transfection of negative control siRNA with scrambled sequences (Table 2), and treated with testosterone (10 nM) or vehicle. In order to evaluate transfection efficiency, we examined relative *PTOV1* mRNAs levels in these cells at 24 h after transfection of the specific siRNAs. The mRNA levels in each VSMC were calculated as a ratio relative to *GAPDH*, and were normalized to the ratio after transfection of negative control siRNA (10 nM).

### Quantitative RT-PCR analysis of *PTOV1* mRNA expression in human aorta

Samples of human abdominal aorta were collected at autopsy from patients without a history of hormone replacement therapy. Autopsies were performed on 32 subjects (16 male, 16 female; mean 60.7 ± 3.3 years) in Tohoku University Hospital (Sendai, Japan) within 2 h post mortem. The Ethics Committee at Tohoku University School of Medicine approved the research protocol for this study. Aortic specimens were tentatively classified into the following four groups according to the sex of the deceased patient and degree of atherosclerosis, as previously described: group A = male, mild atherosclerosis, corresponding to groups I–III in the American Heart Association (AHA) classification; group B = male, advanced atherosclerosis, corresponding to groups IV–VI in the AHA classification; group C = female, mild atherosclerosis; and group D = female, advanced atherosclerosis) [10,11]. The distribution of the cases among these groups was as follows: A, 8 cases (mean 44.3 ± 10.6 years); B, 8 cases (mean 71.3 ± 3.7 years); C, 8 cases (mean 52.0 ± 3.9 years); and D, 8 cases (mean 75.0 ± 2.1 years), respectively. For RT/real-time PCR analysis, these specimens were treated according to our previous report [10]. The mRNA levels for *PTOV1* and *AR* in each sample are given as a ratio relative to *GAPDH*, and evaluated as a ratio (%) compared with that of each control cDNA.

### Immunohistochemical analysis for PTOV1 protein expression in human aorta

Details of immunohistochemical procedures have been previously described [10,11]. We used immunostaining with diaminobenzidine (DAB) for immunohistochemical analysis of PTOV1 protein (using a monoclonal anti-human PTOV1 antibody; Novocastra Laboratories, Newcastle, UK) and AR (using a monoclonal antibody for human AR; Dako Corporation, Carpinteria, CA, USA). We also used double immunostaining with DAB and Vector Blue as colorimetric reagents, with a combination of monoclonal antibodies for α-smooth muscle actin (α-SMA; Dako Corporation), for macrophages (PG-M1, Dako Corporation), and for leukocytes (human leukocyte common antigen antibody (LCA; Dako Corporation) in adjacent tissue sections. After determining the areas for evaluation by simultaneous observation using a multi-headed light microscope, three authors (YN, TS, and HS) independently evaluated immunoreactivity. Scoring of immunoreactivity was performed based on our previous reports with some modifications [10,16]. When PTOV1 protein was immunolocalized to the cytoplasm, the relative immunoreactivity in each specimen was classified into the following three groups: 2 = more than 50% positive cells; 1 = more than 10% and less than 50% positive cells; and 0 = negative or less than 10% positive cells, respectively [16]. When PTOV1 protein immunoreactivity was detected in the nuclei, the relative immunoreactivity in each specimen was evaluated by the percentage of immunoreactivity, ie the labelling index (LI) [10]. When inter-observer differences were >5%, the three aforementioned authors re-evaluated these discrepant immunostained slides simultaneously using a multi-headed light microscope, and the mean value was obtained.

### Statistical analysis

Values for all results were given as the mean ± standard error of the mean (SEM). Results of quantitative RT-PCR, cell count assay, and the relative immunoreactivity for protein in the nuclei were analysed using one-way analysis of variance followed by unpaired *t*-test for comparisons between two groups. Results of immunohistochemistry of cytoplasmic protein were analysed using the $\chi^2$-test. Statistical differences between immunoreactivity for PTOV1 protein and AR were evaluated using Spearman's rank correlation. A $p$ value $<0.05$ was considered significant in this study.

## Results

### Characterization of two VSMC cell lines

By RT-PCR analysis, both HUVS-112D cells and T/G HA-VSMC cells expressed *AR* mRNA (Figure 1A). In addition, AR protein was demonstrated in both of these cell lines by immunoblotting analysis (110 kD) (Figure 1B). Relative levels of AR mRNA and protein expression in these cells were approximately 5–10% of those in LNCaP cells that were examined in parallel (data not shown).

### Gene chip microarray assay

Table 3 summarizes 11 genes that were up-regulated by testosterone treatment in both types of VSMC in duplicated microarray analysis. Among these genes, human prostate tumour overexpressed protein 1, ie *PTOV1* was detected in both of these VSMCs. Recently, *PTOV1* has been reported to be induced by androgen and to be involved in cell cycle regulation [14,15,17]. *AGTR2* was also reported to be associated with androgenic effects, but it is unknown whether *AGTR2* is involved in cell growth [18]. Therefore we focused our subsequent studies on *PTOV1* as

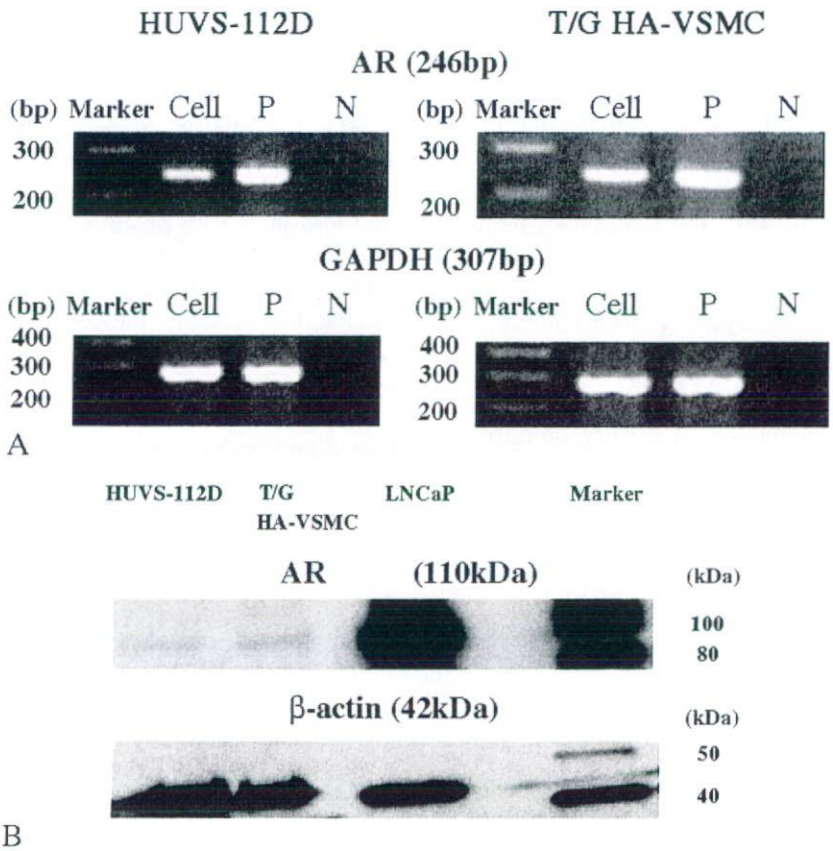


**Figure 1.** (A) Results of RT/real-time PCR analysis for *AR* and *GAPDH* in two cultured human VSMCs (HUVS-112D, and T/G HA-VSMC), positive controls, and negative controls. Cell = each type of cultured vascular smooth muscle cell; P = positive control (LNCaP prostate cancer cell line); N = negative control (no cDNA), respectively). (B) Immunoblotting analysis of AR and $\beta$-actin in HUVS-112D, T/G HA-VSMC, and LNCaP cells. Total protein was extracted, and 60 $\mu$g protein from each cell was loaded. Immunoblotting analysis demonstrated both AR (110 kD) and $\beta$-actin protein (42 kD) in all cells

