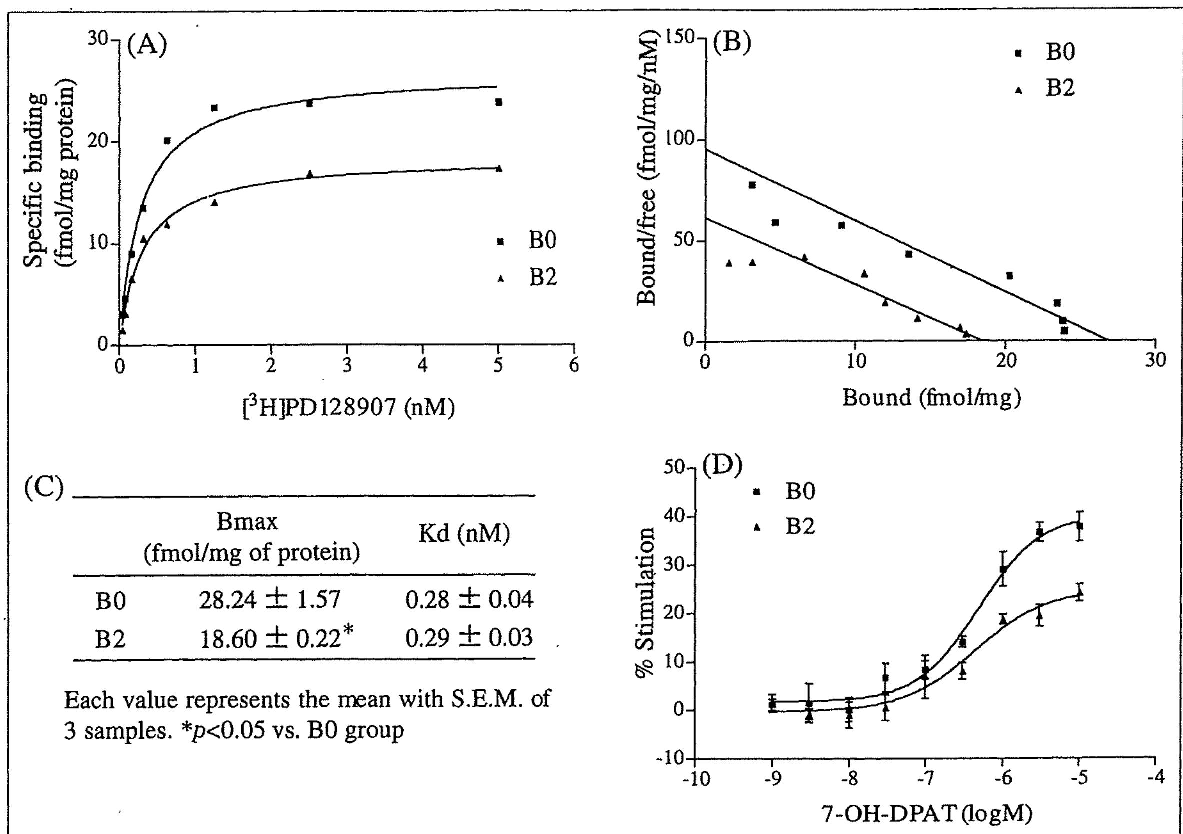

	Bmax (fmol/mg of protein)	Kd (nM)
B0	28.24 ± 1.57	0.28 ± 0.04
B2	18.60 ± 0.22*	0.29 ± 0.03

Each value represents the mean with S.E.M. of 3 samples. *p<0.05 vs. B0 group

図4 Bisphenol-Aの胎児期および授乳期慢性曝露による(A～C)dopamine D_3受容体に対するligandの結合能の変化と(D)dopamine D_3受容体誘発Gタンパク質活性化作用に対する影響

(A～C)Bisphenol-Aの胎児期および授乳期慢性曝露によりdopamine D_3受容体数の有意な低下が認められた．(D)Bisphenol-Aの胎児期および授乳期慢性曝露により，前脳辺縁部領域における7-OH-DPAT誘発Gタンパク質活性化作用の有意な増強が認められた．(A～C)Each value represents the mean with S.E.M. of 3samples. *$p<0.05$ vs. B0 group. (D) $F_{1,174}=30.45$, $p<0.001$ vs. B0 group. Data are expressed as the mean ± S.E.M. of 3 independent samples.

期慢性曝露によりひき起こされたdopamine D_1受容体のup-regulationは，bisphenol-Aのestrogen様作用に起因している可能性が考えられる．しかしながら，bisphenol-Aのestrogen受容体に対する親和性は内因性estrogenである17β-estradiolの1/1,500であり，その estrogen 活性は1/100,000程度に過ぎない[1)18)]．このような背景から，著者らはbisphenol-Aのみならず，17β-estradiolの胎児期および授乳期慢性曝露マウス(Low-BPA：bisphenol-A 3 μg/kg/day p.o., High-BPA：bisphenol-A 200mg/kg/day p.o., 17β-estradiol：17β-estradiol 3 μg/kg/day p.o.)を作成し，依存性薬物に対する感受性の変化について検討を行った．その結果，17β-estradiolの胎児期および授乳期慢性曝露群においては，bisphenol-Aの胎児期および授乳期慢性曝露群で認められたようなmorphine誘発報酬効果の増強は認められなかった．また，著者らはグリア細胞にも着目し，その変化を形態学的に解析することで，神経-グリア細胞間の相互作用に及ぼすbisphenol-A曝露の影響について，マウス中脳部由来初代培養アストロサイトを用いて検討した．その結果，低用量のbisphenol-Aを処置した細胞において，アストロサイトの肥大化，樹状突起の伸展といった形態変化がひき起こされた．さらに，この形態変化がアストロサイトの機能変化を伴うか否かについて検討する目的で，dopamine誘発細胞内Ca^{2+}濃度の変化を測定したところ，無処置群と比較し，bisphenol-A処置群において有意な細胞内Ca^{2+}濃度の上昇がひき起こされた．このようなアストロサイトの形態変化や機能変化は，初代培養アストロサイトに覚せい剤であるmethamphetamineを処置した際と類似した反応である．なお，低用量17β-estradiolの処置では，このよう

な変化が認められなかった．これらのことから，bisphenol-Aはestrogen様作用とは異なった機構により依存性薬物に対する感受性を亢進させ，その機構の一つに，アストロサイトの活性化が関与している可能性が示唆された．

