Prenatal and neonatal exposures to an endocrine disruptor, bisphenol-A, affect central dopaminergic and cholinergic neuron development

2007

Kazuya Miyagawa

A dissertation submitted in partial fulfillment of the requirements leading to the degree of Doctor (Pharmacy) presented to the Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, Tokyo Japan

This dissertation is dedicated to my parents, grandparents and brother.

Table of Contents

Abbreviations

Chemical substances and Drugs

BPA: Bisphenol-A

DAMGO: [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin

E2: 17β-Estradiol

EGTA: Ethylene glycol bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid

GDP: Guanosine-5'-diphosphate

GTPγS: Guanosine-5'-o-(3-thio)triphosphate

ICI182,780: 7α,17β-[9[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]-nonyl]estra-1,3,5(10)-triene-3,17-diol

7-OH-DOAT: 7-Hydroxy-N,N-di-n-propyl -2-aminotetralin

SCH23390: (5R)-8-Chloro-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7-ol

Buffers

BSS: Balanced salt saline

PBS: Phosphate-buffered saline

Brain regions

ac: Anterior commissure

CA: Cornu ammonis

DG: Dentate gyrus

N.Acc: Nucleus accumbens

VTA: Ventral tegmental area

Endogenous substances

ATP: Adenosine triphosphate

cAMP: Cyclic adenosine monophosphate

CREB: cAMP responsive element binding protein

FGF: Fibroblast growth factor

GABA: γ-Aminobutyric acid

GAP-43: Growth associated protein-43

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

Shh: Sonic hedgehog

TGF: Transforming growth factor

Rceptors and Transporters

D_1R: Dopamine D_1 receptor

D_2R: Dopamine D_2 receptor

D_3R: Dopamine D_3 receptor

DAT: Dopamine transporter

RXR: Retinoid receptor

Enzymes

ChAT: Choline acetyltransferase

TH: Tyrosine hydroxylase

Serum

FBS: Fetal bovin serum

NGS: Normal goat serum

HS: Normal horse serum

Injection routes

i.c.v.: Intracerebroventricular

p.o.: Per os

s.c.: Subcutaneous

Instruments

CPP: Conditioned place preference

RT-PCR: Reverse transcription-polymerase chain reaction

Others

ANOVA: Analysis of variance

CNS: Central nervous system

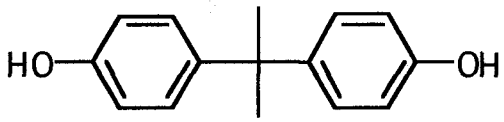
DMEM: Dulbecco's modified Eagle's medium

EDs: Embryonic days

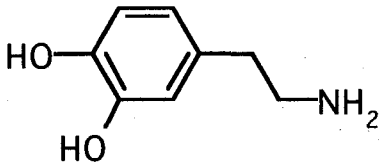
SPEED: Strategic Programs on Environmental Endocrine Disruptors

Structures of drugs using in the present study

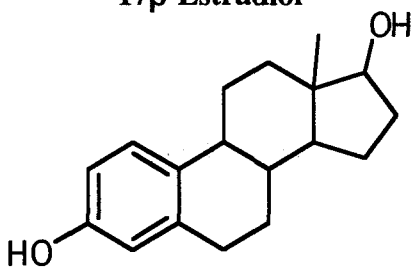
Bisphenol-A



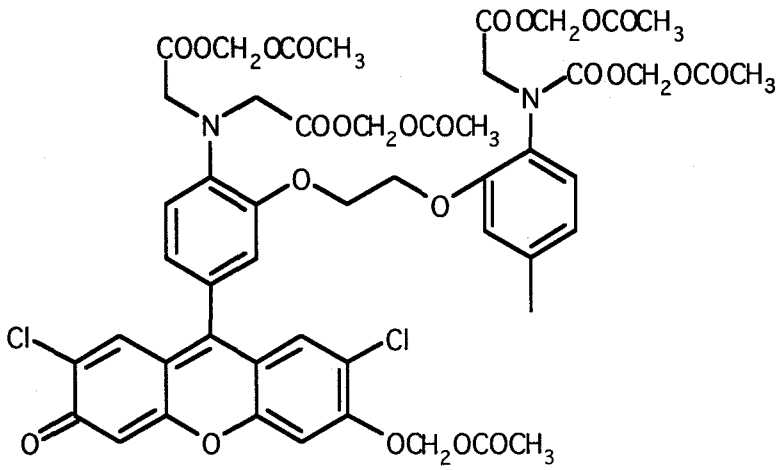
Dopamine



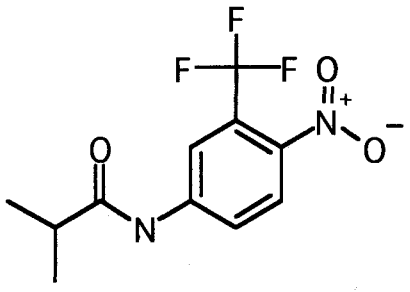
17β-Estradiol



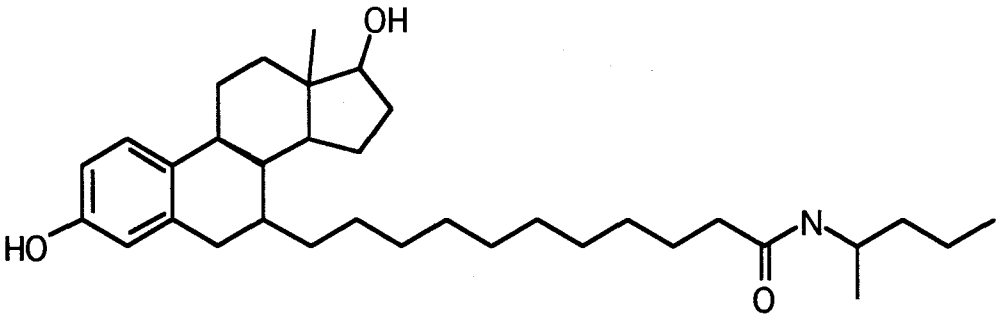
Fluo-3



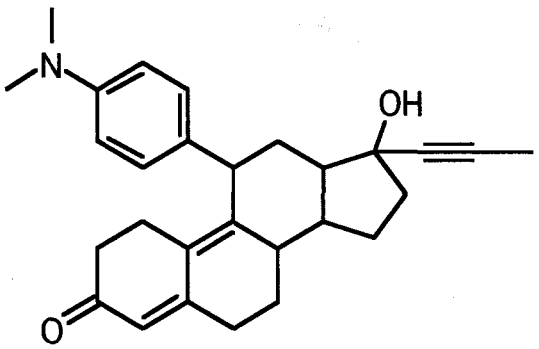
Flutamide



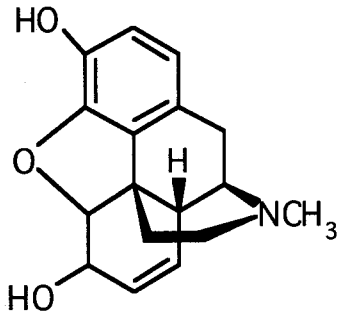
ICI182,780: 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]-nonyl]estra-1,3,5(10)-triene-3.17-diol



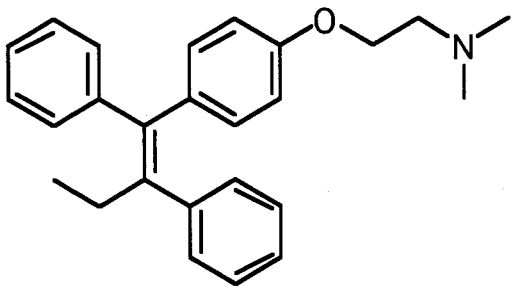
Mifepristone



Morphine



Tamoxifen



General Introduction

Endocrine disruptors

The developing fetus utilizes natural hormonal messages and effects that originate in its own hormone system and that of its mother for developmental guidance. These hormonal messages influence virtually all characteristics of a developing fetus, including those of its nervous system, sex, and reproductive system. "Our Stolen Future" scientifically explores the adverse effects of endocrine disruptors on natural hormones. Experiments on endocrine disruptors in laboratory animals have shown conclusively that fetal exposure to environmental, synthesized, or natural xenoestrogenic chemicals can wreak lifelong damage. Because comprehensive scientific proof is lacking, much more evidence is necessary to justify further efforts in research, public policy draft, and personal safety. A great many researches have indicated that human exposure to endocrine disruptors can be prevented by various courses and routes. Moreover, the chemical exposure levels in humans must be determined to grasp the state of environmental pollution. Therefore an assessment of the extent of exposure to endocrine disruptors is vital to promoting human health and preventive medicine.