**Table 3.** Ratios of gene expression determined by GeneChip microarray analysis after testosterone treatment of cultured VSMCs for 2 h

| Gene symbol | HUVS-112D | T/G HA-VSMC | Function | Association with androgen (reference) |
|---|---|---|---|---|
| *PAK7* | 3.9 | 3.6 | Neurite development | Unknown |
| *PIK3R4* | 1.7 | 3.3 | Cell signalling | Unknown |
| *CELSR1* | 1.9 | 3.1 | Cell adhesion | Unknown |
| *CACNA1G* | 2.5 | 2.9 | Calcium channel | Unknown |
| *AGTR2* | 2.2 | 2.5 | Regulator of aldosterone secretion | Koike *et al* [18] |
| *INVS* | 2.7 | 3.6 | Renal tubular development | Unknown |
| *GPR77* | 3.5 | 2.6 | Cell signalling | Unknown |
| *CASP10* | 3.3 | 2.8 | Apoptosis | Unknown |
| *AP4S1* | 2.1 | 2.5 | Formation of cell structure | Unknown |
| *T1A-2* | 1.7 | 2.7 | Membrane glycoprotein | Unknown |
| *PTOV1* | 1.8 | 2.0 | Cell growth/mitogenesis | Benedit *et al* [14] |

'Ratios' represent the mean ratios of expression levels of each gene mRNA in duplicate experiments compared with control.

an androgen-responsive gene possibly involved in the proliferation of human VSMCs.

### *PTOV1* mRNA expression in VSMCs after androgen treatment

Testosterone significantly increased *PTOV1* mRNA levels in AR-positive VSMCs compared with controls in both of these cell lines ($p < 0.05$) (Figure 2). However, testosterone with flutamide, an AR-blocker (100 nM), did not increase its mRNA expression in either of these cells ($p < 0.05$) (Figure 2).

### *PTOV1* siRNA transfection and cell proliferation assay

Quantitative RT-PCR analysis demonstrated that *PTOV1* mRNA levels were decreased in a dose-dependent manner in the cells transfected with *PTOV1* siRNAs (Figure 3A). After transfection of negative

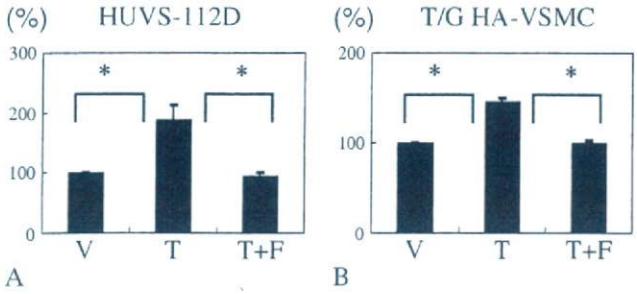


**Figure 2.** Results of RT/real-time PCR analysis for *PTOV1* in HUVS-112D (A) and T/G HA-VSMC cells (B) among cells treated with vehicle (V) (control), testosterone (T) alone (10 nM), and T (10 nM) with flutamide (F), an AR-blocker (100 nM), respectively after 2 h (*$p < 0.05$)

control siRNA (10 nM), testosterone promoted cell proliferation significantly in both of these cell lines ($p < 0.05$) (Figure 3B). However, testosterone with transfection of *PTOV1* siRNA (10 nM and 100 nM) did not increase cell proliferation in these two cell lines (Figure 3B).

### *PTOV1* mRNA expression in human aorta

The results of RT/real-time PCR analysis demonstrated the presence of specific single bands for *AR* and *PTOV1* in human aorta (Figure 4A). The relative abundance of *PTOV1* mRNA was significantly greater in male aorta with a mild degree of atherosclerotic changes (group A) than in those of other groups (groups B, C, and D)($p < 0.05$) (Figure 4B). The relative abundance of *AR* mRNA was also significantly greater in male aorta with a mild degree of atherosclerotic change (group A) than in male aorta with a severe