Bisphenol-Aの慢性曝露による神経行動毒性発現と曝露時期の関連性

脳の機能的な発達過程において，もっとも外界から影響を受けやすいのは胎児期から授乳期にかけてである．成体の脳は血液-脳関門が発達しており，血液から薬物などの脳への侵入を防いでいる．この血液-脳関門は，授乳期以降に発達することが報告されているため[19]，胎児期から授乳期にかけては血液-脳関門がほとんど形成されておらず，未発達であると考えられる．したがって，このような時期にbisphenol-Aの曝露を受けると，成体と比較して容易にbisphenol-Aが脳内へ移行することが推察される．事実，すでに述べたように，bisphenol-Aを胎児期から授乳期にかけて慢性的に曝露することにより脳内dopamine神経系の機能に影響を及ぼすことを明らかにした．さらにbisphenol-Aは，成体に対してはほとんど影響を及ぼさないことがすでに報告されている[20]ことからも，胎児期から授乳期におけるbisphenol-Aの容易な脳移行性が中枢神経系に影響を及ぼす一因となっている可能性が考えられる．

一般に，脳の発達において神経細胞の増殖は胎児期，とくに器官形成期にもっとも盛んに行われることが明らかにされている．そのため，生後の脳重量はほとんど変化しない．一方，脳の機能的な発達，すなわちシナプスの形成およびシナプス密度の増加に伴う神経ネットワークの構築は，出生後の授乳期においてもっとも盛んに行われていることが報告されている．著者らは，このような曝露時期の重要性を考え，bisphenol-Aの曝露時期を妊娠初期から授乳期まで４分割したマウスから生まれた仔を用いて，morphine誘発自発運動促進作用および報酬効果について検討した．その結果，コントロール群と比較して，器官形成期曝露群および授乳期曝露群ではmorphine誘発自発運動促進作用および報酬効果の有意な増強が認められた．さらに，前脳辺縁部領域におけるdopamine誘発Ｇタンパク質活性化作用も同様の期間において有意な増強が認められた．これらのことから，血液-脳関門が未発達な時期であり，神経細胞の増殖および神経ネットワークの構築過程に非常に重要である器官形成期および授乳期におけるbisphenol-Aの慢性曝露は中枢神経系に対して多大な影響を及ぼすものと考えられる．

まとめ

レイチェル・カールソンの『沈黙の春』が出版されて以来，環境中の化学物質は野生生物の個体群に対して，複雑で有害な影響を及ぼすことがあり，ヒトの健康は健全な環境と複雑に関連していることについての認識が深まってきている．とくに過去20年間には，内分泌システムを阻害する可能性のある化学物質への曝露から生じるヒトおよび野生生物における有害影響について，科学的関心，公開討論，メディアの注目などで増大している．一方，内分泌かく乱化学物質の中枢神経系に対する影響についての報告がされるようになったのはごく最近のことである．

生命発生以来30億年の間に存在しなかった何万種類もの化学物質が，近々100年間に環境中に放出されては，いくら生命体が適応能力に富んでいるとしても，それらに対応する時間的余裕はないであろう．このような状況では，内分泌かく乱化学物質が個体発生のプログラムに影響して，正常な発生を妨害する可能性が高い．成熟個体に対しては可逆的に影響する外因性の情報伝達物質も胎児期および授乳期の未熟な個体では不可逆的に作用しうることはサリドマイドの例をみるまでもなく，種の存続に打撃を与えることを意味する．内分泌かく乱化学物質の作用を評価する際，成熟生体と胎児期および授乳期の生体とを分けて，別個の視点から評価する必要があると考えられる．現在までに著者らが明らかにしている結果は，胎児期および授乳期にbisphenol-Aを含め内分泌かく乱化学物質を飲食物とともに摂取した母親から生まれた現代人が，依存性薬物による精神依存の形成に対する脆弱性がひき起こされている可能性を提示する

ものであり，第3次覚せい剤乱用期は，食生活などを背景とした環境因子もかかわっている可能性も否定できないことを示している．

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RESEARCH ARTICLE

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

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Abstract

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

Introduction

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

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

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

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

Materials and method

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

Animals

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

Membrane preparations

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

[^{35}S]GTPγS binding assay

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

Dopamine D_3 receptor binding assay

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

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

RT-PCR assay

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

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

Results

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

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

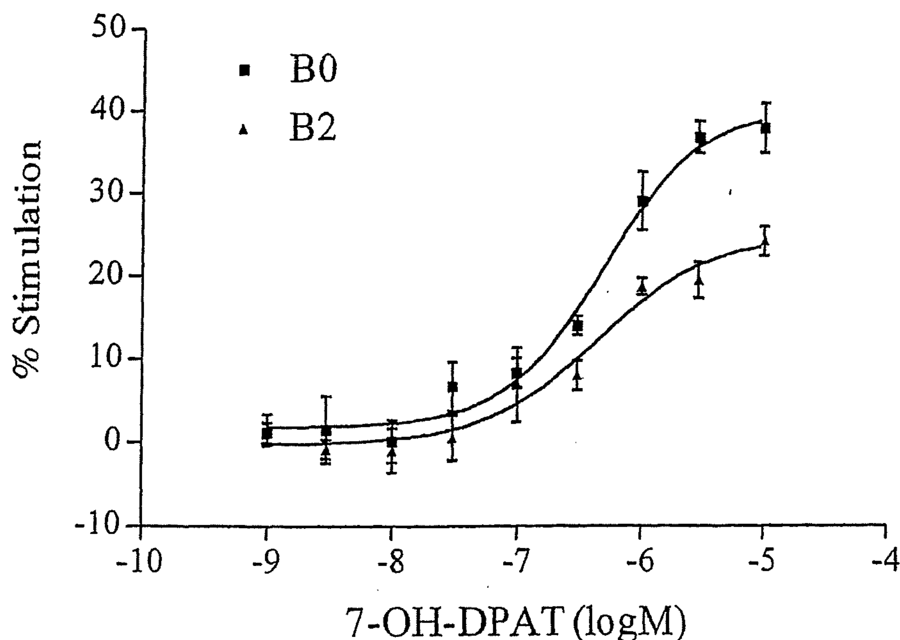

Figure 1. *Comparison of the stimulation of $[^{35}S]GTP\gamma S$ binding by dopamine D_3 receptor agonist 7-OH-DPAT to membranes of the limbic forebrain obtained from control (B0: square) and BPA-treated (B2: triangle) mice. Membranes were incubated with $[^{35}S]GTP\gamma S$ (50 pM) and GDP (30 μM)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