The report entitled "Global Assessment of the State-of-the-Science of Endocrine Disruptors," published by the International Programme on Chemical Safety, is a result of a global comprehensive review of publicly available scientific literature on endocrine disruptors. Furthermore, SPEED'98/JEA's "Strategic Programs on Environmental Endocrine Disruptors '98" from the Environmental Agency and health science research

conducted by the Ministry of Health, Labour and Welfare, which deals with the risk assessment of endocrine disruptors in Japan, have been published.

Bisphenol-A

Bisphenol-A (4,4'-isopropylidene-2-diphenol) is one of the most common endocrine disruptors. It is used as the monomer to manufacture polycarbonate plastic (the resin that is used as linings for most food and beverage cans), as dental sealants, and as an additive in other widely used consumer products. Bisphenol-A is one of the highest-volume chemicals produced worldwide. Heat and contact with either acidic or basic compounds accelerate hydrolysis of the ester bond linking bisphenol-A molecules in polycarbonate and resins. Specifically, the heating of cans to sterilize food, the presence of acidic or basic food or beverages in cans or polycarbonate plastic, and the repeated washing of polycarbonate products have all been shown to result in an increase in the rate of leaching of bisphenol-A[1-4].

It has been reported that the administration of bisphenol-A to pregnant mice on gestation days at a dose within the range typical of the environmental exposure of humans produces significant changes in the postnatal growth rate and brings on early puberty in these mice[5]. Thus bisphenol-A may cause toxicity in the developmental process. Recent findings also demonstrated that chronic treatment with bisphenol-A affects the development of the central nervous systems[6]. Furthermore, it has been reported that prenatal and neonatal exposures to bisphenol-A markedly enhances the pharmacological actions of drugs of abuse associated with the potentiation of the

dopaminergic neurotransmission[7-9].

Implications of the mesolimbic dopamine system in the rewarding effect

Multiple lines of research have indicated the mesolimbic dopamine systems in drug rewards, including psychostimulants and opioids, measured by either self-administration of the drug or the conditioned place preference (CPP) paradigm[10].

It has been reported that the ventral tegmental area (VTA) is involved in the opioid-induced rewarding effect. The microinjection of morphine into the VTA has been shown to consistently produce a rewarding effect either by self-administration or CPP paradigm[11-13].

A great amount of evidence points to a critical role of the nucleus accumbens (N.Acc) in mediating methamphetamine-induced rewarding effects. The microinjection of dopamine receptor antagonists, such as (5R)-8-chloro-3-methyl-5-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7-ol (SCH23390) and sulpiride, into the N.Acc. blocked methamphetamine-induced place preference[14,15].

Taken together, these reports provide evidence that the rewarding effect induced by drugs of abuse appears to depend, at least in part, on mesolimbic dopaminergic neuronal activity.

Dopaminergic neuron development

Dopaminergic neurons in the mammalian brain have received substantial attention in the past, given their fundamental role in several body functions and behaviors. Cells of

the VTA modulate rewarding and cognitive behaviors, and their dysfunction is involved in the pathogenesis of addictive disorders and schizophrenia. The precise time of origin of the first postmitotic dopaminergic neurons remains a matter of debate. It has become widely accepted that the first dopaminergic neuron is born around day embryonic day (ED) 10.5 of mouse development[16,17]. At this time, these cells are characterized by an expression of the nuclear receptor family member Nurr1 and instead should be considered as postmitotic dopaminergic precursors, since these are not yet fully differentiated dopaminergic neurons[18]. In a strict sense, as soon as these precursors start expressing the rate-limiting enzyme for dopaminergic synthesis, tyrosine hydroxylase (TH), they can be considered capable of synthesizing dopamine and thus being truly dopaminergic neurons. This happens 1 day later in embryonic development at ED11.5 in the mouse[18]. Dopaminergic neurogenesis peaks at around day ED12.5 of mouse development and declines thereafter[19,20]. Migration of the dopaminergic neurons to their final positions in the ventral mesencephalon and innervation of their target fields takes place during the first postnatal weeks. In the rat, this aspect of dopaminergic neuron development, which can also be considered as "maturation" of the dopaminergic system, appears to be finished around the third postnatal week[21]. At this time, the dopaminergic system has acquired its adult morphology and functionality. Several reports indicated that enormous factors, such as LIM homeobox transcription factor 1a/b (Lmx1a/b), transforming growth factor α/β (TGFα/β), sonic hedgehog (Shh), fibroblast growth factors (FGFs), Wnt family, Nurr-1, retinoid receptors (RXRs), paired- and homeodomain-containing transcription factor (Pax) family, and trophic factors play

critical roles of induction, specification, terminal differentiation, and maintenance in the dopaminergic neuron development[22-25].

Astrocytes

There are two categories of cells in the CNS; neurons and adjacent glial cells, including astrocytes, microglia, and oligodendrocytes. Cell interactions are obviously the basis of function of the nervous system as much as of any other body system. It was thought for a long time that the relevant cell interactions for brain function were those of neurons with other neurons. Now it is more and more evident that taking into consideration the interactions of neurons with glial cells is essential in understanding how the brain works. Neuron-glia cross talk appears to be fundamental for the most basic phenomenon in the nervous system, that is, the transfer of information by chemical synapses[26]. Astrocytes are the principal type of glial cell and are known to support the proliferation, survival, and maturation of developing neurons. It is not an exception in the dopaminergic neurons. Li *et al.* reported that the activation of dopamine receptors in astrocyte promotes the survival of dopaminergic neurons[27]. Moreover, Johansson and Stromberg reported that astrocytes play a major role in long-term dopaminergic outgrowth, both in axonal elongation and in the branching of neuritis[28].

Aim and Scope

The aim of the present study is to clarify the influence of chronic exposure to an endocrine disruptor, bisphenol-A, on the central nervous system in mice. In my experimental approach, behavioral, neurochemical, and biochemical analysis were performed. The specific aims of the proposed research follow.

Chapter 1:

To ascertain the influence of *in vitro* treatment with low-dose bisphenol-A, I investigated whether *in vitro* treatment with low-dose bisphenol-A could affect the morphology of astrocytes in mouse-purified midbrain astrocytes and neuron/glia cocultures. Furthermore, I also explored the difference between bisphenol-A and 17β-estradiol on the astrocytic and neuronal responses.

Chapter 2:

To ascertain the relationship between the developmental toxicity of bisphenol-A and its exposure period, the morphine-induced hyperlocomotion and rewarding effect were examined in mice chronically exposed to bisphenol-A during several developing periods.

Chapter 3:

To clarify the effect of bisphenol-A on dopaminergic neuron development, I

investigated whether prenatal exposure to it could produce morphological change in dopaminergic neurons and the pattern of expression of genes regulating dopaminergic neuron development. Furthermore, I also explored whether prenatal and neonatal exposures to bisphenol-A induces astrocytic activation associated with the alternation of the dopaminergic neuron development.

Chapter 4:

On the endocrine-disrupting chemical problems, the low-dose effects are serious problems. I investigated whether dopaminergic neurotransmission could be induced by prenatal and neonatal exposures to bisphenol-A at a lower dosage than "Lowest Observed Adverse Effect Level."

Chapter 5:

Since the action site of bisphenol-A remained unclear, it is most likely that prenatal and neonatal exposures to bisphenol-A induces other behavioral abnormalities associated with the alternation of not only the dopaminergic system, but also other neurotransmissions. The present study was then undertaken to investigate whether prenatal and neonatal exposures to bisphenol-A could alter other behavioral abnormalities, such as anxiogenic behavior, motor learning behavior, and memory.

Ethics

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments. Animals were used only once in the present study.

Chapter 1

Dynamic changes in dopaminergic neurotransmission induced by a low concentration of bisphenol-A in neurons and astrocytes

Introduction

Many toxic stimuli activate astrocytes as determined by morphological changes and by an increase in the levels of glial fibrillary acidic protein (GFAP), which is a marker of astrocytes[29,30]. The activation of astrocytes may control the structural and functional plasticity of synapses in the central nervous systems (CNS). It has been reported that long-term exposure to drugs of abuse can induce neuronal plasticity[31,32], and Narita *et al.* reported that the treatment of mouse cortical neuron/glia cocultures with drugs of abuse, such as methamphetamine and morphine, caused morphological changes in astrocytes[33]. Moreover, treatment with methamphetamine increased the sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids[33]. Taken together, these findings indicate that astrocytes may play an important role in the development of dependence on drugs of abuse. It has also been reported that they play a critical role in the survival, differentiation, pharmacological activity, and resistance to the lesion of dopaminergic neurons.

