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

*Prenatal and neonatal exposure to BPA*

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

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

*Place conditioning*

The place-conditioning procedure has been used to evaluate the motivation properties, such as rewarding or aversive effects, of drugs in adult rodents (17). Place conditioning was conducted as previously described (5, 6). The apparatus was a shuttle box (15 × 30 × 15 cm: w × l × h), which was made of acrylic resin board and divided into two equal-sized compartments. One compartment is white with a textured floor, and the other is black with a smooth floor to create equally preferred compartments. For conditioning, groups of mice (seven mice in a group) were confined to one compartment after morphine injections (morphine-paired side) and to the other compartment after saline injection (saline-paired side). The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. Conditioning sessions (3 days for morphine, 3 days for saline) were conducted once daily for 6 days. Immediately after s.c. injection of morphine (1 mg/kg), animals were placed in one compartment for 1 h. On alternate days, animals receiving the vehicle were placed in the other compartment for 1 h. On day 7, tests of conditioning were performed. The partition separating the two compartments was raised to 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. The mice were not treated with either morphine or saline, and then placed on the platform. The time spent in each compartment during a 900-s session was then recorded automatically using an infrared beam sensor (KN-80, Natsume Seisakusyo Co., Tokyo, Japan). All sessions were conducted under conditions of dim illumination (28 lux lamp) and white masking noise.

*Statistical analysis*

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

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

## Results

*BPA, but not $E_2$, causes the activation of astrocytes*

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

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

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

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

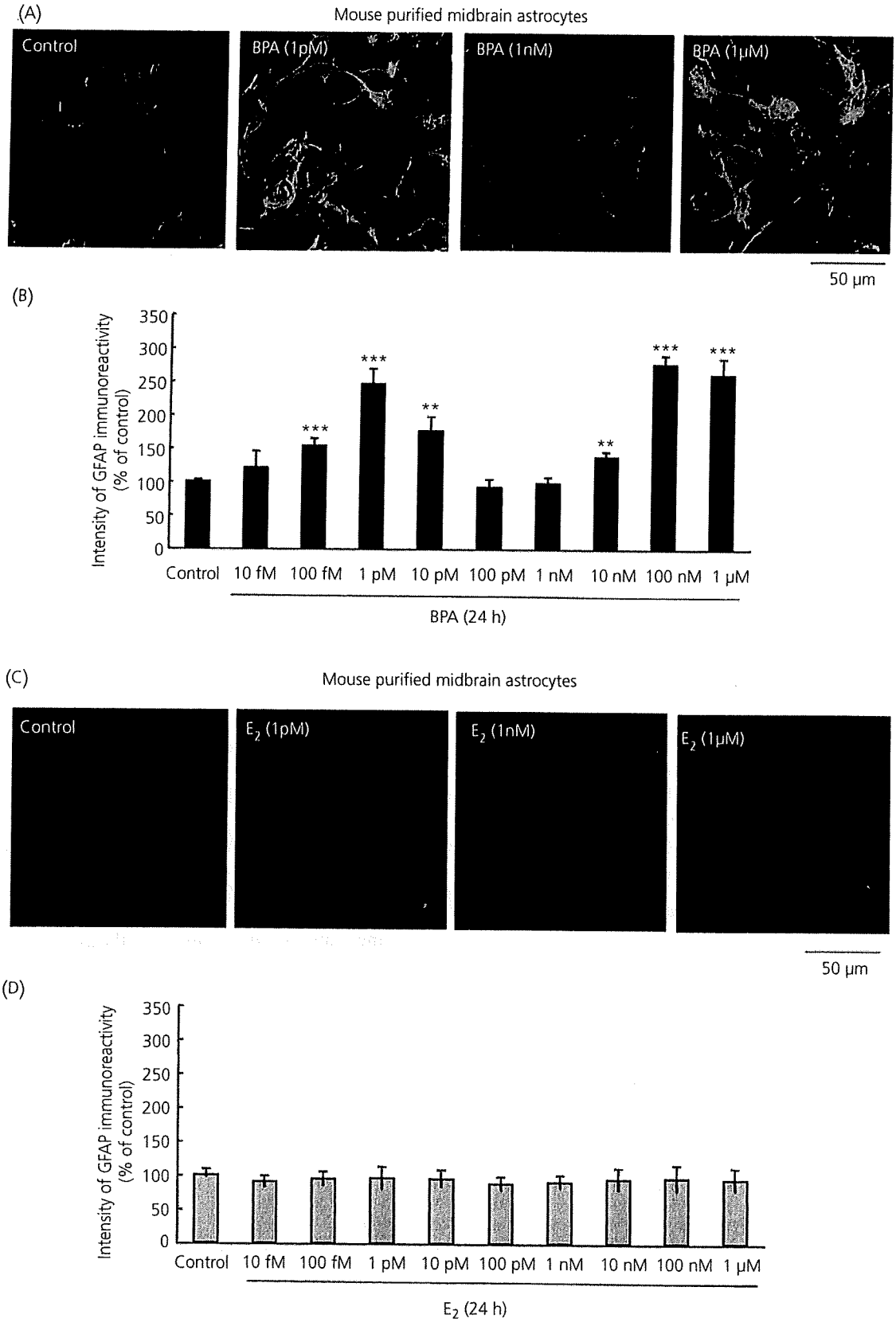
Dopamine (1–100 μM) produced a transient increase in the intracellular $Ca^{2+}$ concentration in mouse purified midbrain astrocytes (Fig. 3). The $Ca^{2+}$ responses to dopamine (100 μM) in astrocytes were significantly enhanced by pretreatment with a low concentration of BPA (1 pM, 24 h, **$P < 0.01$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

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

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

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



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

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

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

### *BPA-induced neuronal cell death*

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

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

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

## Discussion

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

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

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

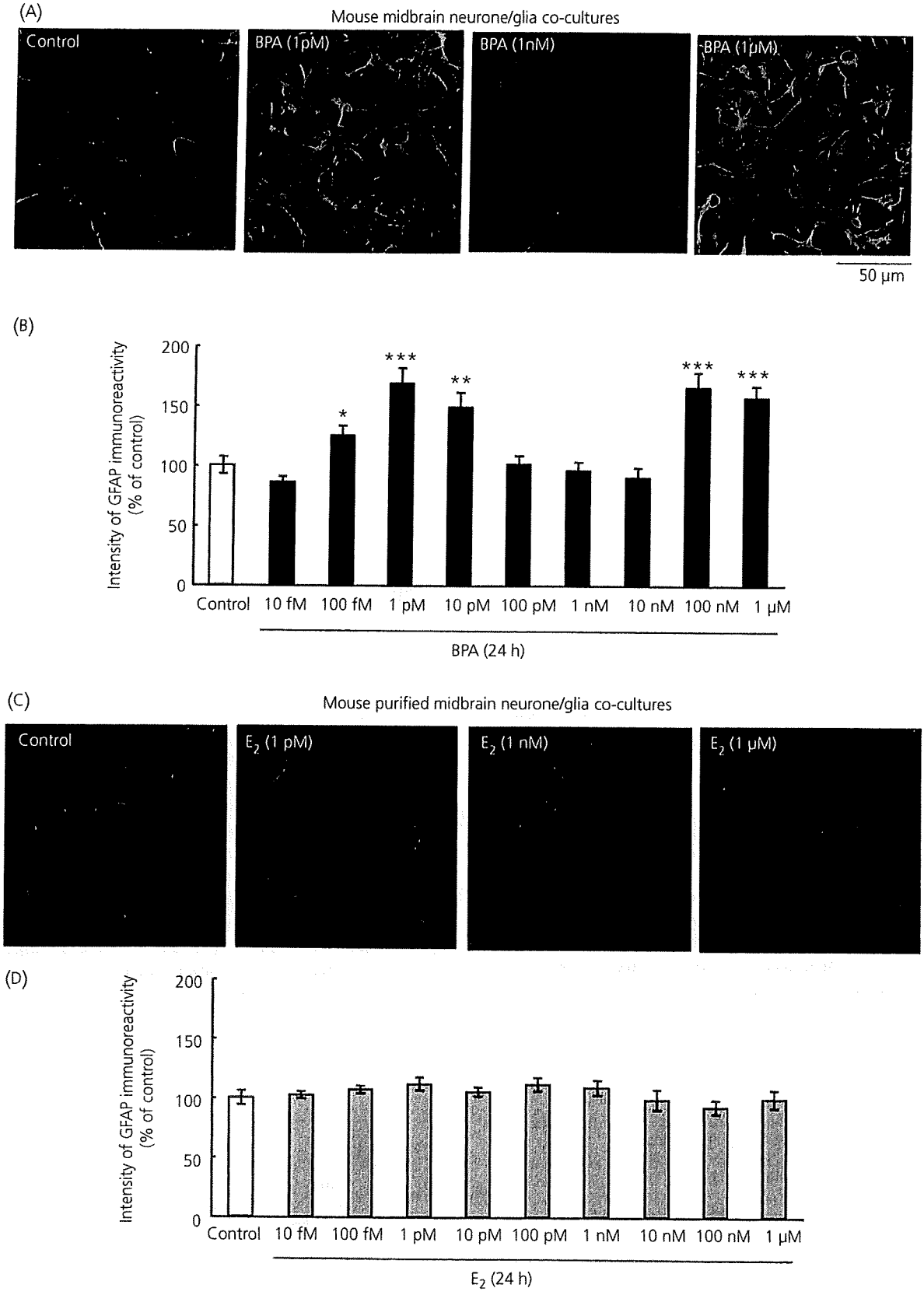
Neurones and astrocytes respond to various and chemical stimuli, including neurotransmitters, neuromodulators and hormones, with an increase in the intracellular $Ca^{2+}$ concentration. These $Ca^{2+}$ responses result from the co-ordinated activity of several molecular cascades responsible for $Ca^{2+}$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