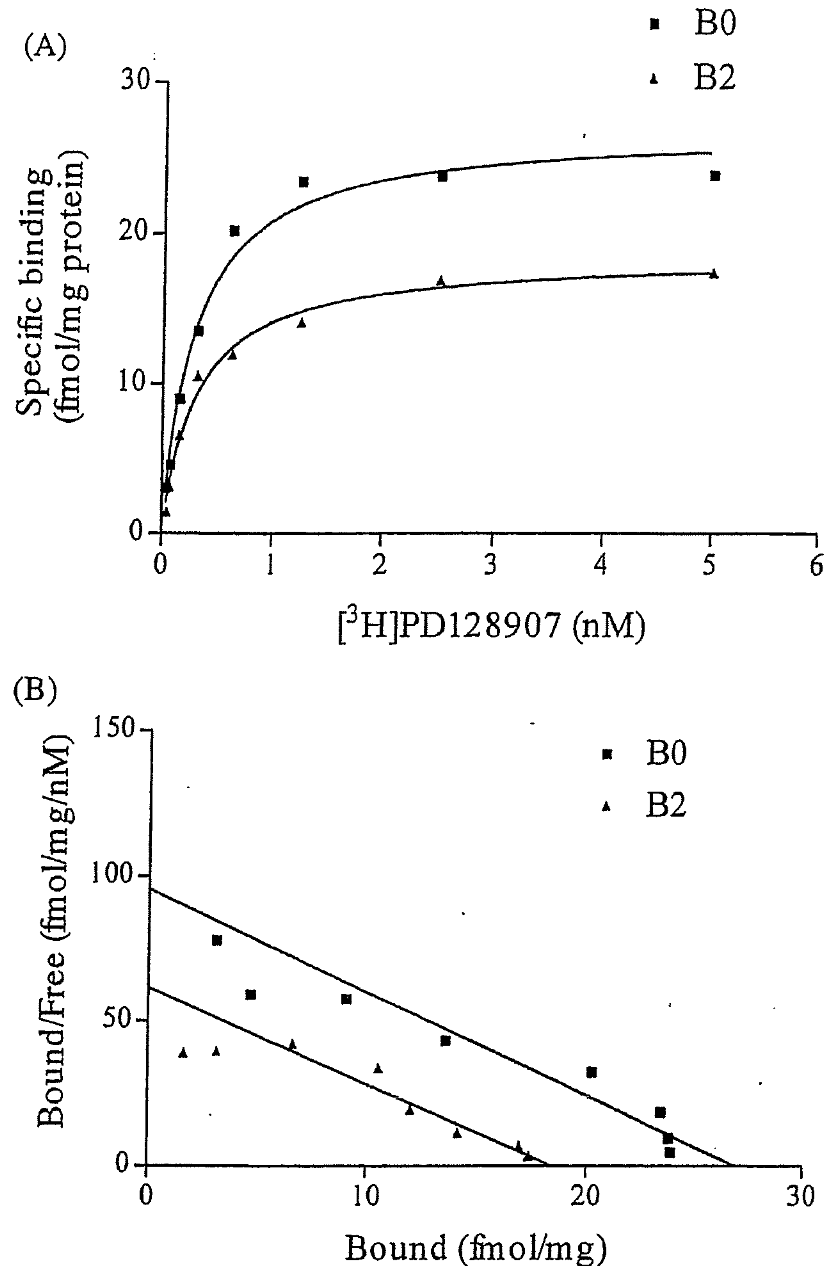

Figure 2. *Decrease of dopamine D3 receptor density in the limbic forebrain obtained from mice prenatally and neonatally expos to BPA. Saturation binding (A) and Scatchard analysis (B) were performed by receptor binding assay using [^{3}H]PD1289(Membranes were incubated with [^{3}H]PD128907 (0.039–5.0 nM) and Gpp(NH)p (100 μm).*

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

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

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

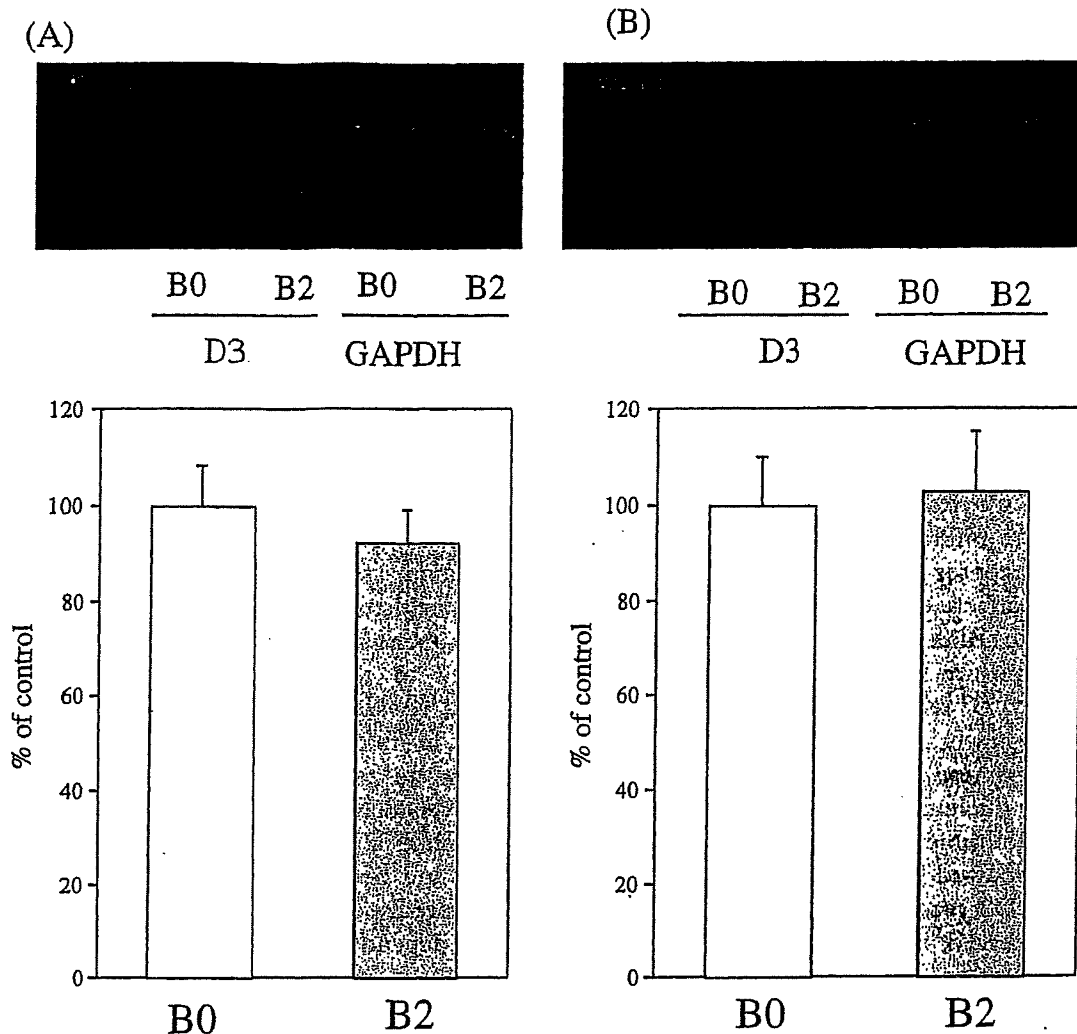

Figure 3. *No changes in the expression of dopamine D3 receptor mRNAs in the (A) limbic forebrain and (B) lower midbrain obtained from B0 and B2 mice. Upper panel: representative reverse transcription-polymerase chain reaction for dopamine D3 receptor mRNAs in the limbic forebrain and lower midbrain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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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Abstract