Recently, although several reports suggest that bisphenol-A may affect dopaminergic signaling in the CNS[7-9], little is known about its role in neuron-astrocyte communication. The purpose of the present study is to clarify its effect on neuron-glia communication. I used mouse midbrain neuron/glia cocultures and purified astrocytes to determine the effects of bisphenol-A on the mesolimbic dopamine system.

Because the sex steroid hormones estrogens and androgens have been shown to exert profound effects on brain differentiation, neural plasticity, and central

neurotransmission[34,35], and because bisphenol-A has an affinity for estrogen receptors, albeit 1:2000 that of 17β-estradiol[36], I also investigated the effect of 17β-estradiol on the astrocytic and neuronal responses.

Materials and methods

Tissue Processing

Purified midbrain astrocytes were grown as follows. Midbrains were dissected from ICR mice at postnatal 1 day (Tokyo Laboratory Animals Science, Tokyo, Japan), minced, and treated with trypsin (0.025 %, Invitrogen, Grand Island, NY, USA) dissolved in phosphate-buffered saline (PBS) solution containing 0.02 % L-cysteine monohydrate (Sigma-Aldrich, St Louis, MO, USA), 0.5 % glucose (Wako Pure Chemicals, Osaka, Japan) and 0.02 % bovine serum albumin (Wako Pure Chemicals). After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (20 min, 1,000 x g). After the centrifugation, cells were plated in a flask (75 cm^2 culture flask, Corning Inc, Corning, NY, USA). Seven days after seeding in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5 % precolostrum newborn calf serum (FBS, Invitrogen), 5 % heat-inactivated (56 °C, 30 min) horse serum (HS, Invitrogen), 10 U/mL penicillin and 10 μg/mL streptomycin in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C, the flask was shaken for 12 hr at 37 °C to remove non-astrocytic cells. After 7 days from seeding, the cells were seeded at a density of 1 x 10^5 cells/cm^3, and maintained for 7 days in DMEM supplemented with 5 % FBS, 5 % HS, 10 U/mL penicillin and 10^{-5} g/mL streptomycin in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C.

Midbrain neuron/glia co-cultures were grown as follows. Midbrains were obtained from newborn ICR mice at postnatal 1 day, minced, and treated with papain (9 U/mL,

Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02 % L-cysteine monohydrate, 0.5 % glucose and 0.02 % bovine serum albumin. After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (10 min, 1,000 x g). The cells were then seeded at a density of 2 x 10^6 cells/cm^3. The cells were maintained for 7 days in DMEM supplemented with 10 % FBS, 10 U/mL penicillin and 10 μg/mL streptomycin.

Drug treatment and immunohistochemistry

At 8 days after seeding, *in vitro*, the cells were treated with either normal medium or medium containing bisphenol-A (10 fM - 1 pM, Wako Pure Chemicals) or 1, 3, 5 [10]-estratriene-3,17β-diol (17β-estradiol; 10 fM - 1 pM, Sigma-Aldrich) for 24 hr. To explore role for steroid hormone receptors in mediating the effects of bisphenol-A (1 pM, 24 hr), cells were pre-treated with either an estrogen receptor antagonist 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]-nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI182,780; 100 nM, 1 μM or 2 μM, Tocris-Cookson, Ellisville, MO, USA), an estrogen receptor agonist/antagonist tamoxifen (100 nM, 1 μM or 10 μM, Sigma-Aldrich), a progesterone receptor antagonist mifepristone (100 nM, 1 μM or 10 μM, Sigma-Aldrich) or an androgen receptor antagonist flutamide (100 nM, 1 μM or 10 μM, Sigma-Aldrich) for 24 hr. Cells were then treated with normal medium or bisphenol-A (1 pM) with or without these steroid hormone receptor ligands for an additional 24 hr. Glial cells were then identified by immunofluorescence using mouse anti-glial fibrillary acidic protein antibody (GFAP; 1:1000, Chemicon Inc., Temecula, CA, USA) followed

by incubation with Alexa 488-conjugated goat anti-mouse IgG (1:4000). Images were collected using a Radiance 2000 laser-scanning microscope (Radiance 2000: BioRad Laboratories, CA, USA).

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

To evaluate the apoptotic neuronal cell death, mouse midbrain neuron/glia co-cultures were treated with normal medium, bisphenol-A (1 pM, 1 nM or 1 μM) or 17β-estradiol (1 pM, 1 nM or 1 μM) for 24 hr. The cells were then identified by immunofluorescence, using rabbit-anti-cleaved caspase-3 antibody (1:100, Cell Signaling Technology Inc., Beverly, MA, USA), followed by incubation with Alexa 488 conjugated goat anti-rabbit IgG (1:10000). Images were collected using a Radiance 2000 (BioRad Laboratories).

Confocal Ca^{2+} imaging

Confocal Ca^{2+} imaging was conducted according to previously reports. Mouse midbrain neuron/glia cocultures or purified astrocytes were incubated for 24 hr with normal medium or medium containing bisphenol-A (1 pM). Cells were then loaded with 10 μM Fluo-3 acetoxymethyl ester (Dojindo Molecular Technologies, Kumamoto, Japan) during incubation for 90 min at room temperature. After a further 20-30 min of de-esterification with the acetoxymethyl ester, the cells which seeded on coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance 2000, Bio-Rad, Richmond, CA, USA). Fluo-3 was excited with the 488 nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths > 515 nm, and average baseline fluorescence (F_0) of each cell was calculated. To compensate for the uneven distribution of Fluo-3, self-ratios were calculated (Ratio: Rs = F/F_0). The amplitude was determined by subtracting the average of baseline fluorescence ratio (F_{basal}/F_0) from the maximum of fluorescence ratio after a drug treatment (F_{max}/F_0). Dopamine (1, 10 or 100 μM, Sigma-Aldrich) was perfused via a plastic tube for 30 sec at 5 mL/min at room temperature in cultured cortical neurons or astrocytes followed by superfusion of basal salt solution (BSS, pH 7.4) containing 150 mM of NaCl, 5 mM of KCl, 1.8 mM of $CaCl_2$, 1.2 mM of $MgCl_2$, 25 mM of N-2-hydroxyethylpiperazine-N'-2-ethaneslufonic acid and 10 mM of D-glucose.

Statistical analysis

The data of 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 ANOVA with Bonferroni/Dunnett's test

Results

A low concentration of bisphenol-A, but not 17β-estradiol, causes the activation of astrocytes

To ascertain the effect of bisphenol-A in mouse purified midbrain astrocytes, I performed immunohistochemical staining with a polyclonal antibody for GFAP. Mouse midbrain purified astrocytes were treated with either normal medium or bisphenol-A (10 fM, 100 fM or 1 pM) for 24 hr. Treatment with bisphenol-A (100 fM, 1 pM,) for 24 hr 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 (***$p < 0.001$ vs. control cells, Fig. 1-1A, B).

Unlike bisphenol-A, treatment with 17β-estradiol (10 fM - 1 pM, 24 hr) failed to produce morphological changes in midbrain astrocytes at all concentrations tested (Fig. 1-1C, D).

I next explored the effect of bisphenol-A on mouse midbrain neuron/glia cocultures. In this culture system, numerous glial cells, especially astrocytes, surround neurons. Mouse midbrain neuron/glia cocultures were treated with either normal medium or bisphenol-A (10 fM - 1 pM) for 24 hr. Treatment with bisphenol-A (100 fM or 1 pM, 24 hr) caused a robust activation of astrocytes in midbrain neuron/glia cocultures (*$p < 0.05$, *** $p < 0.001$ vs. control cells, Fig. 1-1E, F), whereas treatment with 17β-estradiol (10 fM - 1 pM) failed to produce an increase in GFAP-like immunoreactivity in mouse midbrain neuron/glia cocultures at any doses tested (Fig. 1-1G, H).

Enhancement of dopamine-induced Ca^{2+} responses by a low concentration of bisphenol-A

Dopamine (1-100 μM) produced a transient increase in the intracellular Ca^{2+} concentration in mouse purified midbrain astrocytes (Fig. 1-2). The Ca^{2+} responses to dopamine (100 μM) in astrocytes were significantly enhanced by pre-treatment with a low concentration of bisphenol-A (1 pM, 24 hr, $^{**}p < 0.01$ vs. control cells, Fig. 1-2A, B).

On the basis of morphological appearance, neuron-like cells were selected for the Ca^{2+} imaging studies. Under these criteria, dopamine (1-100 μM) produced a transient increase in the intracellular Ca^{2+} concentration in cultured midbrain neuron-like cells (Fig. 1-2C, D). These Ca^{2+} responses were significantly enhanced by treatment with a low concentration of bisphenol-A (1 pM, 24 hr, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control cells, Fig. 1-2C, D).