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

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

activation of the phospholipase C-inositol-1,4,5-triphosphate signalling pathway (21, 22). Dopamine-induced $Ca^{2+}$ responses are also modulated by dopamine $D_2$ receptor (23, 24). On the other hand, dopamine $D_3$ receptor normally coexists with dopamine $D_1$ and $D_2$ receptors (25, 26), which contributes to the inhibitory modulation of dopamine $D_1$



and/or $D_2$ receptor-mediated signalling (7). We previously reported that prenatal and postnatal exposure to BPA (2 mg/g of mother's food) enhanced central dopamine $D_1$ receptor function (5) and attenuated dopamine $D_3$ receptor function in mice (27). Thus, the present data suggest that treatment with 1 pM of BPA may enhance dopamine $D_1$ receptor function and/or attenuate dopamine $D_3$ receptor function, resulting in enhancement of the dopamine-induced $Ca^{2+}$ response in neurones and astrocytes.

In the present study, we observed morphological changes in astrocytes by treatment with either 1 pM or 1 μM of BPA. We also found a difference between 1 pM and 1 μM of BPA: treatment with BPA (1 pM) in midbrain neurone/glia cocultures clearly enhanced dopamine-induced $Ca^{2+}$ responses in neurones, whereas treatment with BPA (1 μM) decreased dopamine-induced $Ca^{2+}$ responses in neurones. Treatment with a high concentration of BPA markedly induced neuronal cell death in midbrain neurone/glia cocultures. Thus, these data suggest that a high concentration of BPA may lead to a dynamic change in the neurone-glia network, resulting in neurotoxicity.

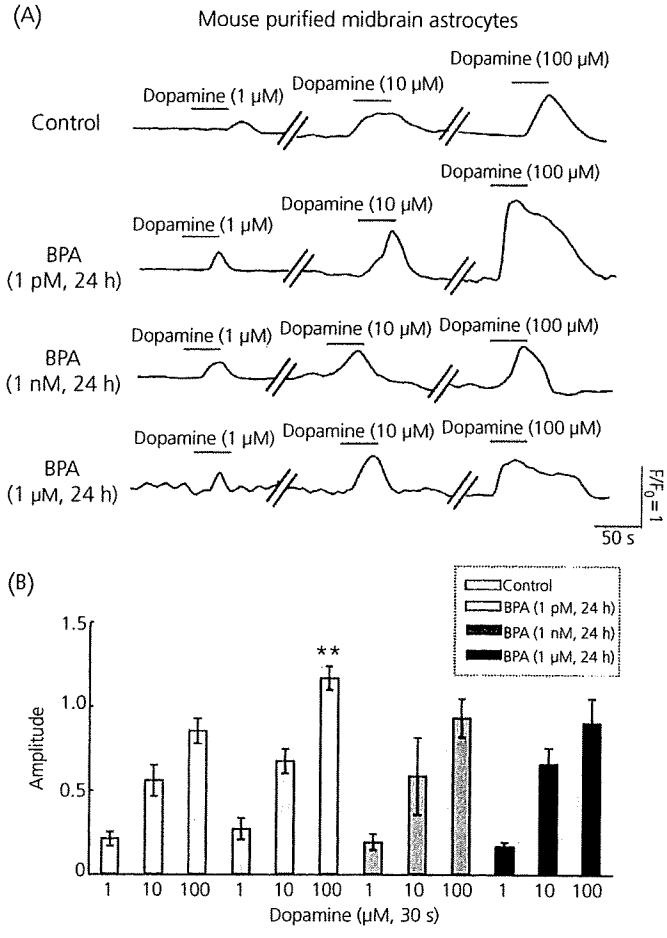



FIG. 3. The $Ca^{2+}$ response to dopamine in astrocytes was significantly enhanced by treatment with a low concentration of bisphenol-A (BPA). (A) Traces show a typical increase in the intracellular $Ca^{2+}$ concentration evoked by dopamine (1–100 μM) in control or BPA (1 pM, 1 nM or 1 μM) treated astrocytes. (B) The $Ca^{2+}$ responses to dopamine (1–100 μM) in control and BPA (1 pM, 1 nM or 1 μM) treated astrocytes are summarised. Data represent the mean ± SEM of 27–63 cells. **P < 0.01 versus control cells.

