に起因した自己免疫疾患の悪化効果があることが判明したものの，ダイオキシンが自己免疫疾患に影響するか否かは不明のままである．筆者らはこれまでに，唾液腺，涙腺を標的臓器とする自己免疫疾患であるシェーグレン症候群(Sjögren's syndrome；SS)のモデルマウスを確立し，その病態に関し研究を進めてきた[27)~29)]．SSの臨床病態は閉経期以降の女性に発症ピークを有し，ドライアイ，ドライマウスなどの乾燥症候群を呈し，血清自己抗体として抗SSAあるいは抗SSB抗体が検出され，小唾液腺の口唇生検により導管周囲性のリンパ球浸潤が