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

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

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

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

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

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

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

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

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

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

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

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

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

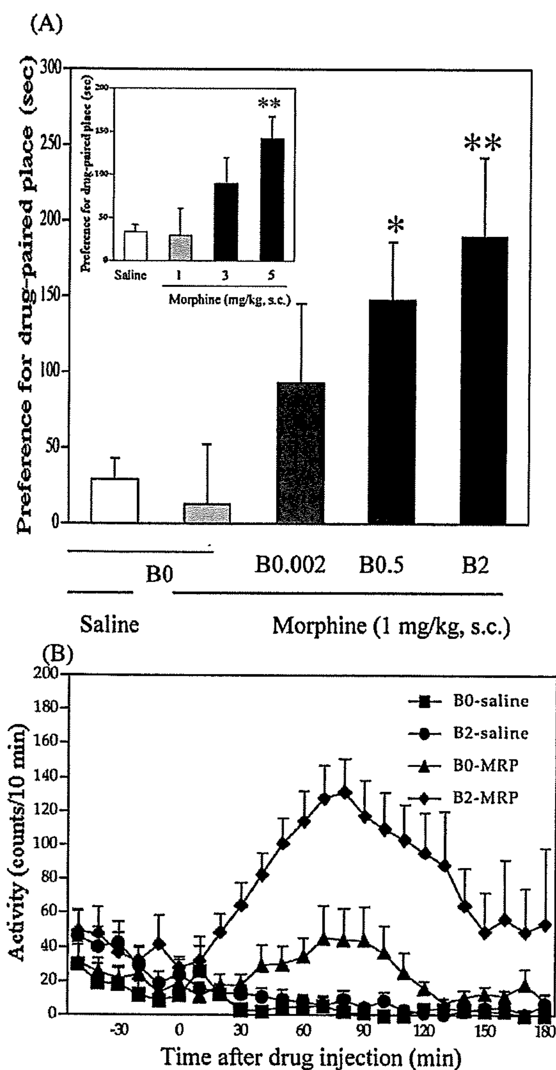

Fig. 1. (A) Effect of prenatal and neonatal exposure to BPA on the rewarding effect of morphine in mice. (Inner) Dose-response for the morphine-induced place preference in control B0 mice (* $P < 0.05$ vs. saline-treated mice). (Outer) Effect of BPA (0.002–2 mg/g of food: B0.002–B2) on the morphine (1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

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

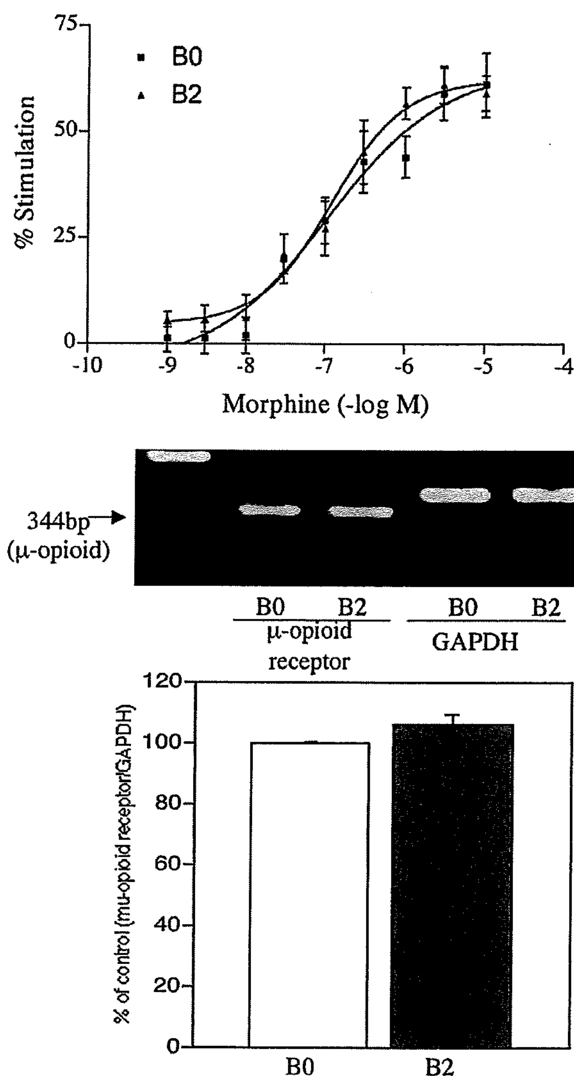

Fig. 2. No changes in the μ-opioid receptor in the mice prenatal and neonatal exposure to BPA. (A) Comparison of the stimulation of [^{35}S]GTPγS binding by morphine to membranes of the lower midbrain obtained from control (B0: square) and BPA-treated (B2: triangle) mice. Membranes were incubated with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

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

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

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

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Industrial Health 2006, 44, 510–524 **Original Article**

Effects of Perinatal Exposure to Bisphenol A on Brain Neurotransmitters in Female Rat Offspring

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Abstract: Pregnant Sprague-Dawley (CD IGS) rats were orally administered doses of bisphenol A (BPA) at 4, 40, and 400 mg/kg, from gestation days 6 to postnatal day 20. Neurotransmitters such as dopamine (DA) and serotonin (5HT) were extracted from the brains of dams and female offspring, and measured using liquid chromatography. BPA at 400 mg/kg was toxic and dosed rats died. At 3 wk after birth, brain levels of 3,4-dihydroxyphenylacetic acid (DOPAC, a DA metabolite), homovanillic acid (HVA, a DA metabolite), 5HT, 5-hydroxyindoleacetic acid (5HIAA, a 5HT metabolite) in female offspring were increased and the HVA/DA ratio was high in some brain areas of BPA-treated groups as compared with controls. At the age of 6 wk, levels of choline (Ch) in BPA-treated groups at 4 and 40 mg/kg were higher than control in all of eight brain areas. No changes were observed in acetylcholine (ACh) contents. In 9-wk-old offspring, changes in monoamines and metabolites were scattered and not great. At 3 wk after delivery, levels of 5HIAA in some brain areas of dams treated with BPA were higher than in control dams. Dose dependent increases in HVA and the HVA/DA ratio of the occipital cortex, and in the HVA/DA ratio of the frontal cortex were observed. The turnover of DA and 5HT was accelerated in 3-wk-old offspring and dams. BPA possesses very weak estrogenic activity. Changes in cerebral neurotransmitters observed in offspring and dams in this study may have been related to the estrogenic activity of BPA. However, further investigation is needed to examine the contribution of hormonal activity to such neurotransmitter changes.