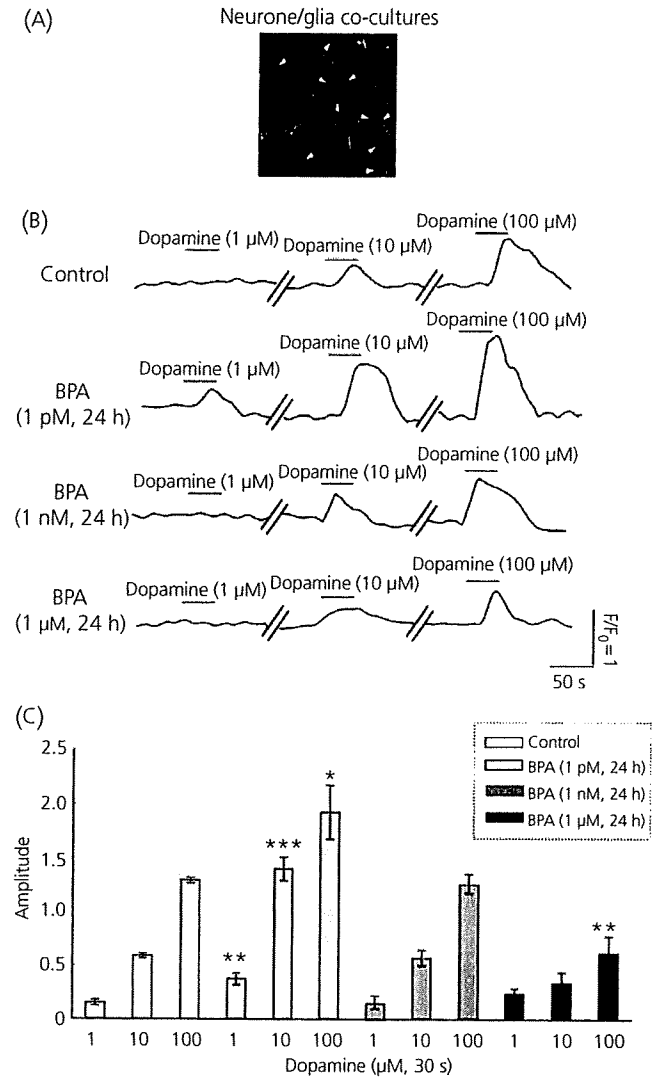



FIG. 4. The $Ca^{2+}$ response to dopamine in neurones was significantly enhanced by treatment with a low concentration of bisphenol-A (BPA). (A) Mouse neurone/glia cocultures were stained with a mouse antibody to anti-neuronal nuclei (Neu-N) (red) and a rabbit antibody to glial fibrillary acidic protein (green). Arrow heads show Neu-N-positive neurones, which we used as cultures neurones for the $Ca^{2+}$ imaging studies. (B) Traces show a typical increase in the intracellular $Ca^{2+}$ concentration in control evoked by dopamine (1–100 μM) or BPA (1 pM, 1 nM or 1 μM) treated neurones. (C) The $Ca^{2+}$ responses to dopamine (1, 10, 100 μM) in control and BPA (1 pM, 1 nM or 1 μM) treated neurones are summarised. Data represent the mean ± SEM of 27–63 cells. *P < 0.05, **P < 0.01, ***P < 0.001 versus control cells.

Similar to other drugs of abuse, the μ-opioid receptor agonist morphine acts as a rewarding stimulus when administered to animals (28). For example, rodents have been shown to intravenously self-administer morphine rather than saline when given a choice (29). Furthermore, μ-opioid receptor agonists can lower the electric current threshold for intracranial electrical self-stimulation, which indicates that opioids can facilitate the central reward mechanism itself. These positive motivational actions of opioids are indicative of their rewarding properties, and are considered to be fundamental for their ability to produce psychological dependence in humans (28).

As mentioned above, humans might be orally exposed to BPA in daily life. In a previous study, we chronically treated pregnant and lactating female mice with BPA-admixed powder food containing 2 mg of BPA/g of food, and this enhanced the development of rewarding effects induced by drugs of abuse in their adult offspring (5, 6). Under these conditions, mother mice received approximately 200 mg/kg/day of BPA. In addition, the blood level of BPA in their pups was approximately 10 ng/ml, which is considered to be more than 30-fold higher than the level for healthy human exposure (5). On the other hand, vom Saal *et al.* (30) estimated that humans are exposed to BPA at a dose of 2–20 μg/kg/day. Based on these reports, we ascertained the effects of oral exposure to BPA. A group of mother mice were orally administered BPA at a dose of 3 μg/kg/day, which is considered to be a suitable dose to reflect environmental exposure to BPA. Another group of mother mice were orally administered BPA at a dose of 200 mg/kg/day, which is considered to be higher than the environmental exposure to BPA. We also investigated the effect of prenatal and neonatal exposure to $E_2$ (3 μg/kg/day) to compare its effects with those of BPA. Their pups, which were prenatally and postnatelly exposed to BPA, were used in the place preference studies.

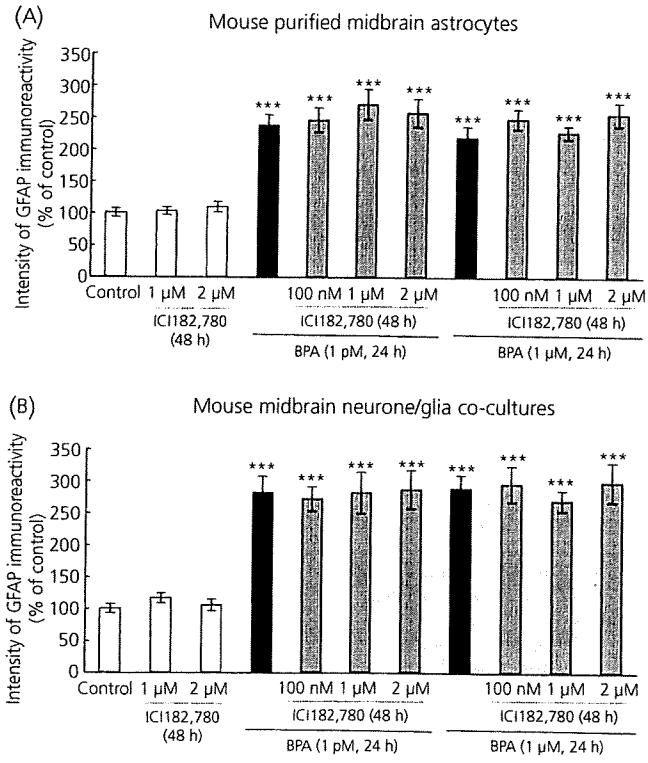



FIG. 5. The effect of ICI182,780 on astrocytic activation induced by bisphenol-A (BPA). Mouse purified midbrain astrocytes (A) or neurone/glia cocultures (B) were treated with normal medium (control) or ICI182,780 (100 nM, 1 μM or 2 μM) for 24 h. Cells were then treated with normal medium, BPA (1 pM or 1 μM) with or without ICI182,780 (100 nM, 1 μM or 2 μM) for an additional 24 h. The cells were stained with a polyclonal antibody to glial fibrillary acidic protein (GFAP). The intensity of GFAP-immunoreactivity was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells. ***P < 0.001 versus control cells. The white bars indicate the levels of GFAP-like immunoreactivity in the cells treated without BPA. The black bars indicate the levels of GFAP-like immunoreactivity in the cells treated with BPA. The grey bars indicate the levels of GFAP-like immunoreactivity in cells treated with BPA and ICI182,780.

One of the most important aspects of the present study was that *in vivo* prenatal and neonatal exposure to BPA (3 μg or 200 mg/kg/day administered to pregnant and lactating dams) clearly enhanced the rewarding effect of morphine in mice. Although BPA at 200 mg/kg/day may be higher than the environmental exposure, BPA may be found in the environment at a level equivalent to 3 μg/kg/day. As mentioned above, *in vitro* experiments indicate that the enhancement of $Ca^{2+}$ responses to dopamine induced by BPA could lead to an increase in the excitability of central dopaminergic neurotransmission in both neurones and astrocytes. These findings suggest that the enhancement of dopaminergic transmission in neurones and astrocytes induced by BPA may, at least in part, lead to enhancement of the development of psychological dependence on morphine.

BPA can modulate gene transcription and numerous biological changes via oestrogenic receptors (1, 31, 32). It has been reported that equal doses of BPA and $E_2$ could activate the transcription factor cAMP-responsive element binding protein (CREB) via nonclassical oestrogen receptor, resulting in the transcriptional activation of CREB-responsive genes (33). On the other hand, obvious differences between BPA and $E_2$ have also been reported. For example, $E_2$ at 10 nM reduced the duration of $Ca^{2+}$ oscillations in mouse oocytes, whereas concentrations of BPA as high as 100 μM were necessary for similar inhibition (34). It has been reported that $E_2$ inhibits the astrocytic uptake of glutamate, which is the most important excitatory neurotransmitter in the CNS, whereas BPA has no such effect (35). Taken together, these observations suggest that BPA and $E_2$ may be coupled to different signalling cascades in the CNS.

Astrocytes are among of the most important target cells for $E_2$. Astrocytes express all types of oestrogen receptors during development and in the adult brain (35–37). However, in the present study, neither the oestrogen receptor antagonist ICI182,780 nor the oestrogen receptor agonist/antagonist tamoxifen failed to block the activation of astrocytes induced by BPA. The progesterone receptor antagonist mifepristone and the androgen receptor antagonist flutamide also had no effect on the activation of astrocytes induced by BPA. Furthermore, $E_2$ had no effect on the activation of astrocytes in both purified astrocytes and neurone/glia cocultures. It appears likely that oestrogen receptors and other steroid hormone receptors may not be critical for the activation of astrocytes induced by BPA. We also found that prenatal and postnatal *in vivo* exposure to $E_2$ failed to enhance the rewarding effect of morphine in mice. These data suggest that oestrogenic neurotransmission is not essential for the enhancement of dopaminergic neurotransmission and hypersensitivity to the morphine-induced rewarding effect induced by exposure to BPA.