Effects of steroid hormone antagonists on the activation of astrocytes induced by a low concentration of bisphenol-A

To explore the involvement of steroid hormone receptor-dependent signalling in the activation of astrocytes, I next investigated whether steroid hormone antagonists could affect the bisphenol-A-induced increase in GFAP expression in midbrain astrocyte or neuronal/glial cultures. The highly selective estrogen receptor antagonist ICI182,780 (100 nM, 1 μM, 2 μM) was administered as pretreatment (24 hr) and cotreatment (24

(100 nM, 1 μM, 2 μM) was administered as pretreatment (24 hr) and cotreatment (24 hr) with a low concentration of bisphenol-A (1 pM) in both mouse purified midbrain astrocyte and neuron/glia cocultures. ICI182,780 failed to attenuate the activation of astrocytes induced by a low concentration of bisphenol-A (1 pM, Fig. 1-3A). Pretreatment (24 hr) and cotreatment (24 hr) with either the estradiol 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 a low concentration of bisphenol-A (1 pM) in both mouse purified midbrain astrocytes (Fig. 1-3C, D, E) and neuron/glia cocultures (Fig. 1-3F, G, H). These results suggest that activation of astrocytes by a low concentration of bisphenol-A was not mediated via estrogen receptors, progesterone receptors or androgen.

Bisphenol-A -induced neuronal cell death

I next investigated whether *in vitro* treatment with either bisphenol-A or 17β-estradiol could induce neuronal cell death. Treatment with a high concentration, but not a low concentration, of bisphenol-A in mouse midbrain neuron/glia cocultures caused the robust activation of caspase-3, which is a marker of neuronal cell death (Fig. 1-4). Unlike bisphenol-A, a high concentration of 17β-estradiol failed to produce caspase-3 activation (Fig. 1-4).

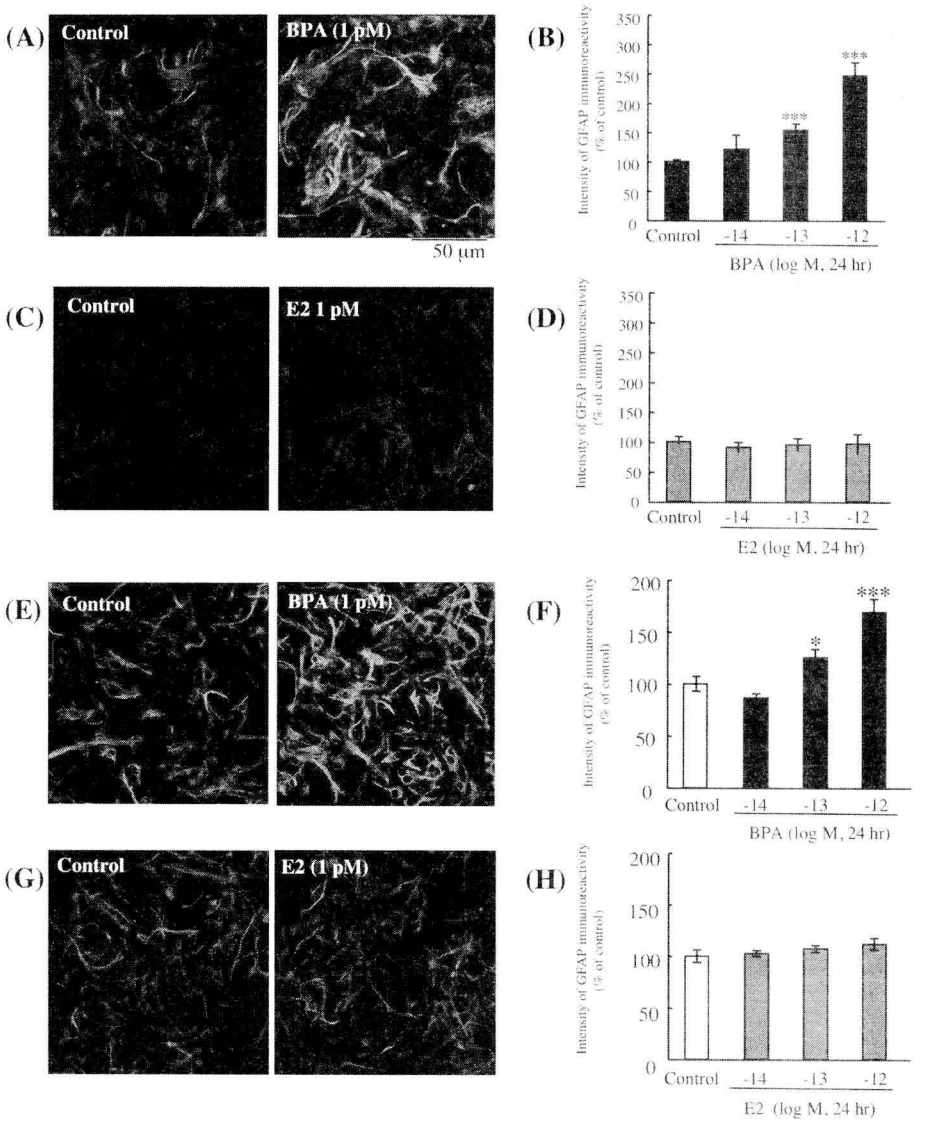


Fig. 1-1 Treatment with bisphenol-A (BPA) for 24 hr caused astrocytic activation in mouse purified midbrain astrocytes and midbrain neuron/glia cocultures (A, E) Mouse purified midbrain astrocytes (A) and midbrain neuron/glia cocultures (E) were treated with normal medium or BPA (1 pM). The cells were stained with a polyclonal antibody to GFAP. (B, F) Mouse purified midbrain astrocytes (B) and midbrain neuron/glia cocultures (F) were treated with normal medium or BPA (10 fM, 100 fM or 1 pM) for 24 hr 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. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control cells. (C, G) Mouse purified midbrain astrocytes (C) and midbrain neuron/glia cocultures (G) were treated with normal medium or 17β-estradiol (E2, 1 pM). The cells were stained with a polyclonal antibody to GFAP. (D, H) Mouse purified midbrain astrocytes (F) and midbrain neuron glia cocultures (H) were treated with normal medium or E2 (10 fM, 100 fM or 1 pM) for 24 hr 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.

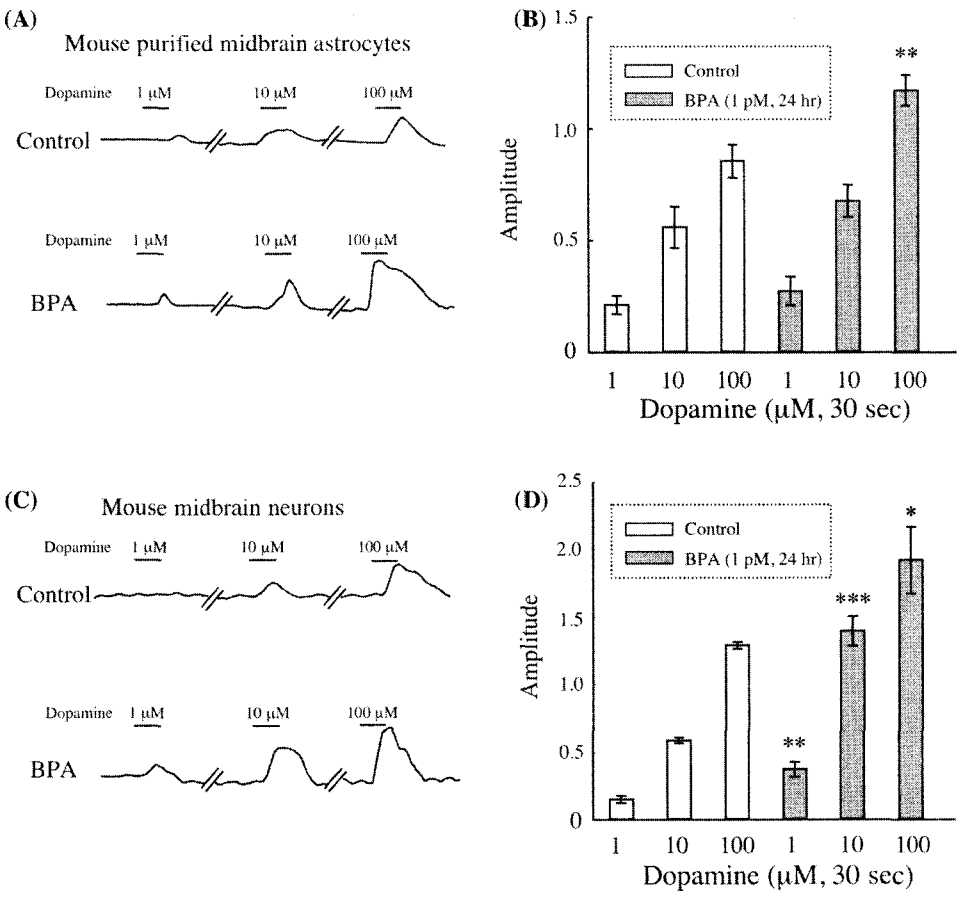


Fig. 1-2 The Ca^{2+} response to dopamine in astrocytes and neurons was significantly enhanced by treatment with a low concentration of bisphenol-A (A, C) Traces show the dopamine (1, 10 or 100 μM)-evoked increase in the intracellular Ca^{2+} concentration in control or BPA (1 pM)-treated astrocytes (A) and neurons (C). (B, D) The Ca^{2+} responses to dopamine (1, 10, 100 μM) in control and BPA (1 pM)-treated astrocytes (B) and neurons (D) are summarized. Data represent the mean± SEM of 27-63 cells. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. control cells.