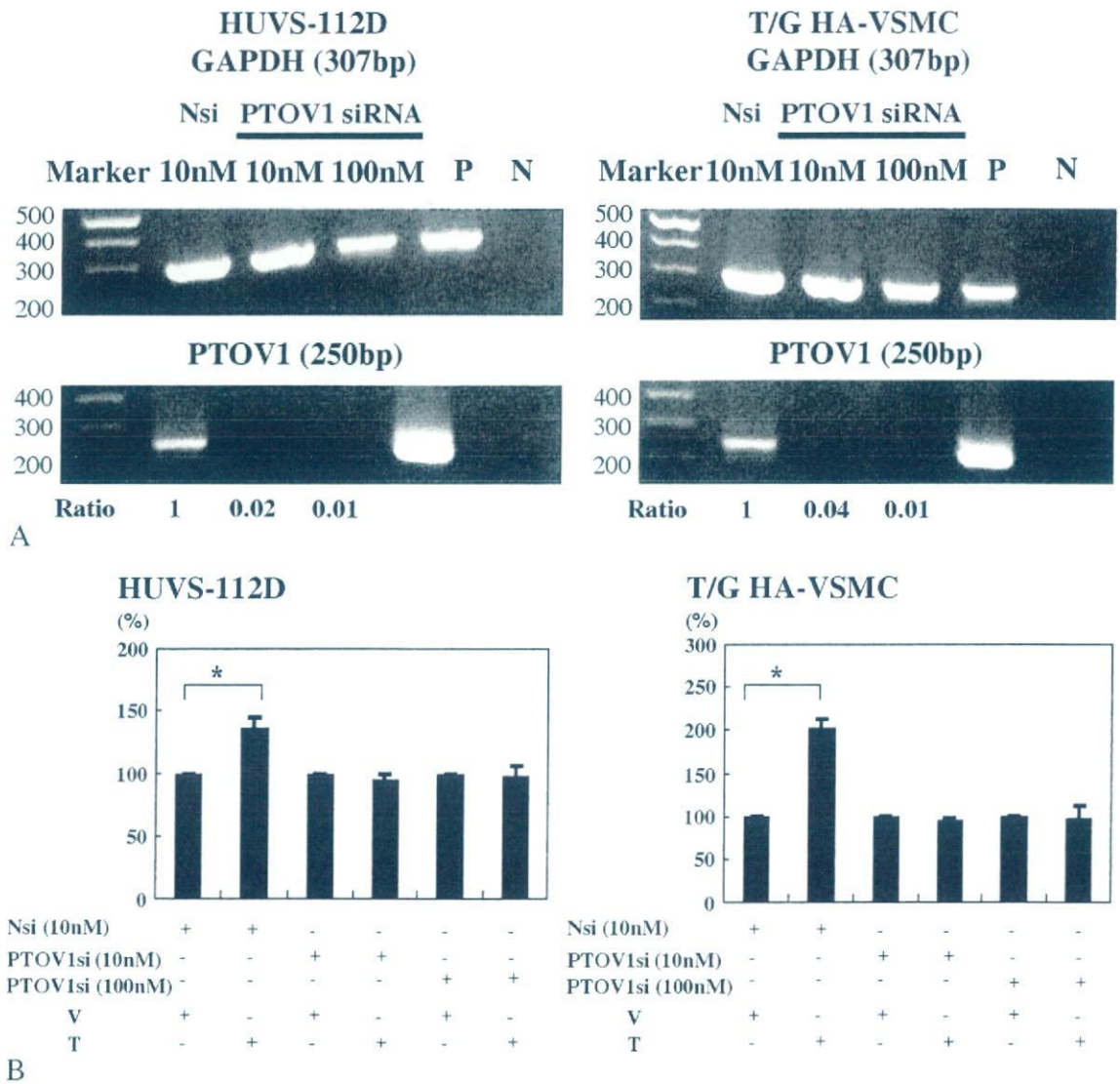


**Figure 3.** (A) Expression of *PTOV1* and *GAPDH* mRNAs at 24 h after transfection of *PTOV1* siRNA (10 nM or 100 nM) or negative control siRNA (Nsi) (10 nM) in HUVS-112D and T/G HA-VSMC cells detected by real-time PCR, respectively. *GAPDH* mRNA expression was monitored as an internal control. The ratio of *PTOV1/GAPDH* was calculated and values were normalized to the ratio obtained from the negative control transfection of Nsi (10 nM). P = positive controls (LNCaP prostate cancer cell lines); N = negative controls (no cDNAs), respectively. (B) The relative levels of cell numbers in HUVS-112D and T/G HA-VSMC cells among cells treated with vehicle (V) (0.1% ethanol) and testosterone (T) alone (10 nM) after transfection of *PTOV1* siRNA (PTOV1si) (10 nM or 100 nM) or negative control siRNA (Nsi) (10 nM) (*$p < 0.05$)

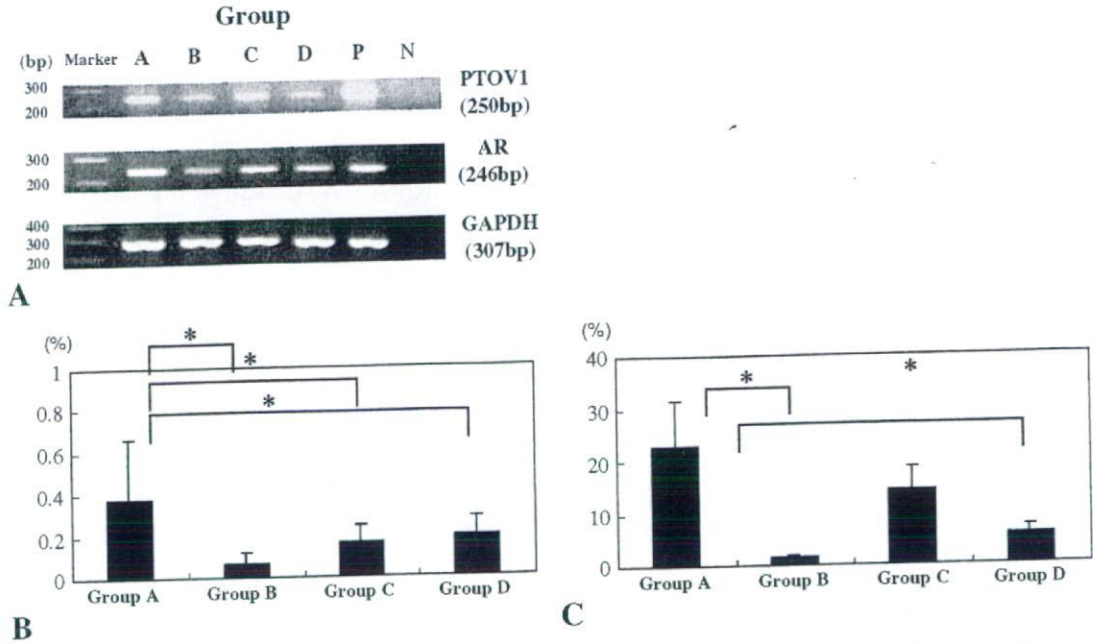


**Figure 4.** (A) Results of RT/real-time PCR analysis for *PTOV1* in human aortas. Bands for PCR products were detected as specific single bands (246 bp for *AR*, 250 bp for *PTOV1*, and 307bp for *GAPDH*). The amplified products were run on a 2% agarose gel stained with ethidium bromide. Representative photographs for these RT/real-time PCR gene products are illustrated. A = aorta from a 38-year-old man with mild atherosclerotic change; B = aorta from a 72-year-old man with severe atherosclerotic change; C = aorta from a 45-year-old woman with mild atherosclerotic change; D = aorta from a 76-year-old post-menopausal woman with severe atherosclerotic change; P = positive controls; N = negative controls. (B) Results for *PTOV1* mRNA expression levels ($^*p < 0.05$). (C) Results for *AR* mRNA expression levels ($^*p < 0.05$)

degree of atherosclerosis (group B) and in female aorta with a severe degree of atherosclerosis (group D) ($p < 0.05$) (Figure 4C).

### Immunohistochemistry for PTOV1 in human aorta

PTOV1 protein was expressed in both the nucleus and the cytoplasm of VSMCs in each group examined (Figures 5 and 6). AR protein was expressed in the nuclei of VSMCs in each group (Figures 5 and 6). However, none of the LCA- or PG-M1-positive cells demonstrated any PTOV1 immunoreactivity (Figure 5). The relative levels of PTOV1 immunoreactivity in the nuclei of neointimal VSMCs were significantly higher in male aorta with a mild degree of atherosclerotic change (group A) than in those of the other groups examined (groups B, C, and D) (Figure 6A). In addition, AR-positive cells in the neointima were also significantly more abundant in male aorta with a mild degree of atherosclerotic changes (group A) than in those of the other groups (groups B, C, and D) ($p < 0.05$) (Figure 6E). There was also a significant positive correlation between AR and PTOV1 immunoreactivity in the nuclei of VSMCs in the neointima ($p < 0.05$) (data not shown). AR-positive cells in the tunica media were significantly more abundant in male aorta with a mild degree of atherosclerotic change (group A) than in male aorta with a severe degree of atherosclerosis (group B) and in female aorta with a mild degree of atherosclerosis (group C) ($p < 0.05$) (Figure 6F). However, there were no significant differences in PTOV1 immunoreactivity in the cytoplasm of cells in the neointima or in the nucleus and/or cytoplasm of cells in the tunica media among these groups (Figure 6B, C, and D).