Key words: Bisphenol A, Perinatal exposure, Offspring, Brain, Neurotransmitters, Dopamine, Serotonin, Acetylcholine, IGS rat

Introduction

Among many stabilizers of plastics, bisphenol A (BPA) is a popular stabilizer that mimics the actions of estrogen and affects the endocrine glands *in vivo* and *in vitro*[1, 2]. Although BPA binds to estrogen receptors to a lesser extent than 17β-estradiol, BPA affects sperm production and the prostate in male offspring, as well as body weight in male and female offspring[3–5]. Low dose effects of BPA and inverted U-shaped dose response relationships have also been reported at 2 to 20 μg/kg and at 0.1 to 50 mg/kg, respectively[6, 7]. The nervous systems of fetuses and newborns are susceptible to chemical effects[8, 9], and the maternal administration of BPA affects the reproductive system and behavior of experimental animal's offspring[10, 11]. These reports strongly suggest that maternal administration of BPA affects the nervous system of offspring. We previously examined how the maternal administration of BPA affects the reproductive organs, sex hormones, learning and memory functions of offspring[12, 13]. We found that the plasma testosterone concentrations of rats at 9 wk of age were significantly elevated in BPA groups compared with

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controls[13]. The content of testosterone in the testes increased in a similar manner to that in plasma. We also studied neurochemical changes in the neonatal brain. Neurotransmitters play key roles in the regulation of brain function. Many mental and nervous diseases are related to disordered function of neurotransmitters, and neuroactive drugs act by altering neurotransmitter levels[14]. Neurochemical changes are also involved in chemical neurotoxicity[15,16].

The present study used a neurochemical approach to investigate how BPA alters brain function in second generation rats. Following the maternal administration of BPA during pregnancy and lactation, we assayed the brain for contents of norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC, a dopamine metabolite), homovanillic acid (HVA, a dopamine metabolite), serotonin (5HT), 5-hydroxyindoleacetic acid (5HIAA, a 5HT metabolite), acetylcholine (ACh), and choline (Ch, an ACh precursor and metabolite) in female rat offspring.

Materials and Methods

Animals and chemicals

The CD (SD) IGS strain of rats was used, and 24 pregnant 9-wk-old rats were purchased from Charles River Japan Inc. at gestation day (GD) 3. GD 0 was confirmed by the presence of a copulatory plug. They were individually housed under a 12/12 h light/dark cycle with lights on at 08:00, with free access to feed (CE-2, Japan Clea, Inc.) and tap water. Room temperature and humidity were maintained at 23 ± 1°C and 55 ± 5%, respectively. Four rat groups, of 6 pregnant rats each, were given standard BPA (>99.8% pure; Cat#: 280-08561, Lot#: HCE9312, Wako Pure Chemicals, Japan), at 0 (control), 4, 40, or 400 mg/kg body weight (BW), respectively. BPA was dissolved in corn oil (10 ml/kg BW).

Administration of BPA to pregnant rats

BPA was administered to rats by oral gavage between 08:30 and 09:30 from GD 6 through postnatal day (PND) 20. The day of birth by 10:00 was considered PND 0. One dam in the control group was not pregnant. Therefore, 5 dams were available for the analysis in the control group. In the 4 and 40 mg/kg BPA groups, 6 dams were available for the analysis, but in the rat group administered with daily doses of BPA at 400 mg/kg, 4 rats died before and after delivery. Therefore, the rat group given 400 mg/kg BPA was not used in the analysis. Pups were sacrificed at 1, 3, 6, and 9 wk of age between 13:00 and 16:00. The litter size was standardized to 10 pups (male:female = 5:5, if possible) for each dam on PND 7. Subsequently, at 3, 6 and 9 wk of age, 4 to 6 pups of each sex were sacrificed from each BPA dose group. Pups chosen for sacrifice in each of the BPA dose groups were culled from different dams. Because of the imbalance of male and female numbers and different pup numbers among dams, some pups remained after the litter size standardization on PND 7. These pups were used for the analysis at 1 wk of age. Therefore, the numbers of male and female pups sacrificed in each dose group at 1 wk of age ranged from 1 to 10.

Offspring were weaned on PND 21, and males and females were separately housed. The highest dose, 400 mg/kg BPA was selected after Kwon *et al.*[17], who observed no effects of BPA at doses of 320 mg/kg/day from GD 11 through PND 20 on maternal body weight. The brain contents of neurotransmitters of offspring were assayed at 1, 3, 6, and 9 wk after birth. The brain neurotransmitters of dams given BPA were assayed at 3 wk after delivery (15 wk old).

Extraction and measurement of brain substances

Pups were sacrificed by decapitation under ice-cold hypothermia at 1 wk after birth to obtain organs in addition to the brain. At 3 and 9 wk after birth, pups were sacrificed by exsanguination from the abdominal vein under ether anesthesia to obtain organs including the brain. No effects of ether anesthesia on the brain monoamines were confirmed. At 6 wk after birth, the pups were sacrificed by microwave exposure (1.5 KW, 0.8 s) focused on the head (Microwave applicator, Muromachi Kikai Co., Tokyo)[18]. Exposure to microwaves rapidly increases the brain temperature and prevents rapid postmortem changes of brain substances. At 1, 3 and 9 wk after birth, microwaves were not used for sacrifice to obtain the other organs. Therefore, the ACh and Ch contents could not be measured in offspring at these ages. At 1 wk after birth, brain substances were analyzed in the whole brain because the brain was too small to divide exactly into individual regions. Three wk after birth, the brains (half brain) were dissected on ice into the forebrain, hindbrain, medulla oblongata, and cerebellum. The forebrain and hindbrain were obtained by cutting the brain vertically at the level of the optic chiasm after removing the cerebellum and medulla oblongata. At 6 wk after birth, the brains were dissected into the frontal cortex, occipital cortex, hippocampus, midbrain, striatum, hypothalamus, medulla oblongata, and cerebellum as described by Glowinski and Iversen[19]. Nine wk after birth, half the brain was used for the measurement of enzyme activity and the other half of the brain was dissected into the four brain regions like the