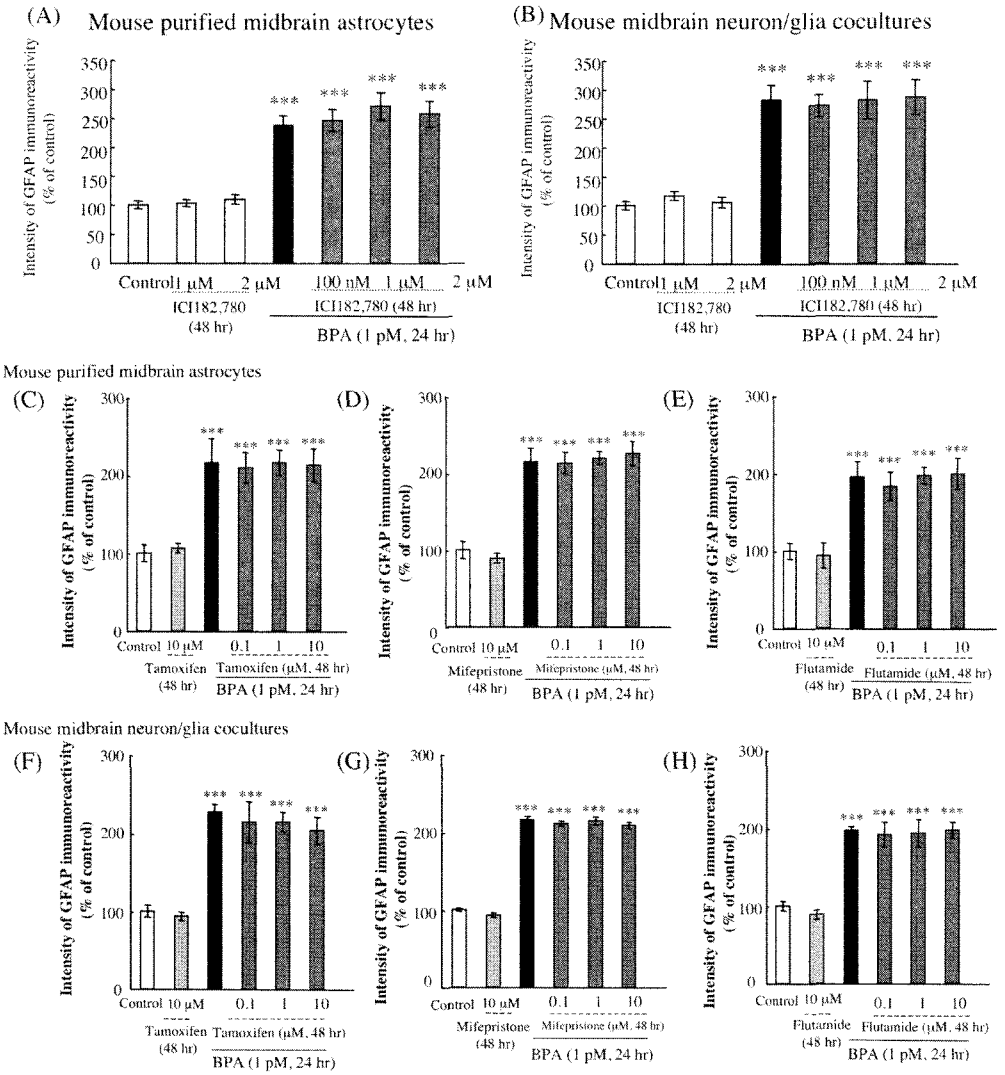


Fig. 1-3 The effects of steroid hormone ligands on astrocytic activation induced by bisphenol-A (A, B) Mouse purified midbrain astrocytes (A) and midbrain neuron/glia cocultures (B) were treated with normal medium (control) or ICI182, 780 (100 nM, 1 μM or 2 μM) for 24 hr. Cells were then treated with normal medium, BPA (1 pM) with or without ICI182, 780 (100 nM, 1 μM or 2 μM) for an additional 24 hr. The cells were 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. ***$p < 0.001$ vs. 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 bisphenol-A. The gray bars indicate the levels of GFAP-like immunoreactivity in cells treated with BPA and ICI182, 780. (C-H) Mouse purified midbrain astrocytes (C, D, E) or mouse midbrain neuron/glia cocultures (F, G, H) 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 hr. Cells were then treated with normal medium, BPA (1 pM) with or without tamoxifen (100 nM, 1 μM or 10 μM, C, F), mifepristone (100 nM, 1 μM or 10 μM, D, G) or flutamide (100 nM, 1 μM or 10 μM, E, H) for an additional 24 hr. The cells were 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. ***$p < 0.001$ vs. 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 gray bars indicate the levels of GFAP-like immunoreactivity in cells treated with BPA and steroid hormone ligands.

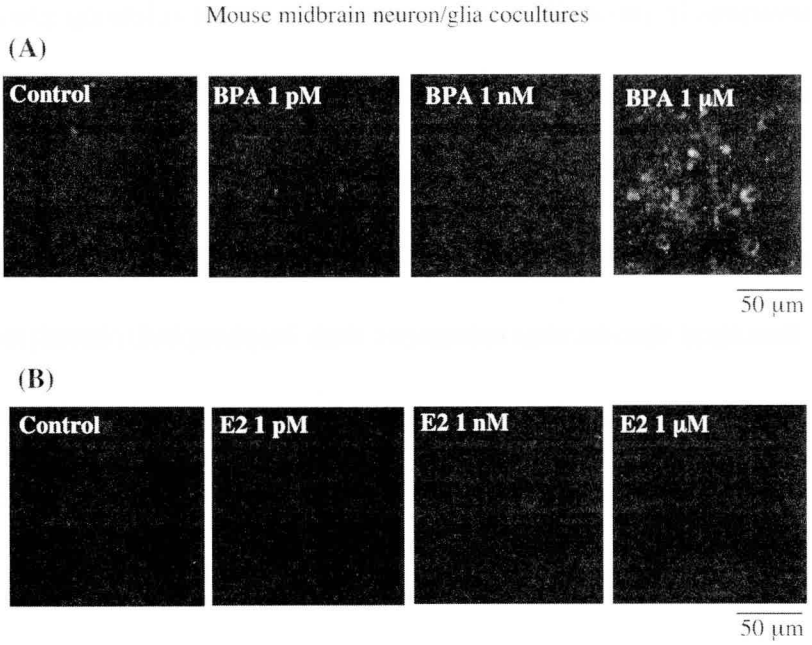


Fig. 1-4 A high concentration of bisphenol-A, but not E2, causes a neuronal cell death in mouse midbrain neuron/glia cocultures. Mouse midbrain neuron/glia cocultures were incubated with normal medium, BPA (1 pM, 1 nM or 1 μM, A) or E2 (1 pM, 1 nM or 1 μM, B) for 24 hr. All cells were stained with a polyclonal antibody to cleaved caspase-3.

Discussion

Growing evidence suggests that astrocytes are important modulators of synaptic transmission. They can respond to neurotransmitters released within the synapse by generating elevations in intracellular Ca^{2+} concentration and releasing glutamate and/or ATP that signal back to neurons[37,38]. Therefore, it is worthwhile to determine the effects of bisphenol-A on astrocytes. In the present study, I investigated the changes in dopaminergic transmission in neurons and astrocytes induced by bisphenol-A. I show here for the first time that *in vitro* treatment with bisphenol-A caused morphological changes in GFAP-positive astrocytes. Inoue *et al.* previously reported that the concentration of bisphenol-A was 0.32 ng/mL (approximately 1.4 pM) in normal human serum[39]. According to this report, it seems quite likely that the amounts of bisphenol-A humans are exposed to results in the exposure of astrocytes to concentrations greater than 1 pM.