In conclusion, the results of the present study suggest that BPA induces dopaminergic amplification in neurones and astrocytes, and may contribute to potentiate the development

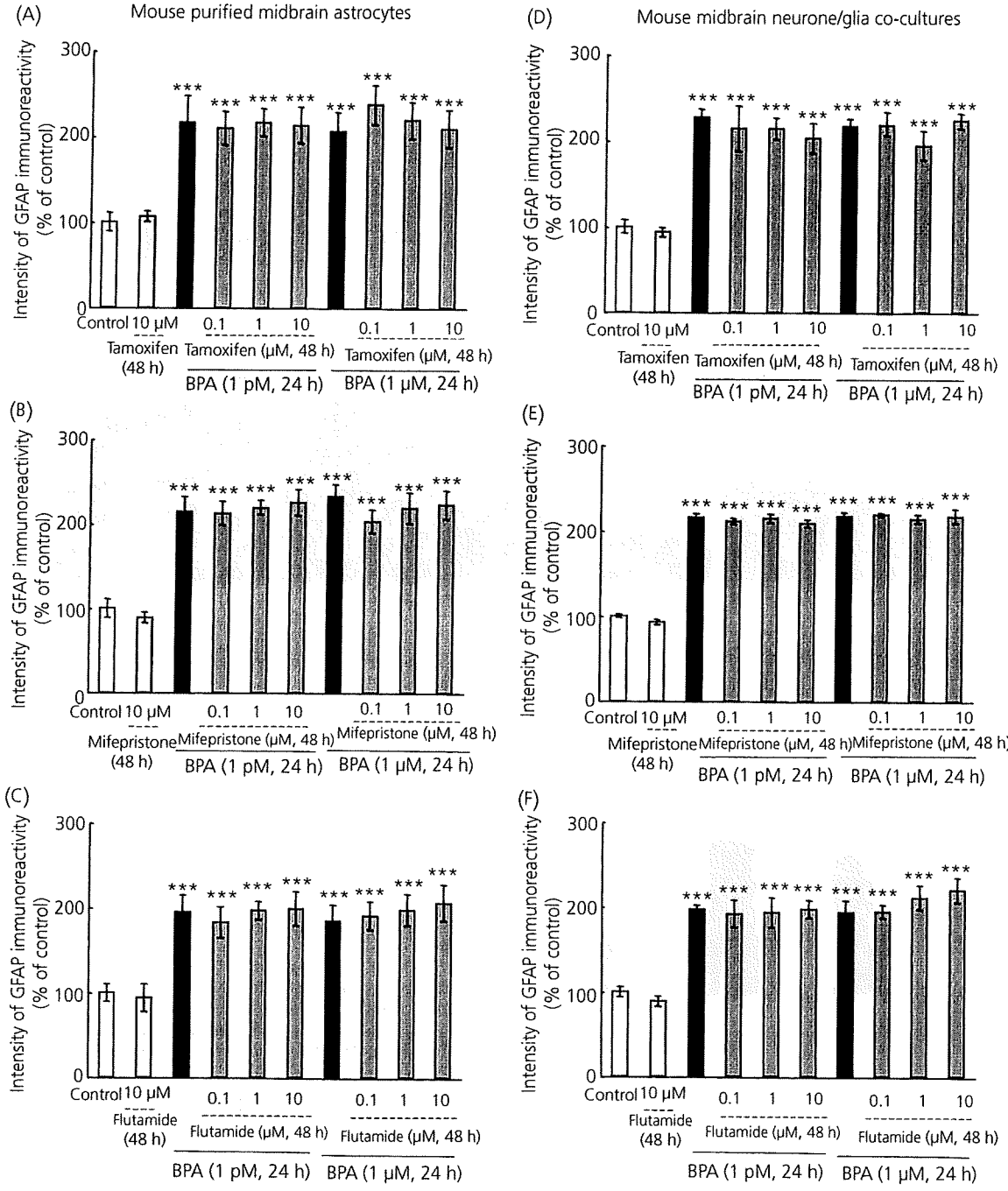



FIG. 6. Effects of steroid hormone ligands on astrocytic activation induced by bisphenol-A (BPA). Mouse purified midbrain astrocytes (A–C) or mouse midbrain neurone/glia cocultures (D–F) were treated with normal medium or tamoxifen (100 nM, 1 μM or 10 μM; A,D), mifepristone (100 nM, 1 μM or 10 μM; B,E) or flutamide (100 nM, 1 μM or 10 μM; C,F) for 24 h. Cells were then treated with normal medium, or medium that had been supplemented with BPA (1 pM, 1 μM) with or without tamoxifen (100 nM, 1 μM or 10 μM; A,D), mifepristone (100 nM, 1 μM or 10 μM; B,E) or flutamide (100 nM, 1 μM or 10 μM; C,F) for an additional 24 h. The cells were stained with a polyclonal antibody to glial fibrillary acidic protein (GFAP). The intensity of GFAP-immunoreactivity was measured using NIH Image. The level of GFAP-like immunoreactivity is expressed as a percent increase (mean ± SEM) with respect to that in control cells. ***$P < 0.001$ versus control cells (without BPA or any antagonists). The white bars indicate the levels of GFAP-like immunoreactivity in cells treated without BPA. The black bars indicate the levels of GFAP-like immunoreactivity in cells treated with bisphenol-A. The grey bars indicate the levels of GFAP-like immunoreactivity in cells treated with BPA and steroid hormone ligands.

of the rewarding effect of morphine. Drug abuse among the young is increasing worldwide. On the other hand, emotional fragility often plays a major role in leading people to drug abuse. Our findings warn that prenatal and postnatal exposure to BPA may be linked to severe health problems in humans, including abnormalities in the CNS, resulting in an emotional sensitivity toward the development of dependence on drugs of abuse.

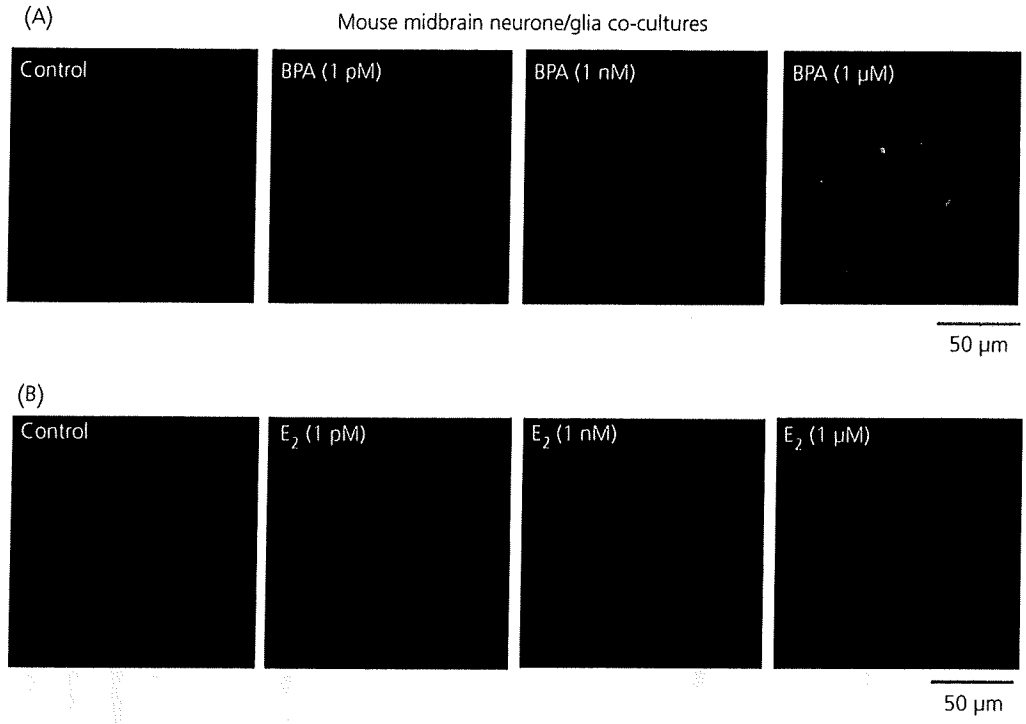



FIG. 7. A high concentration of bisphenol-A (BPA), but not 17β-oestradiol ($E_2$), causes a neuronal cell death in mouse midbrain neurone/glia cocultures. Mouse midbrain neurone/glia cocultures were incubated with normal medium, BPA (1 pM, 1 nM or 1 μM; A) or $E_2$ (1 pM, 1 nM or 1 μM; B) for 24 h. All cells were stained with a polyclonal antibody to cleaved caspase-3.