brain of 3-wk-old rats, to measure monoamine contents. Brains of dams were dissected into eight brain regions as described above according to Glowinski and Iversen. Dams were sacrificed on the day of the weaning of pups between 13:00 and 16:00. They were exsanguinated from the abdominal vein under ether anesthesia to obtain organs in addition to the brain. All brain samples were stored at −80°C and dissolved in 0.2 N $HClO_4$ containing 1 mM EDTA and 5 mM $Na_2S_2O_5$ before disruption using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000 g for 25 min at 4°C, and the supernatant was analyzed by HPLC[20, 21]. Supernatants were divided into two portions (Portion A & B). Portion A was neutralized with potassium acetate and the supernatant was obtained after centrifugation. This supernatant was analyzed by HPLC to determine ACh and Ch contents. Portion B was applied to activated alumina to adsorb NE, DA, and DOPAC (Portion B1). HVA, 5HT, and 5HIAA remained in the eluate after centrifugation (Portion B2). Each portion was passed through a filter of 0.45 μm pore size before application to HPLC.

HPLC analysis

ACh and Ch in portion A and ethylhomocholine (internal standard), were separated by reverse phase ion pair chromatography (Eicompak AC-GEL, Eicom Co., Japan) using a mobile phase comprising 0.1 M phosphate buffer, pH 8.2[22]. One liter of this buffer contained sodium 1-decanesulfonate, tetramethylammonium chloride, and $EDTA \cdot Na_2 \cdot H_2O$. Eluates were passed through a column that fixed ACh esterase and Ch oxidase (AC Enzymepak, Eicom). The column temperature was maintained at 30°C in an oven. The flow rate of the HPLC pump (L-4000, Hitachi Co., Tokyo) was 0.6 ml/min. ACh, Ch, and ethylhomocholine were assayed using an electrochemical detector (ECD-100, Eicom) equipped with a platinum electrode to measure the amount of H_2O_2 produced by the enzyme reaction of the three compounds. The voltage for electrochemical detection was 450 mV.

Monoamines and metabolites were assayed in extracts from the brain homogenates by HPLC equipped with an electrochemical detector (ECD-300, Eicom) and a carbon electrode. Reverse phase ion pair chromatography separated NE, DA, and DOPAC in portion B1 (Eicompak MA-5ODS, Eicom). The mobile phase was citrate-acetate buffer, pH 3.5. The retention time for each component was adjusted by adding sodium 1-octanesulfonate and methanol. An Eicompak MA-5ODS separated HVA, 5HT, and 5HIAA in portion B2 using a mobile phase comprising citrate-acetate buffer, pH 3.9. The separation parameters were as follows: temperature, 25°C; flow rate, between 0.5 and 0.9 ml/min; voltage, between 700 and 800 mV.

Statistics

Means ± SEM of each group were calculated for each of the monoamine or metabolite contents of the brain (nmoles/g tissue). Amine ratios (DOPAC/DA, HVA/DA, 5HIAA/5HT, and ACh/Ch) were calculated for each rat and the mean values of these ratios were obtained for each group. Metabolite/monoamine ratios (DOPAC/DA, HVA/DA, and 5HIAA/5HT) are widely used as markers of turnover of DA and 5HT in cerebral neurons. The statistical significance of differences between the control and dosed groups was examined by Dunnett's multiple *t*-test using statistics software (SPSS Japan Inc.). Differences between groups at $p<0.05$ were considered significant.

Results

Effects of BPA on 1-wk-old offspring

Figure 1 shows how the maternal administration of BPA affected the brain content of neurotransmitters and metabolites in female offspring at 1 wk after birth. In BPA-treated groups, levels of DOPAC and 5HT were low and those of DA and HVA were high, although these changes were less than 20% of each control value and no differences were statistically significant. Metabolite/monoamine ratios (DOPAC/DA, HVA/DA, and 5HIAA/5HT) were calculated for each rat. A significant difference was observed between the DOPAC/DA ratios of the control (0.264, 100%) and 40 mg/kg (0.200, 75.8%) groups. No significant differences were found between the control and BPA-treated groups in other metabolite/monoamine ratios.

Effects of BPA on the offspring at 3 wk of age

Figures 2-1 to 2-3 show levels of neurotransmitters, metabolites, and ratios of DOPAC/DA, HVA/DA, and 5HIAA/5HT in the brains of 3-wk-old rats. Levels of neurotransmitters in the cerebellum were low and the data varied too much to perform statistical analyses. Therefore, data obtained for the cerebellum are not presented. No effects of BPA were observed on the contents of NE and DA in the forebrain, hindbrain, and medulla oblongata. Levels of DOPAC of the 40 mg/kg group and HVA of the 4 mg/kg group were significantly increased in the forebrain compared with controls. A statistical significance was found in the difference of the mean values of the HVA/DA ratio in the forebrain between the control (0.161, 100%) and 4 mg/kg (0.280, 174%) groups. Levels of 5HT and 5HIAA in the

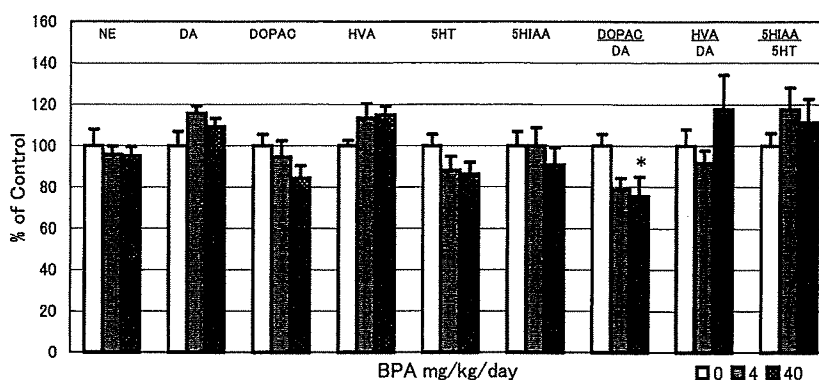

Fig. 1. Effects of perinatal administration of BPA on the neurotransmitter contents of whole brain in 1-wk-old female offspring.

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 0.736 for NE, 1.18 for DA, 0.305 for DOPAC, 0.575 for HVA, 11.8 for 5HT, and 2.69 for 5HIAA; absolute values of ratios for 100% were as follows: 0.264 for DOPAC/DA, 0.522 for HVA/DA, and 0.228 for 5HIAA/5HT. N = 6–8. *: $p<0.05$ by Dunnett's multiple *t*-test.