## Discussion

In our present study, results of both microarray and quantitative RT-PCR analyses all indicated that *PTOV1* is one of the genes induced by testosterone via AR-dependent pathways in cultured human VSMCs. In addition, siRNA analysis demonstrated that *PTOV1* is involved in AR-mediated VSMC proliferation. Results of both quantitative RT-PCR and immunohistochemical studies in human aorta obtained at autopsy further demonstrated that PTOV1, as well as AR, detected in the nuclei of neointimal VSMCs was abundant in relatively young male aorta at an early stage of atherosclerosis.

*PTOV1* has been known to be involved in stimulation of cell proliferation [14,15,17]. This gene is a mitogenic factor that shuttles between nucleus and cytoplasm in a cell cycle-dependent manner in prostate carcinoma cells [14,15,17]. In addition, *PTOV1* overexpression induced cell proliferation and facilitated entry of prostate carcinoma cells into the S phase [14,15,17]. Therefore, these findings all indicated that *PTOV1* may play a very important role in the proliferation of VSMCs. However, it is also true that other atherogenic effects on human VSMCs, such as promotion of PDGF-induced VSMC proliferation,

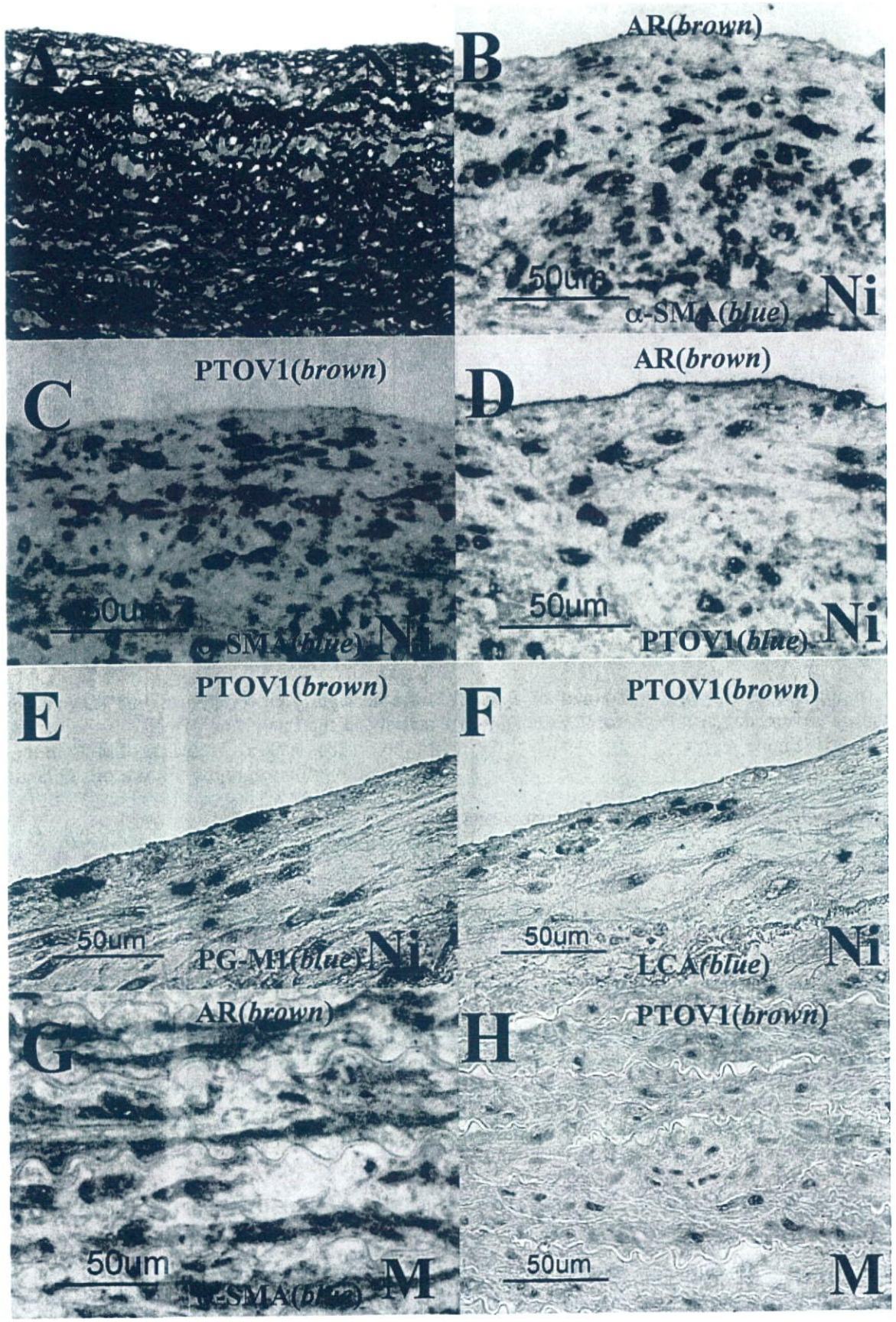


**Figure 5.** Modified Masson Goldner's stains (A), double-immunohistochemical staining for AR and α-muscle actin (α-SMA) (B), for PTOV1 and α-SMA (C), for AR and PTOV1 (D), for PTOV1 and PG-M1 (E), for PTOV1 and leukocyte common antigen (LCA) (F) in the neointima, double-immunohistochemical staining for AR and α-SMA (G) and immunohistochemical staining for PTOV1 (H) in the media of an abdominal aorta specimen obtained from a 38-year-old man with a mild degree of atherosclerosis (group A). Immunopositive cells appear brown as a result of DAB colorimetric reaction and blue as a result of Vector Blue colorimetric reaction. Double-immunopositive cells are confirmed. Ni = neointima; M = media