Neurons 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 coordinated 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. I have demonstrated here that the dopamine-induced Ca^{2+} responses in mixed cultures of neurons and astrocytes were significantly enhanced by treatment with a low concentration of bisphenol-A (1 pM, 24 hr). These findings strongly support the idea that the enhancement of Ca^{2+} responses to

dopamine induced by a low concentration of bisphenol-A 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 the activation of the phospholipase C-inositol-1,4,5-triphosphate signaling pathway[40,41]. Dopamine-induced Ca^{2+} responses are also modulated by a dopamine D_2 receptor[42,43]. On the other hand, the dopamine D_3 receptor normally coexists with dopamine D_1 and D_2 receptors[44], which contribute to the inhibitory modulation of dopamine D_1 and/or D_2 receptor-mediated signaling[45]. Also, it has been reported that prenatal and neonatal exposures to bisphenol-A enhanced central dopamine D_1 receptor function[7] and attenuated dopamine D_3 receptor function in mice[9]. Thus the present data suggest that treatment with 1 pM of bisphenol-A may enhance the dopamine D_1 receptor function and/or attenuate the dopamine D_3 receptor function, resulting in an enhancement of the dopamine-induced Ca^{2+} response in neurons and astrocytes.

As mentioned above, many toxic stimuli activate astrocytes[29,30]. I next investigated whether the astrocytic activation was associated with neurotoxicity. In the present study, treatment with a high (1 μM) concentration of bisphenol-A markedly induced neuronal cell death in midbrain neuron/glia cocultures. Treatment with a low concentration (1 pM) did not. Therefore the astrocytic activation mediated by treatment with a low concentration of bisphenol-A was associated without neurotoxicological action, probably with the pharmacological action of bisphenol-A.

It has been reported that prenatal and neonatal exposures to bisphenol-A induced the

potentiation of dopamine receptor functions in the mouse limbic area, resulting in supersensitivity to methamphetamine- and morphine-induced pharmacological actions[7,9]. My findings suggest that the enhancement of dopaminergic transmission in neurons and astrocytes induced by bisphenol-A may, at least in part, lead to an enhancement of the development of psychological dependence on drugs of abuse.

Bisphenol-A can modulate gene transcription and numerous biological changes via estrogenic receptors[36,46,47]. It has been reported that equal doses of bisphenol-A and 17β-estradiol could activate the transcription factor cAMP-responsive element-binding protein (CREB) via nonclassical estrogen receptor, resulting in the transcriptional activation of CREB-responsive genes[48]. On the other hand, obvious differences between bisphenol-A and 17β-estradiol have been also reported. For example, 17β-estradiol at 10 nM reduced the duration of Ca^{2+} oscillations in mouse oocytes, whereas concentrations of bisphenol-A as high as 100 μM were necessary for similar inhibition[49]. It has been reported that 17β-estradiol inhibits the astrocytic uptake of glutamate, which is the most important excitatory neurotransmitter in the CNS, whereas bisphenol-A has no such effect[50]. Taken together, these observations suggest that bisphenol-A and 17β-estradiol may be coupled to different signaling cascades in the CNS.

It has been reported that astrocytes are among the most important target cells for 17β-estradiol. In fact, they express all types of estrogen receptors during development and in the adult brain[50-52]. In the present study, however, neither the estrogen receptor antagonist ICI182,780 nor the estrogen receptor agonist/antagonist tamoxifen failed to block the activation of astrocytes induced by bisphenol-A. The progesterone receptor

antagonist mifepristone and the androgen receptor antagonist flutamide also had no effect on the activation of astrocytes induced by bisphenol-A. Furthermore, 17β-estradiol had no effect on the activation of astrocytes in purified astrocytes or neuron/glia cocultures. It seems very likely that estrogen receptors and other steroid hormone receptors may not be critical for the activation of astrocytes induced by bisphenol-A.

In conclusion, the present data provide evidence that *in vitro* treatment with a low concentration of bisphenol-A induces dopaminergic amplification in neurons and astrocytes without steroid-hormonergic effects. These effects may contribute to potentiate the development of the rewarding effect of drugs of abuse in mice prenatally and neonatally exposed to bisphenol-A. Further *in vivo* investigation is necessary to fully understand the role of astrocytes in the potentiation of the dopaminergic neurotransmission in mice prenatally and neonatally exposed to bisphenol-A. As mentioned above, it has been reported that astrocytes play a critical role in the survival, differentiation, pharmacological activity, and resistance to the lesion of dopaminergic neurons. Thus it is possible that the astrocytic activation induced by exposure to bisphenol-A affect not only the dopaminergic neurotransmission, but also such things as dopaminergic neuron development.

Chapter 2

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

Introduction

It has been reported that administrations of bisphenol-A to pregnant mice on gestation days in doses within the range typical of the environmental exposure of humans produces significant changes in the postnatal growth rate and brings on early puberty in these mice[5]. Bisphenol-A also inhibits the differentiation of oligodendrocyte precursor cells induced by exposure to the thyroid hormone[53]. These results support the idea that bisphenol-A may cause toxicity in the developmental process. Also, recent findings demonstrated that prenatal and neonatal exposures to bisphenol-A markedly enhance the rewarding effects induced by drugs of abuse, such as methamphetamine[7] and morphine[8]. Furthermore, prenatal and neonatal exposures to bisphenol-A enhances central dopamine D_1 receptor function[7] and attenuates dopamine D_3 receptor function[9] in mice. These findings indicate that exposure to bisphenol-A may cause alterations in dopaminergic neurotransmission in the central nervous system (CNS), resulting in the enhancement of drug rewards. The aim of the present study was to further investigate the relationship between the effects of bisphenol-A on dopamine-related behaviors and the bisphenol-A exposure period, and to determine the most sensitive period in prenatal and neonatal exposures to bisphenol-A in mice.

Materials and methods

Animals

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

Measurement of locomotor activity

The locomotor activity of mice was measured by an ambulometer according to previous report [54]. Briefly, a mouse was placed in a tilting-type round activity cage 20 cm in diameter and 19 cm high. Any slight tilt of the activity cage, which was caused by horizontal movement of the mouse, was detected by three microswitches. Total activity counts were automatically recorded for 3 hr following the injection of saline (10 mL/kg, s.c.) or morphine (10 mg/kg, s.c.; Sankyo Co., Tokyo, Japan).

Place conditioning

Place conditioning was conducted according to previous reports [55,56]. The apparatus was a shuttle box (15 x 30 x 15 cm: w x l x h), which was made of an acrylic resin board and divided into two equal-sized compartments. One compartment is white with a textured floor, and the other is black with a smooth floor to create equally preferred compartments. For conditioning, mice were confined to one compartment after drug injections and to the other compartment after saline injection. The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. Conditioning sessions (three days for morphine, three days for saline) were conducted once daily for six days. Immediately after s.c. injection of morphine (1 mg/kg), animals were placed in one compartment for 1 hr. On alternate days, animals receiving vehicle were placed in the other compartment for 1 hr. On day seven, tests of conditioning were performed as follows. The partition separating the two compartments was raised to 7 cm above the floor, and a neutral platform was inserted along the seam separating the compartments. The mice were not treated with either morphine or saline, and then placed on the platform. The time spent in each compartment during a 900-sec session was then recorded automatically using an infrared beam sensor (KN-80, Natsume Seisakusyo Co., Tokyo, Japan). The preference for drug-paired place was shown as a mean difference between the time spent during the drug-conditioning compartment and saline-conditioning compartment. All sessions were conducted under conditions of dim illumination (28 lux lamp) and white masking noise.

[^{35}S]GTPγS binding assay

In the membrane preparation, mice were killed by decapitation and the limbic forebrain was then dissected according to previous report [56]. The limbic forebrain was rapidly excised at 4 °C, and the tissues were homogenized using a Potter-Elvehjem tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris-Mg^{2+} buffer containing 50 mM Tris-HCl (pH 7.4), $MgCl_2$ and 1 mM EGTA for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 4 °C for 10 min at 48,000 x g. The pellet was resuspended in [^{35}S]GTPγS binding assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4 °C for 10 min at 48,000 x g. The resultant pellet was resuspended in [^{35}S]GTPγS binding assay buffer and stored at -70 °C until used. The membrane homogenate (3-8 μg protein/assay) was incubated at 25 °C for 2 hr in 1 mL of assay buffer with various concentrations of dopamine (Sigma-Aldrich, St Louis, MO, USA), 30 μM guanosine-5'-diphosphate (GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL, USA). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris-HCl (pH 7.4) and 5 mM $MgCl_2$ at 4 °C for 2 hr. Filters were then washed three times with 5 mL of an ice-cold Tris-HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 mL of Soluene-350 (Packard Instrument Company, Meriden, CT, USA) and 4 mL of Hionic Fluor (Packard Instrument Company), equilibrated for 12 hr, and the radioactivity in the samples was determined with a liquid scintillation analyser. Non-specific binding was measured in the presence of 10 μM

unlabeled GTPγS.