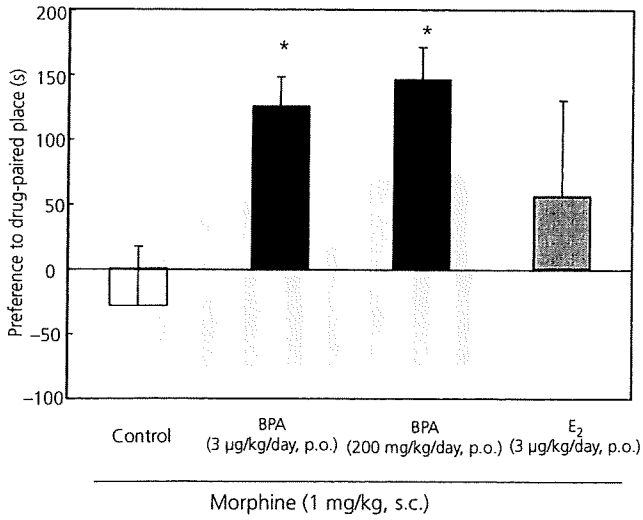



FIG. 8. Enhancement of the morphine-induced rewarding effect in mice that were prenatally and neonatally exposed to bisphenol-A (BPA). The control group did not show any place preference or place aversion with morphine (1 mg/kg s.c.). The BPA (3 μg or 200 mg/kg/day) treated group showed a significant place preference induced by morphine (*$P < 0.05$ versus control group). The 17β-oestradiol ($E_2$) (3 μg/kg/day) treated group did not show any place preference or place aversion with morphine. Each column represents the mean ± SEM place preference score of seven mice.

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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 17$\beta$-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 $\mu$g/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 $\mu$m 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·$Na_2$·$H_2O$. 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

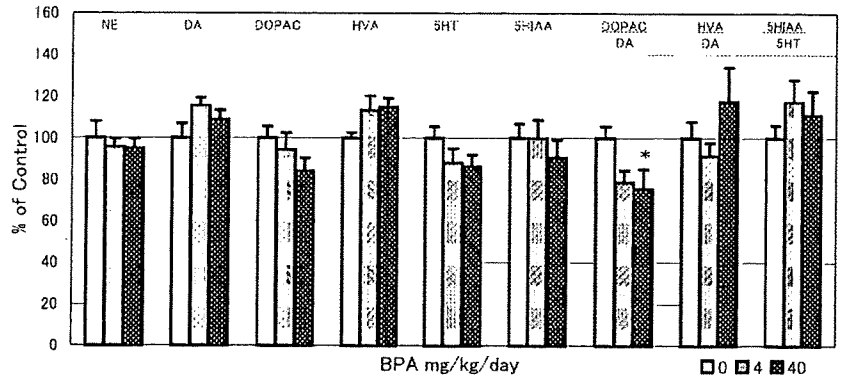


**Fig. 1. Effects of perinatal administration of BPA on the neurotransmitter contents of whole brain in 1-wk-old female offspring.**

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.

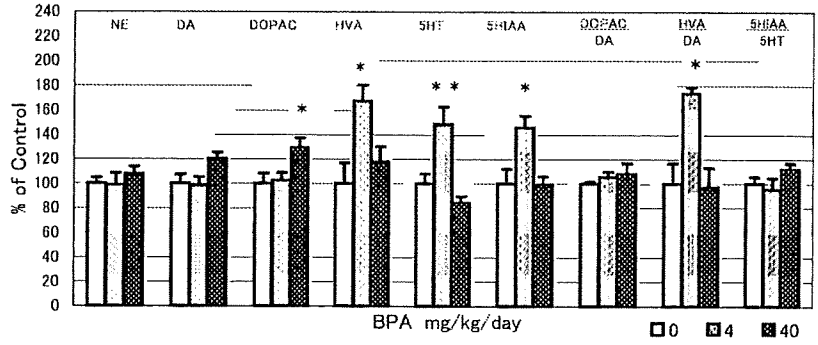


**Fig. 2-1. Effects of perinatal administration of BPA on the neurotransmitter contents of forebrain in 3-wk-old female offspring.**

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.

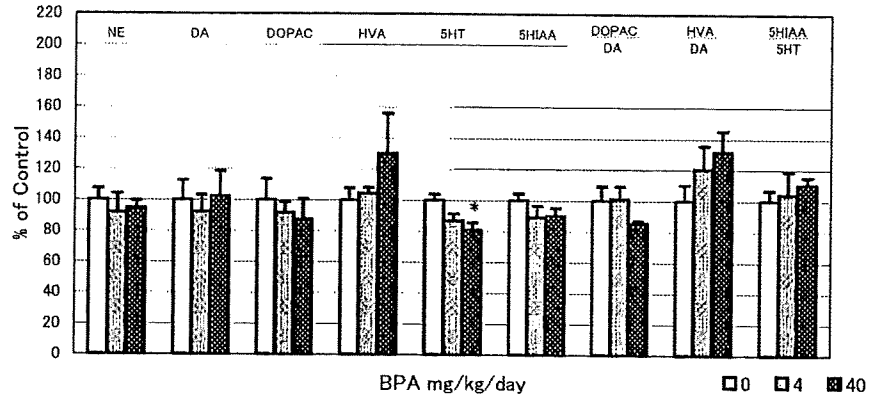


**Fig. 2-2. Effects of perinatal administration of BPA on the neurotransmitter contents of hindbrain in 3-wk-old female offspring.**

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.

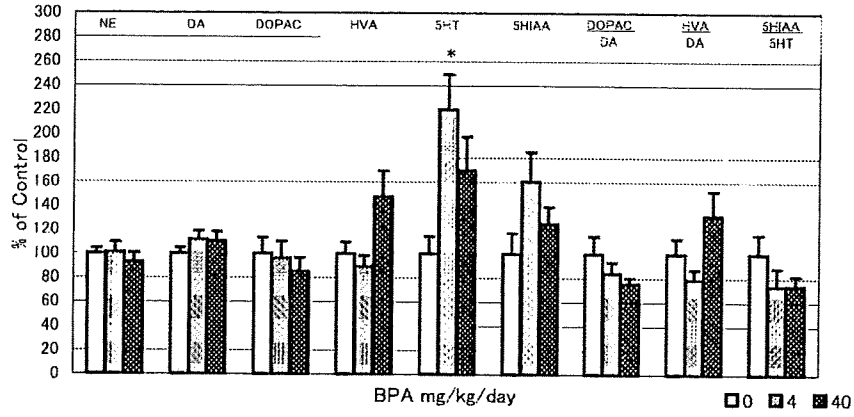


**Fig. 2-3. Effects of perinatal administration of BPA on the neurotransmitter contents of medulla oblongata in 3-wk-old female offspring.**