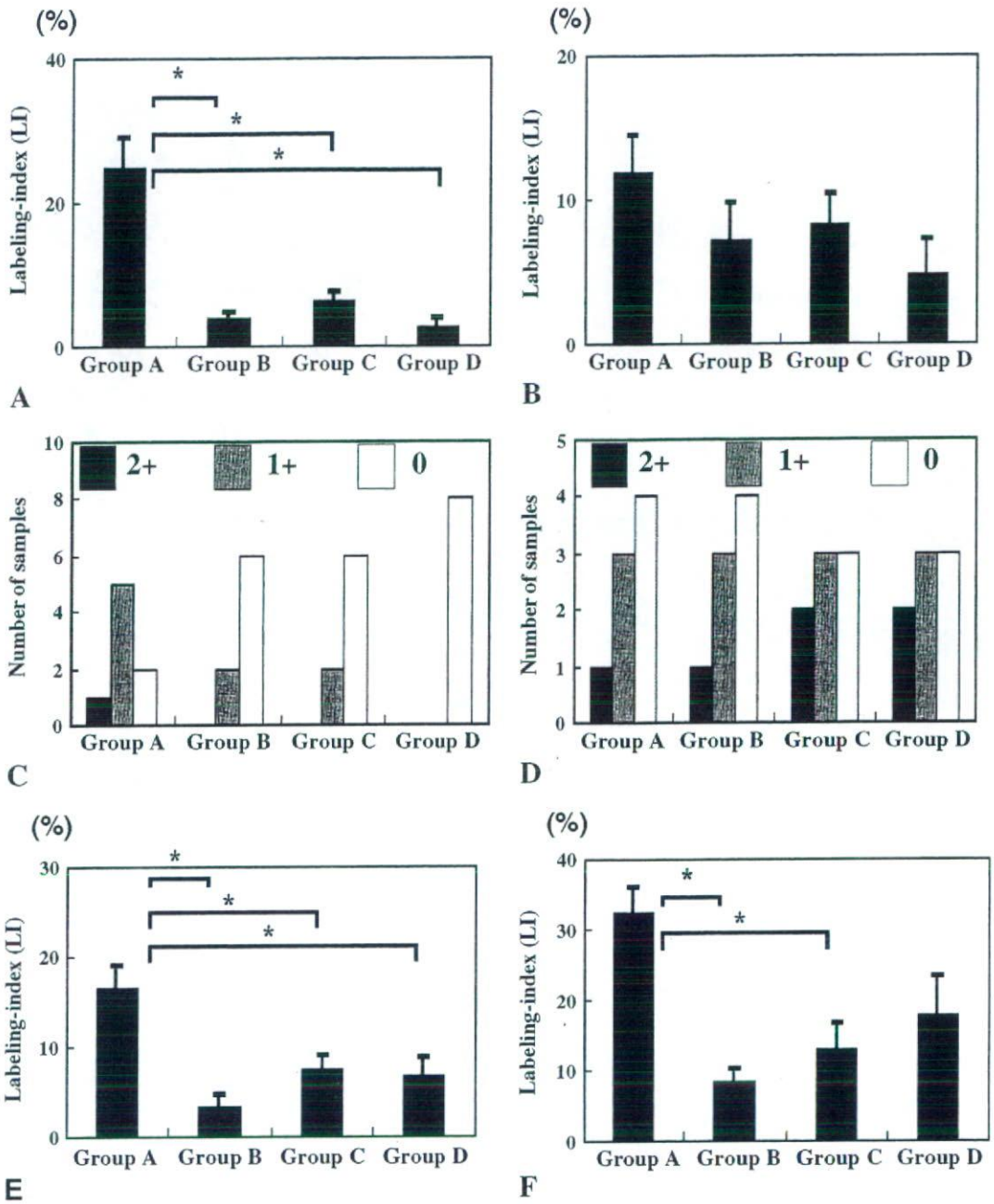


**Figure 6.** (A, B) The relative immunoreactivity of PTOV1 in the nuclei of VSMCs in the neointima (A) and media (B) was evaluated by the labelling index (LI) in each group (0–100), respectively. Data are the mean ± SEM. $^*p < 0.05$, a significant difference between two groups. (C, D) The relative immunoreactivity of PTOV1 in the cytoplasm of VSMCs in the neointima (C) and media (D) was evaluated by the percentage of positive cells (0, 1+, and 2+) in each group, respectively. (E, F) The relative immunoreactivity of AR present in the nuclei of VSMCs in the neointima (E) and media (F) was evaluated by the LI in each group (0–100), respectively. Data are the mean ± SEM. $^*p < 0.05$, a significant difference between two groups

have been reported as the mechanism for androgen-induced effects [19,20]. Therefore, further investigations are required to clarify how these pathways interact in exerting androgenic effects on VSMC proliferation in the human vascular system. Our present siRNA study demonstrated that *PTOV1* may be involved in testosterone-induced VSMC proliferation. However, further investigation is required to clarify the correlation between PTOV1 expression and testosterone-induced VSMC proliferation by reconstituting *PTOV1* expression after transfection of *PTOV1* siRNA.

Quantitative RT-PCR analysis in our present study also demonstrated that flutamide, an AR-blocker, suppressed androgen-induced *PTOV1* mRNA expression. The chromosomal region where *PTOV1* is located, 19q13.3–13.4, has also been demonstrated to harbour a large number of genes whose expression is modulated by androgens [14]. In addition, the expression of *PTOV1* was reported to be induced by exposure to androgens in LNCaP, an androgen-dependent prostate carcinoma cell line [14,17]. Therefore, these findings all indicate that *PTOV1* should also be considered one of the testosterone-induced genes in AR-positive VSMCs.

We also demonstrated that PTOV1 immunoreactivity in the nuclei of neointimal VSMCs was abundant in relatively young male aorta associated with early stage atherosclerosis. High levels and nuclear localization of PTOV1 have also been associated with cell proliferation in prostate carcinoma cells [14,17]. Neointimal VSMCs are, therefore, considered to play very important roles in the development of atherosclerosis in humans, particularly at an early stage, compared with VSMCs in the tunica media [10,11]. Therefore, higher expression of PTOV1 in these VSMCs is possibly related to the development of atherosclerosis. Levels of PTOV1 and AR were higher in male aorta with mild atherosclerosis than in female aorta with mild atherosclerosis. Men are generally considered to have a higher risk of developing cardiovascular disease than similarly aged women because of prolonged exposure to higher androgen concentrations [19,21]. It has also been shown recently that androgens upregulate atherosclerosis-related genes in macrophages from men, but not from women, which reflects the complexity of gender-related atherogenesis [19,22].

Our present study also demonstrated that the relative abundance of AR and PTOV1 in neointimal VSMCs was significantly higher in younger male aorta with mild atherosclerotic changes than in male aorta with severe atherosclerotic changes. However, these findings appear to contradict the hypothesis that, if PTOV1 is induced by androgens and implicated in androgenic effects on atherosclerosis, its expression should be higher in male aorta with more severe atherosclerosis than in male aorta with mild atherosclerosis owing to a longer exposure to elevated serum testosterone levels. There are two possible reasons for this: firstly, decreased AR and PTOV1 expression in the neointima of male aorta with severe atherosclerosis may be induced by the age-related decrease in serum testosterone levels [23]; and, secondly, when neointimal formation progresses, VSMCs with AR expression become less abundant than those without AR and these cells are therefore not necessarily influenced by androgenic atherogenic effects. Therefore, PTOV1 expression in the neointimal VSMCs in the aortas of men with high serum androgens levels may be associated with the androgen-induced onset of atherosclerosis; this may be important for formation of the neointima in the early stages of atherogenesis in the male aorta. However, recently, low concentrations of testosterone have been associated with an increased risk of cardiovascular disease in men [24]. Androgens are also known to be a coronary vasodilator, and a study of postmenopausal women demonstrated that endogenous androgens correlated inversely with carotid neointimal thickness, which suggests that androgens have potential beneficial effects on the human vascular system [19,25,26]. These different effects of androgens may depend on differences in the androgen-responsive genes induced, but further investigations are required to clarify possible direct androgenic effects on the human cardiovascular system.