Statistical analysis

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

Results

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

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

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

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

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

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

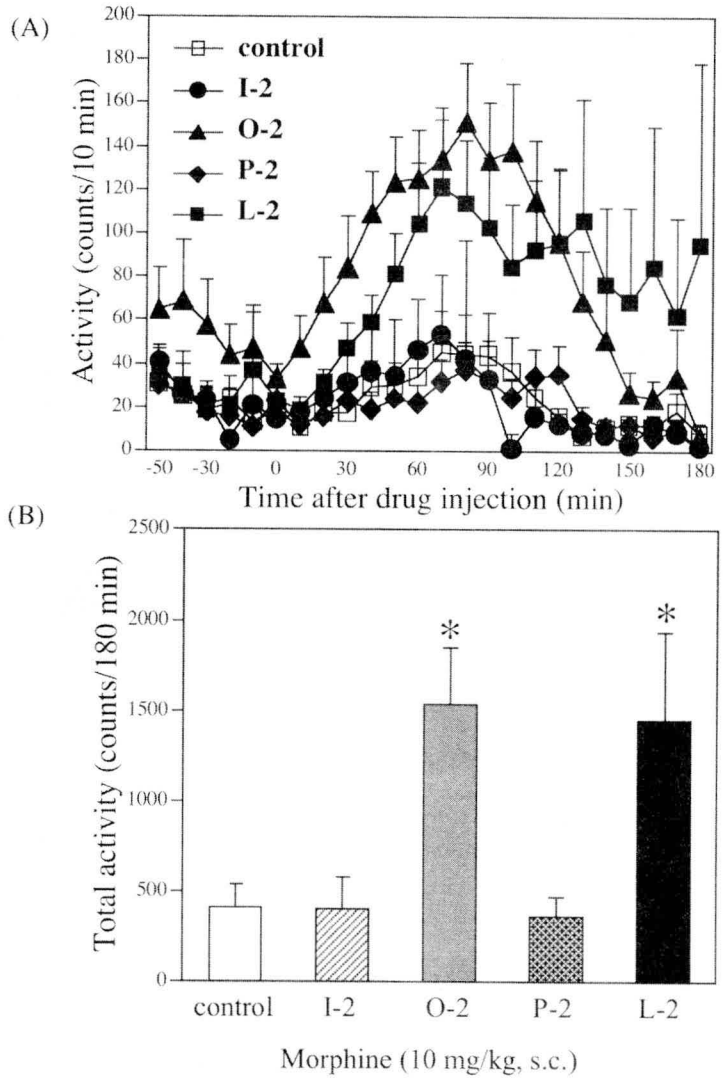


Fig. 2-1 Enhancement of the morphine-induced hyperlocomotion in mice exposed to bisphenol-A during organogenesis or lactation (A) Time-course changes in the morphine-induced hyperlocomotion in control and bisphenol-A-treated (I-2, O-2, P-2 and L-2) mice (I: implantation, O: organogengsis, P: parturition, L: lactation). Each point represents the mean activity counts for 10 min with SEM of 9-10 mice. O-2 (triangle); $F_{(1,299)}$=11.4, $p<0.01$ vs. control (open square), L-2 (circle); $F_{(1,299)}$=4.1, n.s. (B) Total activity in the morphine-induced hyperlocomotion in control and bisphenol-A-treated (I-2, O-2, P-2 and L-2) mice. Mice exposed to bisphenol-A in each period are shown as I-2, O-2, P-2 and L-2 groups. Each column represents the mean total activity counts for 180 min with SEM of 9-10 mice/group. *$p<0.05$ vs. control.

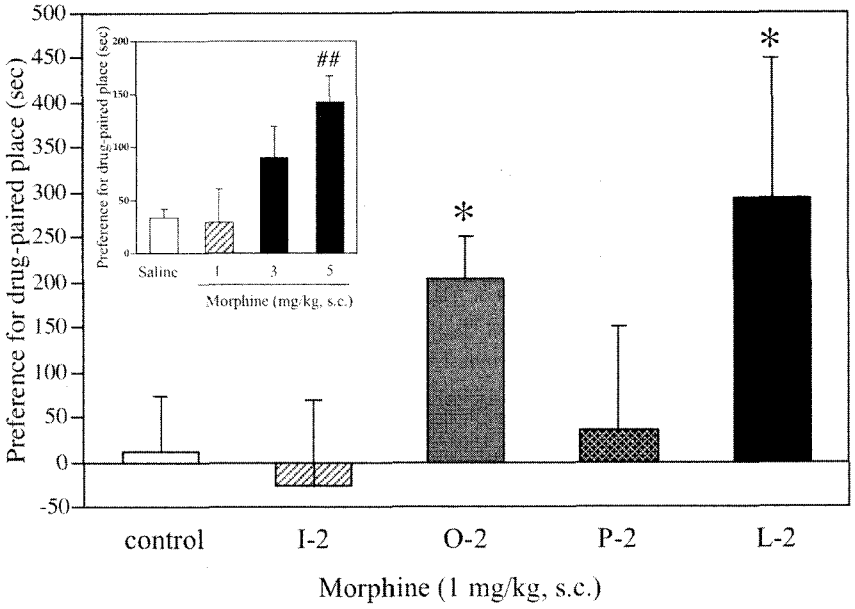


Fig. 2-2 Enhancement of the morphine-induced rewarding effect in mice exposed to bisphenol-A during organogenesis or lactation (Inner) Dose-response for the morphine-induced place preference in control mice (##p<0.01 vs. saline-treated mice). (Outer) Mice exposed to bisphenol-A in each period are shown as I-2, O-2, P-2 and L-2 groups (I: implantation, O: organogengsis, P: parturition, L: lactation). Each column represents the mean conditioning score with SEM of 6-16 mice/group. *p<0.05 vs. control.

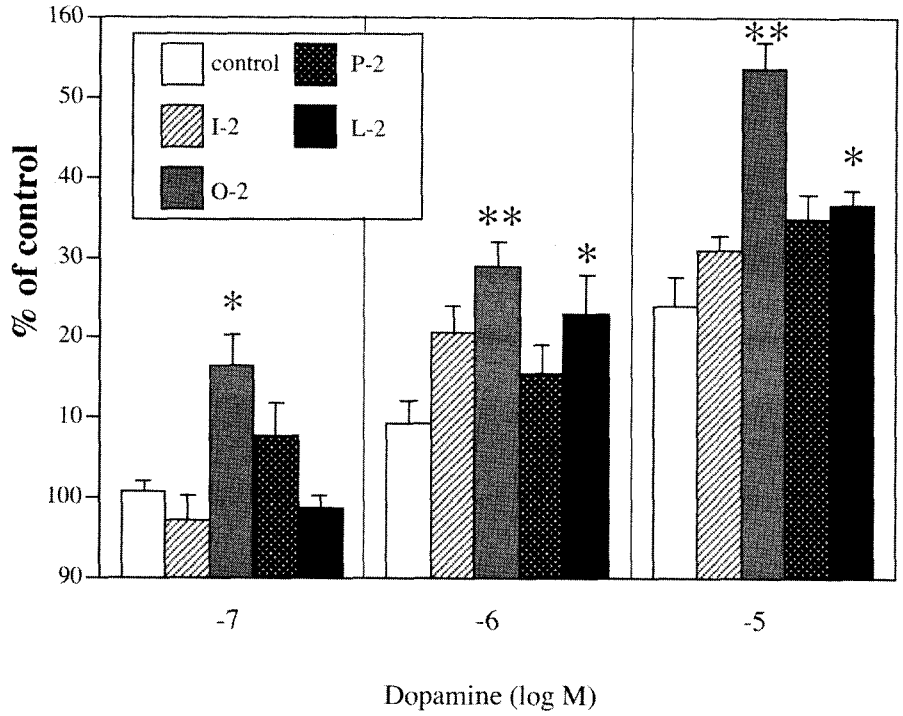


Fig. 2-3 Comparison of the stimulation of [^{35}S]GTPγS binding to membranes from the limbic forebrain by dopamine between control and bisphenol-A-treated mice Membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with dopamine. The data are shown as the percentage of basal [^{35}S]GTPγS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