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

**Table 2-1. Effects of maternal administration of BPA on neurotransmitter contents of 6-wk-old female offspring (striatum, midbrain, and hypothalamus)**

| | | NE | DA | DOPAC | HVA | DOPAC/DA | HVA/DA |
|---|---|---|---|---|---|---|---|
| Striatum | Control | 100 ± 4.3 | 100 ± 8.4 | 100 ± 15.2 | 100 ± 12.4 | 100 ± 6.3 | 100 ± 6.1 |
| | 4 mg/kg | 97.1 ± 15.5 | 95.9 ± 3.7 | 90.4 ± 5.7 | 93.4 ± 5.1 | 96.1 ± 5.4 | 98.1 ± 3.8 |
| | 40 mg/kg | 96.4 ± 7.4 | 92.4 ± 4.2 | 87.0 ± 2.7 | 88.9 ± 4.4 | 96.5 ± 3.3 | 97.2 ± 4.5 |
| | A100 | 1.01 | 32.0 | 2.94 | 3.57 | 0.090 | 0.111 |
| Midbrain | Control | 100 ± 15.8 | 100 ± 15.0 | 100 ± 14.4 | 100 ± 29.4 | 100 ± 2.9 | 100 ± 32.1 |
| | 4 mg/kg | 102.5 ± 1.8 | 92.1 ± 10.6 | 91.0 ± 11.0 | 87.4 ± 15.0 | 99.2 ± 5.9 | 96.4 ± 22.8 |
| | 40 mg/kg | 98.8 ± 6.5 | 101.9 ± 11.8 | 99.6 ± 12.0 | 74.2 ± 17.0 | 97.6 ± 2.7 | 76.4 ± 23.5 |
| | A100 | 2.50 | 1.15 | 0.214 | 0.862 | 0.187 | 0.800 |
| Hypothalamus | Control | 100 ± 6.2 | 100 ± 6.7 | 100 ± 8.1 | 100 ± 26.2 | 100 ± 4.0 | 100 ± 27.0 |
| | 4 mg/kg | 107.6 ± 6.7 | 105.0 ± 8.0 | 93.3 ± 8.0 | 120.0 ± 2.8 | 89.8 ± 7.7 | 111.5 ± 6.1 |
| | 40 mg/kg | 97.1 ± 10.5 | 99.1 ± 7.9 | 93.1 ± 10.5 | 122.3 ± 10.6 | 93.6 ± 5.0 | 121.0 ± 13.1 |
| | A100 | 6.50 | 2.68 | 0.440 | 1.43 | 0.164 | 0.555 |

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

**Table 2-2. Effects of maternal administration of BPA on neurotransmitter contents of 6-wk-old female offspring (striatum, midbrain, and hypothalamus)**

| | | 5HT | 5HIAA | 5HIAA/5HT | ACh | Ch | ACh/Ch |
|---|---|---|---|---|---|---|---|
| Striatum | Control | 100 ± 3.6 | 100 ± 7.9 | 100 ± 6.3 | 100 ± 6.4 | 100 ± 8.2 | 100 ± 7.9 |
| | 4 mg/kg | 108.8 ± 8.7 | 98.9 ± 7.3 | 92.0 ± 6.0 | 110.9 ± 8.8 | 167.1* ± 18.6 | 68.7 ± 11.0 |
| | 40 mg/kg | 104.4 ± 5.2 | 99.0 ± 4.2 | 96.2 ± 7.2 | 94.9 ± 5.2 | 121.7 ± 22.6 | 83.8 ± 11.6 |
| | A100 | 3.25 | 3.33 | 1.02 | 36.2 | 20.3 | 1.82 |
| Midbrain | Control | 100 ± 9.7 | 100 ± 4.3 | 100 ± 10.4 | 100 ± 8.7 | 100 ± 18.5 | 100 ± 12.9 |
| | 4 mg/kg | 85.8 ± 2.9 | 108.6 ± 9.8 | 122.4 ± 12.7 | 102.9 ± 6.8 | 178.8 ± 31.3 | 56.8* ± 10.0 |
| | 40 mg/kg | 78.0 ± 5.3 | 110.6 ± 21.0 | 138.3 ± 27.9 | 91.3 ± 11.9 | 143.8 ± 31.1 | 62.7* ± 8.1 |
| | A100 | 29.0 | 10.6 | 0.380 | 21.7 | 16.8 | 1.42 |
| Hypothalamus | Control | 100 ± 32.2 | 100 ± 37.4 | 100 ± 31.2 | 100 ± 18.9 | 100 ± 8.2 | 100 ± 11.5 |
| | 4 mg/kg | 93.9 ± 6.9 | 80.3 ± 26.4 | 72.5 ± 21.8 | 100.1 ± 2.4 | 108.6 ± 8.4 | 95.4 ± 7.3 |
| | 40 mg/kg | 115.8 ± 26.4 | 111.4 ± 38.4 | 79.1 ± 14.1 | 106.3 ± 13.0 | 128.1 ± 24.3 | 94.2 ± 17.2 |
| | A100 | 18.2 | 10.2 | 0.648 | 16.5 | 11.0 | 1.47 |

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

cerebellum showed no changes in the BPA-treated groups, however, a significant difference was observed between the mean values of the 5HIAA/5HT ratio in the cerebellum of the control (1.356, 100%) and 40 mg/kg (1.083, 80%) groups.

*Effects of BPA on the dams*

Effects of BPA administration on the brain substances of dams are presented in Figs. 4-1 to 4-8. Variances in HVA and HVA/DA in the hippocampus, midbrain, and medulla oblongata were too large, therefore these data were not statistically analyzed. 5HT and 5HIAA were increased with statistical significance in the frontal cortex of the 4 mg/kg group, however, 5HIAA/5HT ratios in BPA-treated groups did not differ from those of the control. A tendency of increase in HVA and HVA/DA (229% of the control at 40 mg/kg) in the occipital cortex was observed in the BPA-treated groups, but these increases were not statistically significant when compared with the control. The level of DA was significantly increased in the hippocampus of the 4 mg/kg group. In the striatum, the level of 5HIAA was significantly increased in the 40 mg/kg group. No changes in monoamine contents and metabolite/monoamine ratios were observed in the midbrain, hypothalamus, medulla oblongata, and cerebellum of the BPA-treated groups.

**Table 3-1. Effects of maternal administration of BPA on neurotransmitter contents of 6-wk-old female offspring (medulla oblongata and cerebellum)**

| | | NE | DA | DOPAC | HVA | DOPAC/DA | HVA/DA |
|---|---|---|---|---|---|---|---|
| Medulla oblongata | Control | 100 ± 5.9 | 100 ± 6.3 | 100 ± 5.0 | 100 ± 8.7 | 100 ± 6.1 | 100 ± 5.3 |
| | 4 mg/kg | 96.1 ± 11.2 | 96.2 ± 11.9 | 88.8 ± 9.1 | 97.7 ± 8.2 | 92.2 ± 3.1 | 100.5 ± 13.9 |
| | 40 mg/kg | 91.9 ± 3.2 | 105.6 ± 13.8 | 91.6 ± 13.2 | 96.2 ± 7.5 | 85.4 ± 3.8 | 92.0 ± 14.3 |
| | A100 | 3.38 | 0.492 | 0.119 | 0.281 | 0.244 | 0.600 |
| Cerebellum | Control | 100 ± 4.4 | 100 ± 5.4 | 100 ± 18.2 | 100 ± 12.2 | 100 ± 19.1 | 100 ± 16.6 |
| | 4 mg/kg | 104.4 ± 10.8 | 107.9 ± 10.4 | 69.3 ± 8.6 | 102.5 ± 7.4 | 64.7 ± 7.8 | 92.9 ± 4.5 |
| | 40 mg/kg | 97.3 ± 3.5 | 142.5 ± 25.9 | 86.4 ± 16.6 | 118.0 ± 14.3 | 65.8 ± 7.8 | 92.9 ± 18.8 |
| | A100 | 1.23 | 0.0636 | 0.0204 | 0.181 | 0.307 | 2.94 |