In summary, *PTOV1* is considered to be one of the testosterone-induced genes involved in AR-mediated stimulation of VSMC proliferation in the aortic neointima and may play important roles in androgen-related atherogenesis in the male human aorta.

## Acknowledgements

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# Memory impairment associated with a dysfunction of the hippocampal cholinergic system induced by prenatal and neonatal exposures to bisphenol-A

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**Abstract**

One of the most common chemicals that behaves as an endocrine disruptor is the compound 4,4′-isopronylidenediphenol, called bisphenol-A. In the previous study, we reported that exposure to bisphenol-A induced the abnormality of dopamine receptor functions in the mouse limbic area, resulting in a supersensitivity of drugs of abuse-induced pharmacological actions. The present study was undertaken to investigate whether prenatal and neonatal exposures to bisphenol-A could alter other behavioral abnormalities such as anxiogenic behavior, motor learning behavior, or memory. In the present study, adult female mice were chronically treated with bisphenol-A-admixed powder food from mating to weaning. All experiments were performed using male pups. Here we found that prenatal and neonatal exposures to bisphenol-A failed to induce anxiogenic effects and motor-learning impairment using the light-dark test, elevated plus maze test, and rota-rod test. On the other hand, we found that prenatal and neonatal exposures to bisphenol-A induced the memory impairment using the step-through passive avoidance test. Immunohistochemical study showed the dramatic reduction in choline acetyltransferase-like immunoreactivity, which is a marker of acetylcholine (ACh) production, in the hippocampus of mice prenatally and neonatally exposed to bisphenol-A. These results suggest that chronic exposures to bisphenol-A could induce the memory impairment associated with the reduction in ACh production in the hippocampus.


*Keywords:* Bisphenol-A; Memory; Choline acetyltransferase; Hippocampus; Endocrine disrupting chemicals

Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. One of the most common endocrine disruptors is the compound 4,4′-isopronylidenediphenol, called bisphenol-A, which is used in the manufacture of many types of products.

Our recent studies suggest that exposure to bisphenol-A during prenatal and postnatal development has long-lasting effects on central dopaminergic systems linked to rewarding effects, as well as drug addiction induced by drugs of abuse [13–16,21,22]. The fetus uses natural hormonal messages that originate in its own hormone system and that of its mother for developmental guidance. Although bisphenol-A, has weak estrogenic activity, prenatal and neonatal exposures to 17β-estradiol failed to induce the supersensitivity of morphine [13]. Furthermore, although it is well known that bisphenol-A disrupts thyroid hormone, prenatal and neonatal exposures to propylthiouracil, an inhibitor of thyroid hormone synthesis, reduced the activation of dopaminergic neurons (unpublished observation). These findings indicate that the disruption of dopaminergic neuron development induced by prenatal and neonatal exposures to bisphenol-A can be mediated by nonhormonergic actions of bisphenol-A. Furthermore, our preliminary biochemical studies showed that bisphenol-A has an affinity for nonspecific binding sites. Thus since its action site remained unclear, it is most likely that prenatal and neonatal exposures to bisphenol-A induce other behavioral abnormalities associated with the alternation not only of the dopaminergic system, but also of other neurotransmissions. The present study was then undertaken to investigate whether prenatal and neonatal exposures to bisphenol-A could alter other behavioral abnor-

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

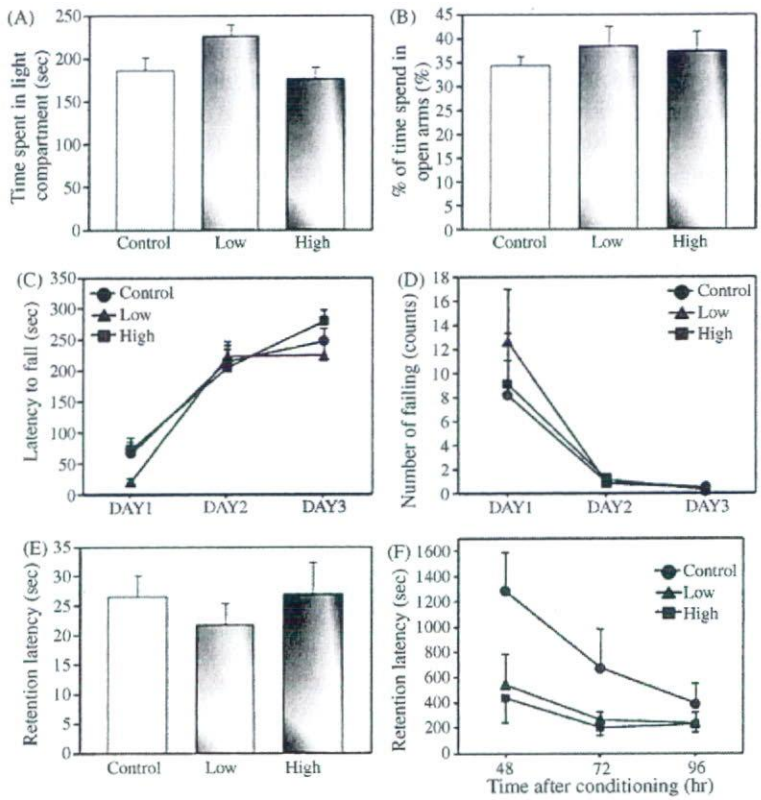


Fig. 1. Behavioral analysis in adult mice prenatally and neonatally exposed to bisphenol-A. (A) Lack of anxiogenic effects by prenatal and neonatal exposures to bisphenol-A using the light–dark test procedure. Prenatal and neonatal exposures to bisphenol-A failed to change the time spent in the lighted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