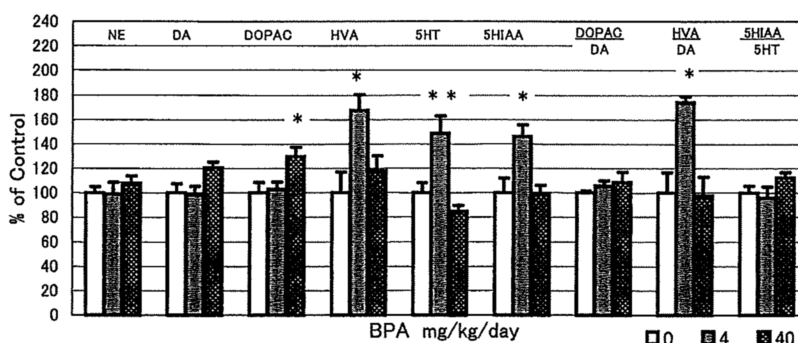

Fig. 2-1. Effects of perinatal administration of BPA on the neurotransmitter contents of forebrain in 3-wk-old female offspring.

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 0.944 for NE, 6.59 for DA, 1.30 for DOPAC, 1.08 for HVA, 1.64 for 5HT, and 1.12 for 5HIAA; absolute values of ratios for 100% were as follows: 0.197 for DOPAC/DA, 0.161 for HVA/DA, and 0.717 for 5HIAA/5HT. N = 4–5. *: $p<0.05$; **: $p<0.01$ by Dunnett's multiple *t*-test.

forebrain of the 4 mg/kg group were significantly increased compared with controls. There was no difference in the 5HIAA/5HT ratio in the forebrain between the control and 4 mg/kg groups. The level of HVA in the hindbrain of the 40mg/kg group was higher than that of the control (130% of the control). The HVA/DA ratios in the hindbrain of BPA-treated groups were higher than that of the control (120 and 132% of the control at 4 and 40 mg/kg, respectively), although statistical significance was not found in these differences. The decrease in 5HT in the hindbrain of BPA-treated groups was within 20% of the control, and was statistically significant for the 40 mg/kg group. There were no significant changes in the 5HIAA/5HT ratios in the hindbrains of the 4 and 40 mg/kg groups. In the medulla oblongata, levels of HVA of the 40 mg/kg group, and 5HT and 5HIAA of the 4 and 40 mg/kg groups, were higher than those of the control. Among them 5HT of the 4 mg/kg group was significantly increased compared with the control. The HVA/DA ratio of the 40 mg/kg group was higher than that of the control, however, the difference was not statistically significant.

Effects of BPA on the offspring at 6 wk of age

Tables 1-1 to 3-2 summarize the results of neurotransmitter analysis of offspring at 6 wk after birth.

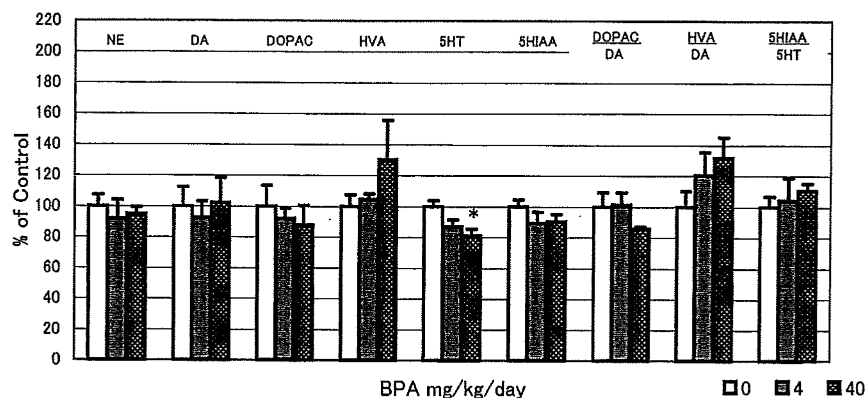

Fig. 2-2. Effects of perinatal administration of BPA on the neurotransmitter contents of hindbrain in 3-wk-old female offspring.

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 1.32 for NE, 1.08 for DA, 0.272 for DOPAC, 0.206 for HVA, 11.9 for 5HT, and 3.32 for 5HIAA; absolute values of ratios for 100% were as follows: 0.254 for DOPAC/DA, 0.188 for HVA/DA, and 0.281 for 5HIAA/5HT. N = 4–5. *: $p<0.05$ by Dunnett's multiple *t*-test.

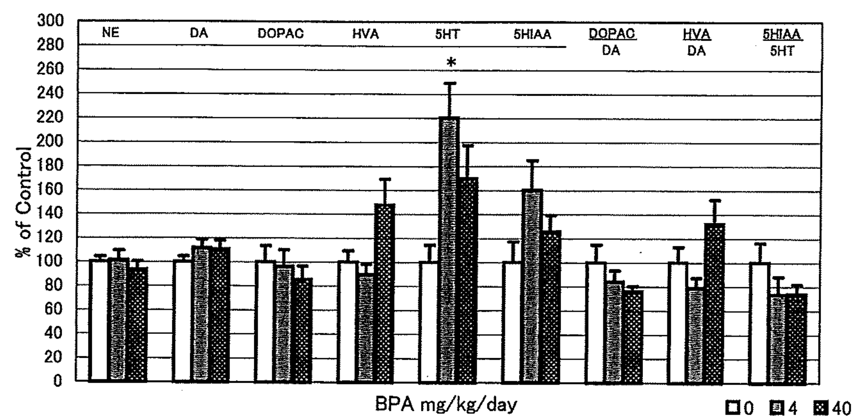

Fig. 2-3. Effects of perinatal administration of BPA on the neurotransmitter contents of medulla oblongata in 3-wk-old female offspring.

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 2.56 for NE, 0.193 for DA, 0.106 for DOPAC, 0.214 for HVA, 3.14 for 5HT, and 3.63 for 5HIAA; absolute values of ratios for 100% were as follows: 0.556 for DOPAC/DA, 1.14 for HVA/DA, and 1.20 for 5HIAA/5HT. N = 4–5. *: $p<0.05$ by Dunnett's multiple *t*-test.