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

**Table 3-2. Effects of maternal administration of BPA on neurotransmitter contents of 6-wk-old female offspring (medulla oblongata and cerebellum)**

| | | 5HT | 5HIAA | 5HIAA/5HT | ACh | Ch | ACh/Ch |
|---|---|---|---|---|---|---|---|
| Medulla oblongata | Control | 100 ± 12.5 | 100 ± 14.1 | 100 ± 9.8 | 100 ± 7.5 | 100 ± 17.8 | 100 ± 13.1 |
| | 4 mg/kg | 115.3 ± 21.8 | 116.4 ± 11.1 | 121.2 ± 17.0 | 90.4 ± 8.6 | 182.0 ± 30.0 | 50.3* ± 10.8 |
| | 40 mg/kg | 63.1 ± 5.2 | 95.3 ± 7.0 | 172.6* ± 18.3 | 90.8 ± 5.5 | 147.5 ± 34.6 | 68.1 ± 14.6 |
| | A100 | 31.5 | 7.10 | 0.202 | 19.3 | 30.5 | 0.693 |
| Cerebellum | Control | 100 ± 29.3 | 100 ± 26.8 | 100 ± 25.1 | 100 ± 13.0 | 100 ± 21.3 | 100 ± 16.6 |
| | 4 mg/kg | 90.4 ± 4.9 | 77.7 ± 17.9 | 86.3 ± 21.9 | 108.8 ± 16.9 | 152.0 ± 26.0 | 66.5 ± 11.6 |
| | 40 mg/kg | 114.5 ± 13.9 | 103.9 ± 15.3 | 93.3 ± 15.7 | 97.6 ± 14.3 | 128.8 ± 17.0 | 70.9 ± 13.1 |
| | A100 | 1.35 | 0.251 | 0.188 | 3.47 | 12.3 | 0.326 |

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

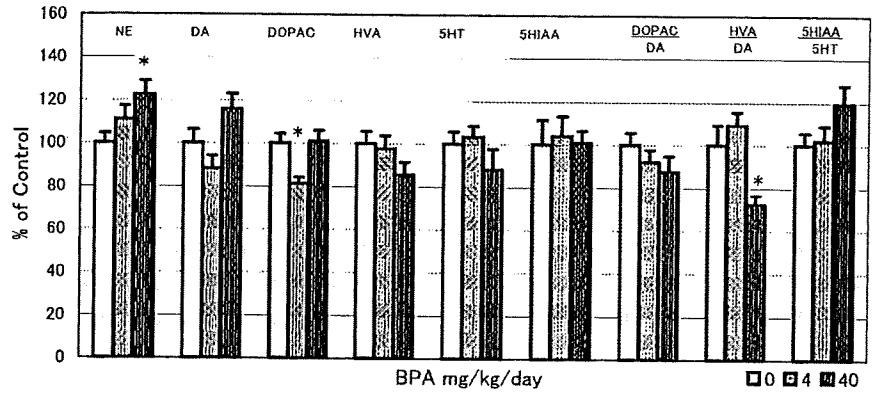


**Fig. 3-1. Effects of perinatal administration of BPA on the neurotransmitter contents of forebrain in 9-wk-old female offspring.**

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 0.722 for NE, 6.44 for DA, 0.945 for DOPAC, 1.32 for HVA, 12.0 for 5HT, and 1.35 for 5HIAA; absolute values of ratios for 100% were as follows: 0.148 for DOPAC/DA, 0.210 for HVA/DA, and 0.111 for 5HIAA/5HT. N = 5. *: $p<0.05$ by Dunnett's multiple *t*-test.

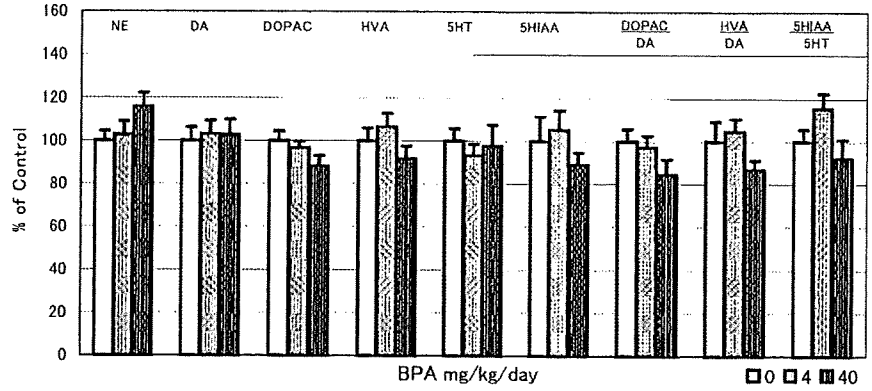


**Fig. 3-2. Effects of perinatal administration of BPA on the neurotransmitter contents of hindbrain in 9-wk-old female offspring.**

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 1.65 for NE, 1.64 for DA, 0.203 for DOPAC, 0.570 for HVA, 9.58 for 5HT, and 3.87 for 5HIAA; absolute values of ratios for 100% were as follows: 0.126 for DOPAC/DA, 0.361 for HVA/DA, and 0.420 for 5HIAA/5HT. N = 5.

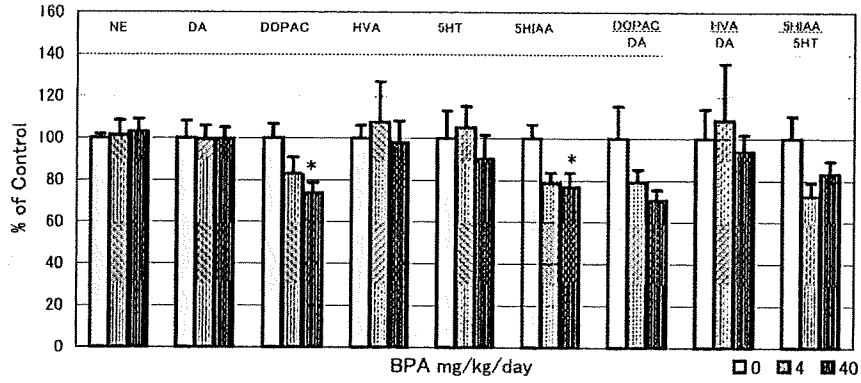


**Fig. 3-3. Effects of perinatal administration of BPA on the neurotransmitter contents of medulla oblongata in 9-wk-old female offspring.**

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 1.98 for NE, 0.193 for DA, 0.0881 for DOPAC, 0.695 for HVA, 2.47 for 5HT, and 3.30 for 5HIAA; absolute values of ratios for 100% were as follows: 0.479 for DOPAC/DA, 3.76 for HVA/DA, and 1.41 for 5HIAA/5HT. N = 5. *: $p<0.05$ by Dunnett's multiple *t*-test.

## Discussion

The reproductive effects of BPA have been studied in detail[23], but little is understood about the effects of BPA on the nervous system. Both positive and negative effects of BPA on the reproductive and other functions of offspring after perinatal exposure have been reported[7, 10, 24, 25]. In our present study, changes in monoamine and metabolite levels due to BPA treatment were not observed in the brains of 1-wk-old female rat pups. Increases in DA metabolite, DOPAC and HVA, were observed in the female rat pups at 3 wk of age, and these increases were statistically significant in the forebrain, DOPAC in the 40 mg/kg group and HVA in the 4 mg/kg group. HVA contents in the hindbrain and medulla oblongata of the 40 mg/kg group were greater than the control. The HVA/DA ratio was significantly high in the frontal cortex of the 4 mg/kg group and it was also high in the occipital cortex and medulla oblongata of the 40 mg/kg group, but the differences were not significant. These results mean that the turnover of DA was accelerated in the BPA-treated groups, and suggest that the release of DA from nerve endings was increased in these groups. Significant increases in 5HT and 5HIAA were observed in the forebrain, and in 5HT in the medulla oblongata of the 4 mg/kg group. The 5HT level

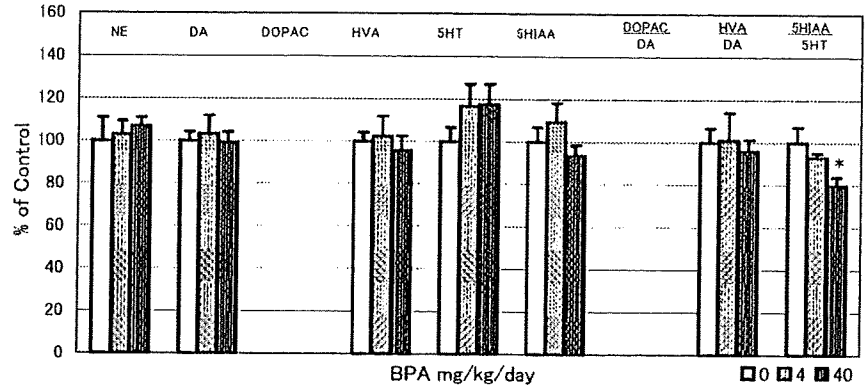


**Fig. 3-4. Effects of perinatal administration of BPA on the neurotransmitter contents of cerebellum in 9-wk-old female offspring.**

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 0.635 for NE, 0.0355 for DA, 0.411 for HVA, 0.346 for 5HT, and 0.464 for 5HIAA; absolute values of ratios for 100% were as follows: 11.7 for HVA/DA, and 1.36 for 5HIAA/5HT. N = 5. *: $p<0.05$ by Dunnett's multiple *t*-test.