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

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

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

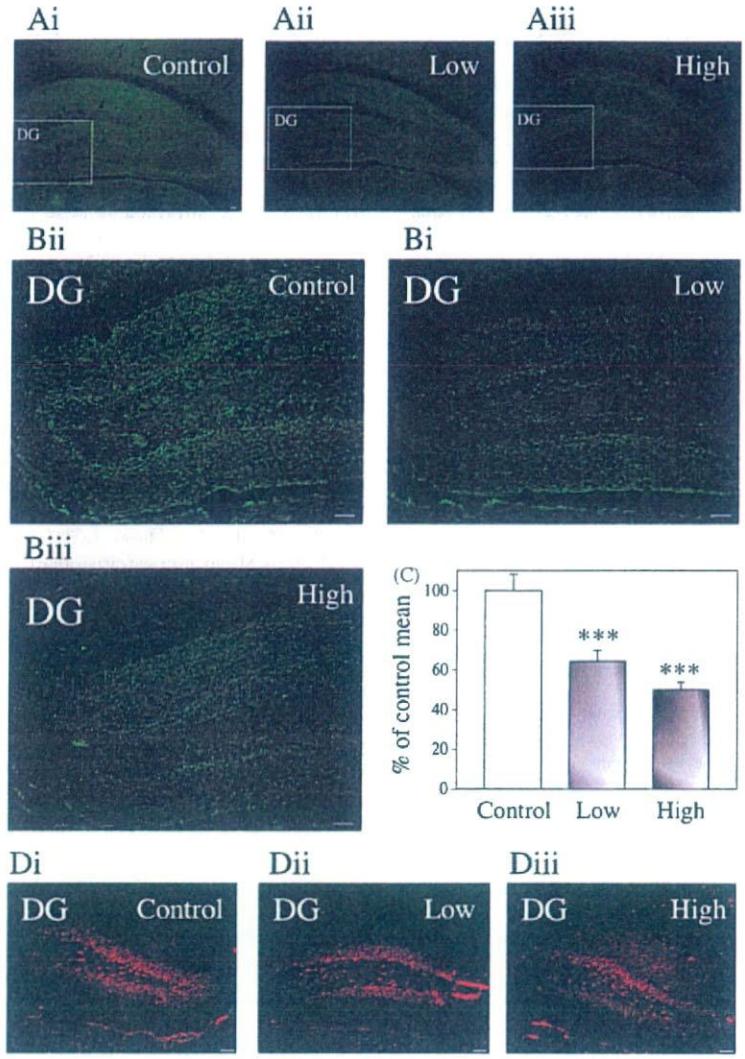


Fig. 2. Dramatic reduction in ChAT-like immunoreactivity in the hippocampus of the mice prenatally and neonatally exposed to low and high doses of bisphenol-A. (Ai–iii) Prenatal and neonatal exposures to low (Aii) and high doses (Aiii) of bisphenol-A dramatically decreased the level of ChAT-IR in the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

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

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

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〔Original Article〕

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

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

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

In recent years there has been increasing public concern that chemicals in the environment may affect the endocrine function of humans and wildlife (Colborn, 1995). Bisphenol-A is an environmental endocrine-disrupting chemical that affects reproduction in wildlife. Bisphenol-A is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins, which are used in food cans and found as a contaminant not only in the liquid of the preserved foods, but also in the water autoclaved in the cans (Brotons et al, 1995). This chemical is also released from polycarbonate flasks during autoclaving (Krishnan et al, 1993). Moreover, it has been reported that significant amounts of bisphenol-A are detected in the saliva of dental patients treated with fissure sealants (Olea et al, 1996).

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

In addition, many recent findings have supported the idea that astrocytes, which are a subpopulation of glial cells, play a critical role in neuronal transmission in the CNS. Their activation may control the structural and functional plasticity of synapses in the CNS. On the other hand, long-term exposure to drugs of abuse can induce neuronal plasticity, and we have shown that treatment of mouse cortical neuron/glia cocultures with methamphetamine or morphine caused morphological changes in astrocytes (Narita et al, 2005). Moreover, treatment with methamphetamine increased the

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

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

## MATERIALS AND METHODS

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

### Animals

All experiments were performed using 10–14-week-old male C57BL/6J mice (Japan SLC, Inc., Shizuoka, Japan) that had been prenatally and neonatally exposed to bisphenol-A (Wako Pure Chemical Industries Ltd., Osaka, Japan). Adult female mice (10 weeks old) were chronically treated with bisphenol-A-admixed powdered food containing 0 (control) or $2 \times 10^3$ $\mu$g bisphenol-A/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. In addition, RT-PCR was also performed using embryonic C57BL/6J mice that had been prenatally exposed to bisphenol-A from mating to embryo 14 days (same concentration as mentioned above).

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

### RT-PCR

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

### Immunohistochemistry

In the immunohistochemical approach, mice were deeply anaesthetized with sodium pentobarbital (70 mg/kg, ip) and perfusion-fixed with 4% paraformaldehyde (pH 7.4). The brains were then quickly removed after perfusion, and thick coronal sections of the midbrain including the ventral tegmental area (VTA) or the limbic forebrain including the nucleus accumbens were initially dissected using Brain Blocker. The brain coronal sections were postfixed in 4% paraformaldehyde for 2 hr. After the brains were permeated with 20% sucrose for 1 day and 30% sucrose for 2 days, they were frozen in embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at 30°C until use. Frozen 8-$\mu$m-thick coronal sections were cut with a cryostat (CM1510; Leica, Heidelberg, Germany) and thaw mounted on poly-L-lysine-coated glass slides. The brain sections were blocked in 10% normal horse serum (NHS) in 0.01 M PBS for 1 hr at room temperature. Each primary antibody was diluted in 0.01 M PBS containing 10% NGS [1:1000 tyrosine hydroxylase (TH) (Chemicon, Temecula, CA), 1:10 GFAP (NICHIREI, Tokyo, Japan) and 1:2500 DAT (Chemicon)] and incubated for 2 days at 4°C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 and Alexa 546 for 2 hr at room temperature. The slides were then coverslipped with PermaFluor Aqueous mounting medium (Immunon, Pittsburgh, PA). Fluorescence immunolabeling was detected using a light microscope (AX-70; Olympus Optical, Tokyo, Japan) and photographed with a digital camera (Polaroid PDMCII/OL; Olympus Optical).