Amine ratios (DOPAC/DA, HVA/DA, 5HIAA/5HT, and ACh/Ch) were calculated for each rat. There were no significant changes in monoamine contents and amine ratios in the frontal and occipital cortices of the BPA-treated groups. In the hippocampus, DA and DOPAC increased by 40 to 50% in the 40 mg/kg group compared to the control. These changes were not significant; however, the increase in Ch of the 4 mg/kg group was significant. Striatal Ch of the 4 mg/kg group was increased significantly compared to the control. Levels of Ch in the midbrain were high in BPA-treated groups and the ACh/Ch ratios of the 4 and 40 mg/kg groups were significantly smaller than those of the control. In the medulla oblongata, compared to the control the 5HT level of the 40 mg/kg group was low and Ch levels in the 4 and 40 mg/kg groups were high, however, none of these changes were statistically significant. The 5HIAA/5HT ratio was high in the 40 mg/kg group and the ACh/Ch ratio was low in the 4 mg/kg group, and these ratios significantly differed from the control. In the cerebellum, the DA content was high in the 40 mg/kg group (143% of the control), and DOPAC levels and DOPAC/DA ratios were low in the 4 and 40 mg/kg groups. Ch contents were

Table 1-1. Effects of maternal administration of BPA on neurotransmitter contents of 6-wk-old female offspring (frontal cortex, occipital cortex, and hippocampus)

		NE	DA	DOPAC	HVA	DOPAC/DA	HVA/DA
Frontal cortex	Control	100 ± 13.5	100 ± 15.6	100 ± 14.1	100 ± 5.0	100 ± 2.9	100 ± 16.4
	4 mg/kg	99.3 ± 4.0	90.3 ± 8.7	89.0 ± 4.8	89.3 ± 10.2	98.8 ± 6.0	90.0 ± 9.9
	40 mg/kg	90.7 ± 9.6	95.0 ± 10.0	90.2 ± 8.7	103.1 ± 5.0	94.5 ± 3.0	104.3 ± 15.5
	A100	1.75	6.31	1.39	0.779	0.222	0.137
Occipital cortex	Control	100 ± 9.5	100 ± 10.5	100 ± 8.9	100 ± 6.5	100 ± 3.1	100 ± 10.2
	4 mg/kg	94.1 ± 5.8	87.0 ± 4.8	78.0 ± 8.3	97.1 ± 7.1	88.2 ± 4.8	108.9 ± 9.1
	40 mg/kg	95.6 ± 4.6	83.4 ± 13.3	86.7 ± 10.4	99.6 ± 8.2	107.9 ± 11.4	123.6 ± 15.3
	A100	1.39	0.899	0.101	0.321	0.113	0.369
Hippocampus	Control	100 ± 5.2	100 ± 13.0	100 ± 4.8	100 ± 8.5	100 ± 9.5	100 ± 22.6
	4 mg/kg	122.1 ± 11.5	101.8 ± 10.9	88.0 ± 4.5	110.1 ± 5.9	84.5 ± 8.5	99.8 ± 13.5
	40 mg/kg	111.9 ± 8.6	141.5 ± 20.4	149.2 ± 28.9	109.0 ± 4.9	101.1 ± 7.8	73.6 ± 9.6
	A100	1.34	0.364	0.0291	0.389	0.0752	1.21

Results are shown as means ± SEM (%). A100, Absolute values for 100% (N = 4–5 for frontal and occipital cortex and N = 3–5 for hippocampus, nmoles/g tissue).

Table 1-2. Effects of maternal administration of BPA on neurotransmitter contents of 6-wk-old female offspring (frontal cortex, occipital cortex, and hippocampus)

		5HT	5HIAA	5HIAA/5HT	ACh	Ch	ACh/Ch
Frontal cortex	Control	100 ± 7.4	100 ± 12.0	100 ± 7.2	100 ± 12.9	100 ± 8.4	100 ± 10.1
	4 mg/kg	94.6 ± 2.9	93.8 ± 5.7	100.6 ± 6.3	106.8 ± 6.5	129.2 ± 8.6	84.5 ± 9.3
	40 mg/kg	107.3 ± 3.1	109.0 ± 5.5	102.7 ± 3.0	103.9 ± 16.1	126.4 ± 22.6	85.7 ± 10.2
	A100	7.76	0.918	0.117	10.1	11.9	0.848
Occipital cortex	Control	100 ± 5.5	100 ± 3.2	100 ± 8.2	100 ± 9.6	100 ± 17.4	100 ± 19.3
	4 mg/kg	103.9 ± 1.68	113.2 ± 7.2	107.4 ± 8.3	99.2 ± 4.4	129.1 ± 25.2	73.7 ± 11.3
	40 mg/kg	95.5 ± 10.5	103.4 ± 6.4	109.2 ± 7.4	90.3 ± 10.9	102.3 ± 18.9	85.3 ± 15.2
	A100	15.2	2.74	0.183	12.7	29.2	0.493
Hippocampus	Control	100 ± 5.3	100 ± 8.0	100 ± 5.1	100 ± 5.8	100 ± 16.4	100 ± 22.4
	4 mg/kg	109.1 ± 5.8	113.1 ± 13.4	102.8 ± 7.7	103.2 ± 13.4	155.5* ± 9.8	59.5 ± 10.3
	40 mg/kg	99.4 ± 11.7	88.6 ± 13.7	88.2 ± 7.0	108.2 ± 3.8	113.5 ± 10.4	86.4 ± 9.3
	A100	7.47	2.91	0.389	18.3	26.3	0.797

Results are shown as means ± SEM (%). A100, Absolute values for 100% (N = 4–5, nmoles/g tissue). *: $p < 0.05$ by Dunnett's multiple *t*-test.

high in the 4 and 40 mg/kg groups, however, none of these changes were statistically significant.

Effects of BPA on 9-wk-old offspring

Figures 3-1 to 3-4 show levels of neurotransmitters, metabolite, and metabolite/monoamine ratios at 9 wk after birth. The level of NE in the forebrain of the 40 mg/kg group was increased significantly compared with the control, although the increase was not great (123% of control). Compared to the control, the level of DA was unchanged, whereas that of DOPAC was significantly decreased (81% of control) in the forebrain of the 4 mg/kg group. The ratio of HVA/DA in the forebrain of the 40 mg/kg group was significantly low (73% of control). There were no significant changes in monoamine levels and metabolite/monoamine ratios in the hindbrain. Levels of DOPAC and 5HIAA were significantly decreased in the medulla oblongata of the 40 mg/kg group compared to the control. Ratios of DOPAC/DA and 5HIAA/5HT were lower than those of the control group, though differences were not significant. The concentration of DOPAC in the cerebellum was too low to detect consistently. Therefore, the DOPAC and DOPAC/DA data in the cerebellum were omitted from Fig. 3-4. Compared to the control, the 5HT and 5HIAA levels of the