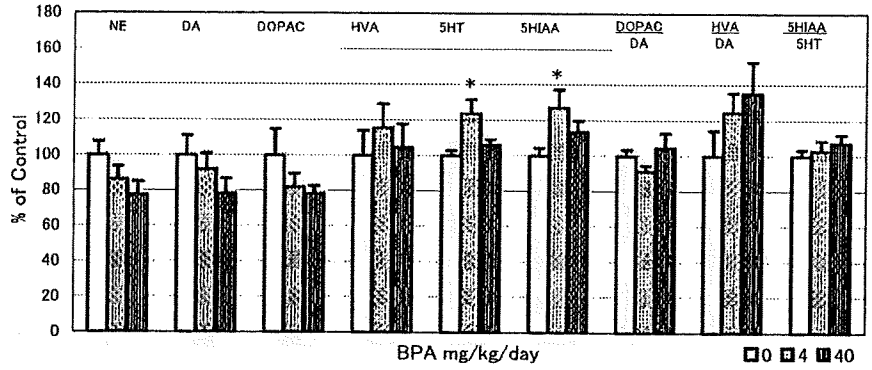


**Fig. 4-1. Effects of perinatal administration of BPA on the neurotransmitter contents of frontal cortex in maternal rats.**

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 1.65 for NE, 4.74 for DA, 1.11 for DOPAC, 0.771 for HVA, 13.8 for 5HT, and 1.70 for 5HIAA; absolute values of ratios for 100% were as follows: 0.232 for DOPAC/DA, 0.166 for HVA/DA, and 0.123 for 5HIAA/5HT. N = 5–6. *: $p<0.05$ by Dunnett's multiple *t*-test.

in the medulla oblongata of the 40 mg/kg group was higher than the control; however, no changes were observed in 5HT and 5HIAA in the forebrain of the 40 mg/kg group. The 5HT and 5HIAA levels in the hindbrain of the BPA-treated groups were less than control, although the degree of the decrease was small. Though 5HT and 5HIAA increased in the forebrain and medulla oblongata, changes in 5HT and 5HIAA seemed to be dependent on brain area and dose of BPA. In 6-wk-old offspring, increases in Ch levels were observed in all of the eight brain areas of the BPA-treated groups, but there were no accompanying changes in ACh levels. Synthesis or uptake into the synaptosome of Ch seems to have been accelerated in the BPA-treated groups. Among changes in catecholamine, serotonin, and their metabolites, DA and DOPAC in the hippocampus, and DA in the cerebellum increased in the 40 mg/kg group by 40 to 50% compared with the control. In 9-wk-old offspring, significant changes in monoamines and metabolites were observed in the forebrain, medulla oblongata, and cerebellum of the BPA-treated groups, however, these changes were scattered and not great.

BPA treatment affected the monoamine and metabolite contents of the brain of dams. Large and dose dependent increases in HVA and in the HVA/DA ratio occurred in the

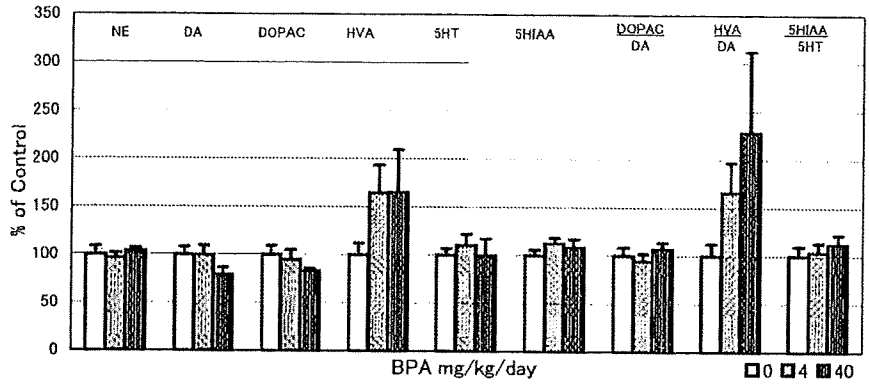


**Fig. 4-2. Effects of perinatal administration of BPA on the neurotransmitter contents of occipital cortex in maternal rats.**

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 1.59 for NE, 3.44 for DA, 0.609 for DOPAC, 0.0749 for HVA, 2.83 for 5HT, and 1.59 for 5HIAA; absolute values of ratios for 100% were as follows: 0.179 for DOPAC/DA, 0.0221 for HVA/DA, and 0.576 for 5HIAA/5HT. N = 5–6.

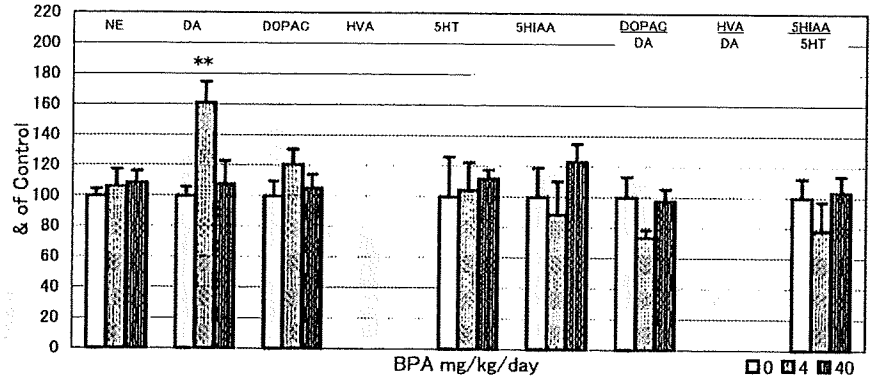


**Fig. 4-3. Effects of perinatal administration of BPA on the neurotransmitter contents of hippocampus in maternal rats.**

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 1.37 for NE, 0.123 for DA, 0.0673 for DOPAC, 2.06 for 5HT, and 1.97 for 5HIAA; absolute values of ratios for 100% were as follows: 0.564 for DOPAC/DA, and 1.02 for 5HIAA/5HT. N = 5–6. **: $p<0.01$ by Dunnett's multiple *t*-test.

occipital cortex of the BPA-treated groups, although they were not statistically significant. A dose dependent increase in the HVA/DA ratio was also observed in the frontal cortex. These findings suggest that DA turnover was accelerated in specific brain areas following BPA treatment. 5HT and 5HIAA increased in the frontal cortex of the 4 mg/kg group and 5HIAA increased in the striatum of the 40 mg/kg group. Everitt *et al.* reported that the serotonin turnover was accelerated in female rats by estrogen administration[26]. According to Shimizu and Bray, estradiol administration increased the ratio of DOPAC/DA but decreased the 5HIAA/5HT ratio in the nucleus accumbens of ovariectomized rats when measured by microdialysis[27]. Our findings are consistent with the changes found by Everitt *et al.* following estrogen treatment, because 5HIAA in the frontal cortex and striatum increased in BPA-treated dams. BPA possesses very weak estrogenic activity and the 5HIAA increase observed in our experiment may be related to the estrogenic activity of BPA. Our experimental conditions were very different from those of Shimizu and Bray, therefore, it is difficult to compare our findings with their results. They measured extracellular neurotransmitters and metabolites in a microdialysis study, whereas, we measured them following the homogenization of the brain, in which both intracellular