### Drugs

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

### Statistical analysis

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

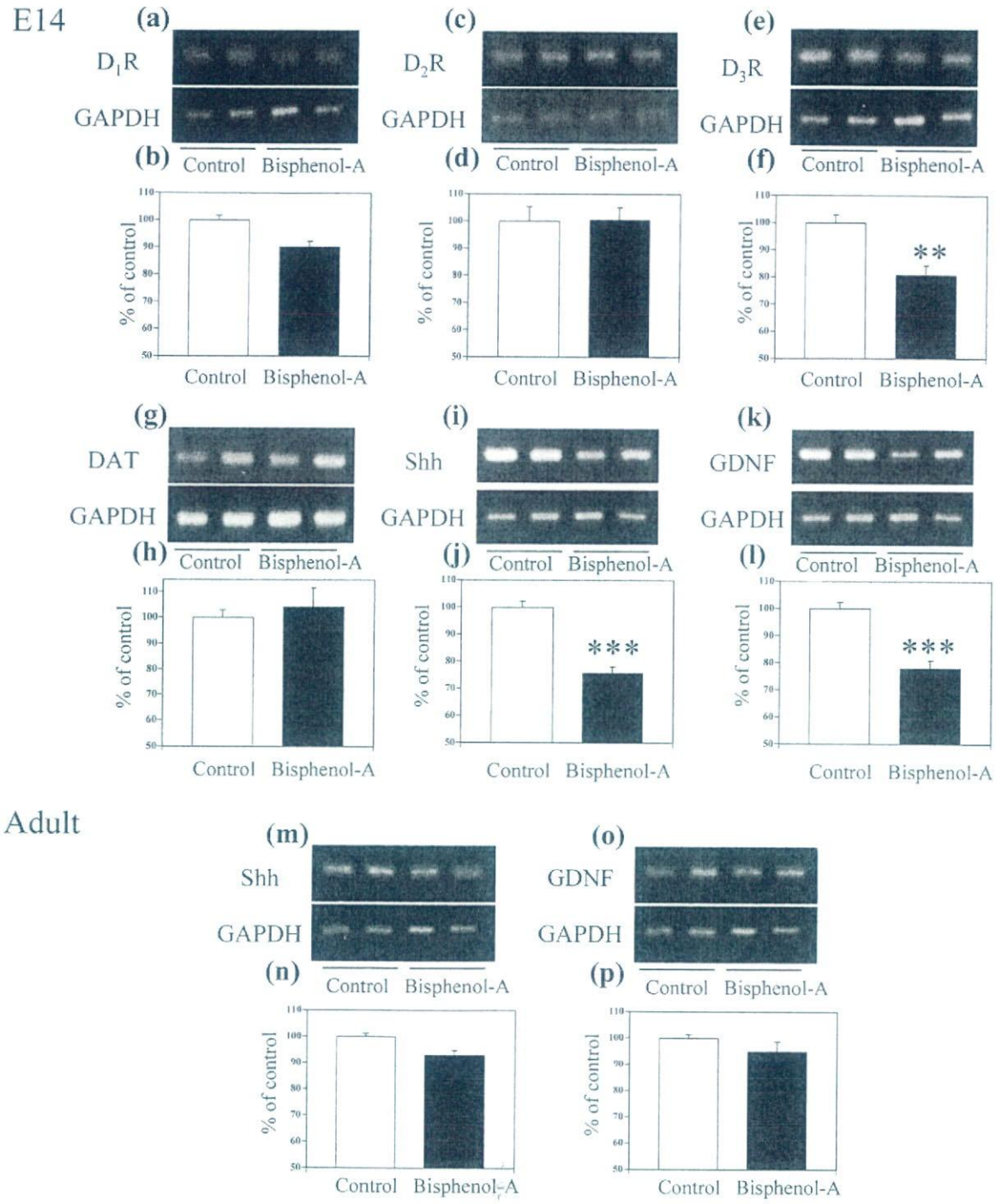


**Fig. 1** Down-regulation of the expression of dopamine $D_3$ receptor, Shh and GDNF mRNAs in the whole brain obtained from embryonic mice prenatally exposed to bisphenol-A. (a, c, e, g, i, k) Representative RT-PCR for the dopamine $D_1$ receptor ($D_1R$; a), dopamine $D_2$ receptor ($D_2R$; c), dopamine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

## RESULT

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

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

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

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

## DISCUSSION

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

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

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

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

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

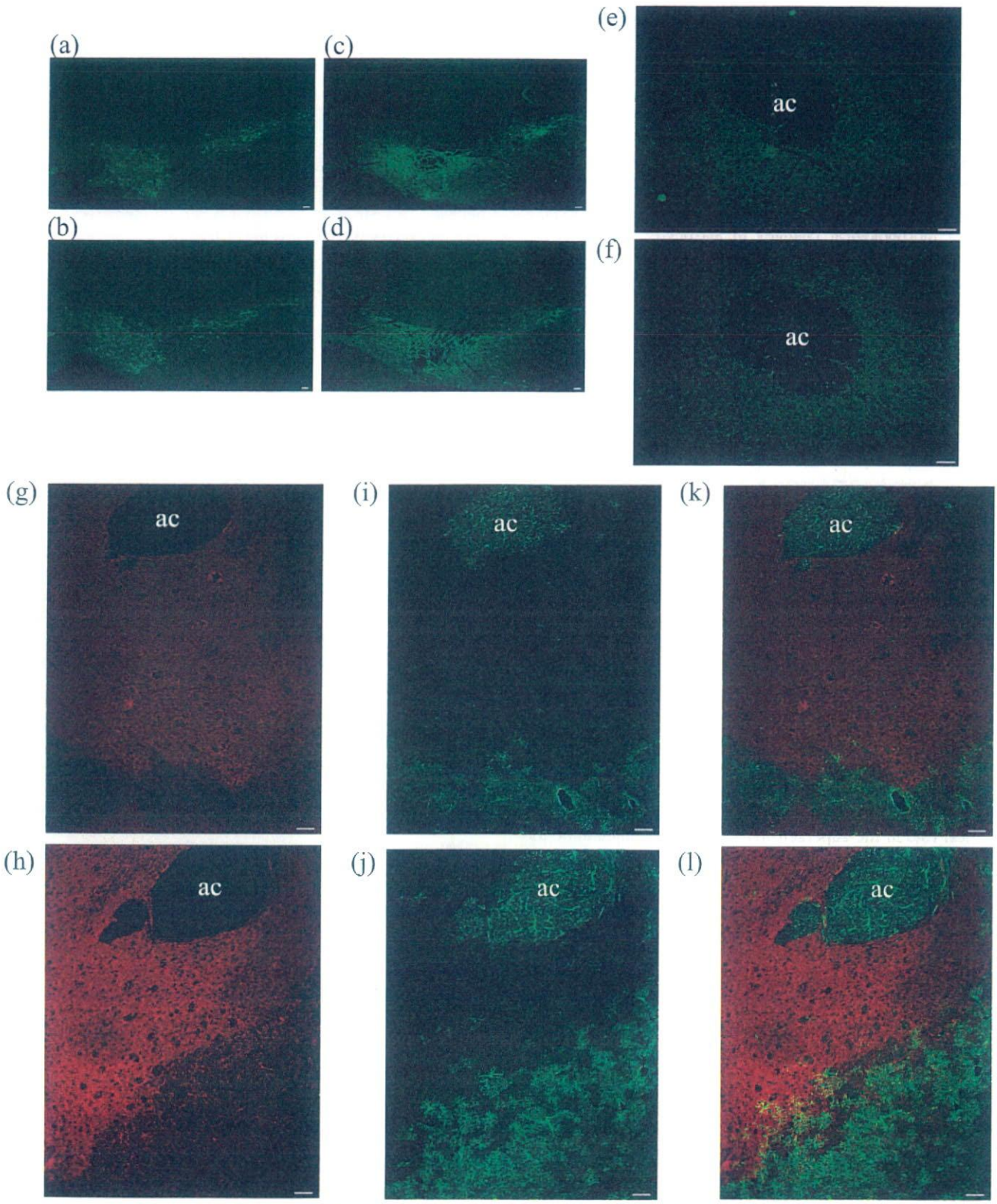


**Fig. 2** Increase in DAT-, TH- and GFAP-IRs in the nucleus accumbens or ventral pallidum of mice prenatally and neonatally exposed to bisphenol-A. (a, b) The TH-IR in the ventral tegmental area (VTA) did not change in mice prenatally and neonatally exposed to bisphenol-A (b) compared to control mice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

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

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

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

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

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