Discussion

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

Many studies have suggested that the mesolimbic dopaminergic system, which projects from the ventral tegmental area (VTA) to the nucleus accumbens, is critical for the initiation of opioid reinforcement and hyperlocomotion[57-59]. Either Tyr-D-Ala-Gly-[N-Me-Phe]-NH$(CH_2)_2$-OH (DAMGO)- or morphine-induced place preference can be blocked by dopamine receptor antagonists[60,61]. Moreover, hyperlocomotion induced by morphine can be blocked by treatment with dopamine receptor antagonists in the nucleus accumbens[62,63]. These findings indicate that the dopamine-containing neuron of the midbrain VTA, which has a high density of μ-opioid receptors, plays a critical role in the rewarding effects and hyperlocomotion by μ-opioid receptor agonists. In terms of dopamine receptor involvement at the terminal site of the mesolimbic dopamine system, the rewarding effects of an abused drug have been shown to be mediated by dopamine D_1 receptors[54,61]. Also, the dopamine D_3 receptor cloned by Sokoloff and colleagues has been characterized extensively[64]. The dopamine D_3 receptor shows a distinct distribution in limbic areas of the brain,

olfactory tubercle[64]. Several pharmacological studies with dopamine D_3 receptor-preferring agonists, such as 7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OH-DPAT), suggest that the dopamine D_3 receptor regulates the inhibitory effect to produce hyperlocomotion in rodents[65,66]. Furthermore, Narita *et al.* reported that the morphine-induced rewarding effect and hyperlocomotion were markedly enhanced in mice lacking the dopamine D_3 receptor gene[7]. These findings suggest that the dopamine D_3 receptor plays a critical role in mediating drug-induced effects on dopamine neurotransmission.

Recent studies showed that prenatal and neonatal exposures to bisphenol-A enhance the rewarding effects of drugs of abuse, which are associated with the up-regulation of central dopamine D_1 receptor functions and the down-regulation of the functional dopamine D_3 receptors in mice[7-9]. In the present study, I investigated the relationship between these neuronal changes and the exposure period for bisphenol-A. The exposure to bisphenol-A during either organogenesis or lactation significantly enhanced the morphine-induced hyperlocomotion and rewarding effect. These findings suggest that prenatal and neonatal, especially organogenetical and lactational exposures to bisphenol-A, lead to the supersensitivity of the drugs of abuse-induced pharmacological actions.

Morphine has been shown to indirectly activate dopamine neurons in the VTA as a consequence of inhibiting nondopaminergic neurons, presumably γ-aminobutyric acid (GABA)-containing neurons, leading to an increased dopamine release in the nucleus accumbens. Mizuo *et al.* reported that prenatal and neonatal exposures to bisphenol-A failed to enhance μ-opioid receptor-mediated G-protein activation by morphine in the

lower midbrain[8]. Moreover, the expression of μ-opioid receptor mRNA was not changed by chronic bisphenol-A treatment, suggesting that μ-opioid receptor function is unaffected in this region[8]. Therefore I next investigated the influence of prenatal and neonatal exposures on bisphenol-A in the mesolimbic dopaminergic function using [^{35}S]GTPγS binding assay. The exposure to bisphenol-A during either organogenesis or lactation also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These results indicate that either organogenesis or lactation is more sensitive to the bisphenol-A-induced neuronal toxicity than any other periods such as implantation and parturition.

Recently, several investigations have provided evidence that the treatment of adult animals with bisphenol-A could not affect the reproductive function or social behaviors[67,68]. I have already confirmed that the acute administration of bisphenol-A with adult mice could not affect the dopamine-related behaviors (data not shown). On the other hand, several investigations clarified the behavioral abnormalities by prenatal and neonatal exposures to bisphenol-A. These findings indicate that prenatal and neonatal exposures to bisphenol-A may cause neuronal toxicity specifically in the developmental process. In the present study, I focused on the relationship between these developmental changes and the exposure period for bisphenol-A. The exposure to bisphenol-A during either organogenesis or lactation significantly enhanced the morphine-induced hyperlocomotion and rewarding effect. Furthermore, the exposure to bisphenol-A during either organogenesis or lactation also produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain. These

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

Generally, it is well known that in cerebral development the proliferation, differentiation, or migration of nerve cells and glia cells is carried out most briskly at organogenesis[69]. Furthermore, the functional development of the CNS, synaptogenesis, and the construction of the nerve network are carried out most briskly at lactation[69]. Therefore, these reports strongly support the present results that these periods are the most sensitive for the influence of bisphenol-A exposure in the development of the CNS. These results suggest that exposure to bisphenol-A during organogenesis could affect the differentiation or migration of neuronal stem cells. Furthermore, an exposure to bisphenol-A during lactation affects the functional development of the CNS, including synaptogenesis and the construction of the neuronal network.

Taken together, the present data may explain that although adult animals treated with bisphenol-A could not affect the reproductive function and social behaviors, the prenatal and neonatal exposures, especially either organogenesis or lactation, to bisphenol-A induced developmental neuronal toxicity in the midbrain of rodents. Although further investigation is necessary to fully understand the molecular mechanism of disruption of dopaminergic neuron development by bisphenol-A, my findings warn that exposure to bisphenol-A during either organogenesis or lactation may predispose their children to the development of dopamine-related disorders.

Chapter 3

Changes in central dopaminergic systems with the expression of Shh and GDNF in mice exposed to bisphenol-A during developmental periods

Introduction

It has been reported that prenatal and neonatal exposures to bisphenol-A markedly enhanced the rewarding effects or hyperlocomotion induced by methamphetamine[7] and morphine[8,70]. These findings indicate that exposure to bisphenol-A during development alters the 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. As described in chapter 2, 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 effects. 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. It has been wildly accepted that the first dopaminergic neurons are born around ED 10.5 of mouse development. Moreover, dopaminergic neurons of the ventral tegmental area (VTA) are generated from approximately ED 10 to ED 14[24]. Therefore, chronic treatment with bisphenol-A could disrupt dopaminergic neuron development. The purpose of the present study was then to clarify the effect of bisphenol-A on the dopaminergic neuron development in mice.

Also, 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 the treatment of mouse cortical neuron/glia cocultures with methamphetamine or morphine causes morphological changes in astrocytes[33]. Moreover, treatment with methamphetamine increased the sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids[33]. Furthermore, astrocytes play a critical role in dopaminergic neuron development. As described in chapter 1, the *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 the levels of GFAP. Therefore, I also investigated whether prenatal and neonatal exposure to bisphenol-A induces astrocytic activation associated with the alternation of the dopaminergic neuron development.

Materials and Methods

Animals

All experiments were performed using 10-14 weeks 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 zero (control), 2 x 10^3 μg bisphenol-A/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. In addition, RT-PCR was also performed using embryonic C57BL/6J mice that had been prenatally exposed to bisphenol-A from mating to embryo 14 days (same concentration as mentioned above).

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 according to previous report[71], and the targeted genes were amplified in 50 μL of a PCR solution containing $MgCl_2$, dNTP mix and DNA polymerase (Invitrogen, Carlsbad, CA, USA) 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 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 ventral tegmental area (VTA) or the limbic forebrain including the nucleus accumbens/ventral pallidum were initially dissected using Brain Blocker. The brain coronal sections were postfixed in 4% paraformaldehyde for 2 hr. After the brains were permeated with 20% sucrose for 1 day and 30% sucrose for 2 days, they were frozen in embedding compound (Sakura Finetechnical, Tokyo, Japan) on isopentane using liquid nitrogen and stored at -30°C until use. Frozen 8-μm-thick coronal sections were cut with a cryostat (CM1510; Leica, Heidelberg, Germany) and thaw mounted on poly-L-lysine-coated glass slides. The brain sections were blocked in 10% normal horse serum (NHS) in 0.01M 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, USA), 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, USA). 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).

Statistical analysis

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

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. 3-1E, F), Shh ($p<0.001$, Fig. 3-1I, J) and GDNF ($p<0.01$, Fig. 3-1K, L) mRNA production in the whole brain obtained from embryonic mice (ED 14). On the other hand, no changes in mRNA levels of Shh (Fig. 3-1M, N) and GDNF (Fig. 3-1O, P) mRNA 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. 3-1A, B), dopamine D_2 receptor (Fig. 3-1C, D) and DAT (Fig. 3-1G, 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

I first investigated the possible morphological changes in dopaminergic neuron. The DAT or TH-like immunoreactivity (DAT-IR or TH-IR) in the VTA was observed by immunohistochemical analysis (Fig. 3-2A-D). Prenatal and neonatal exposure to bisphenol-A failed to induce morphological changes in dopamine cell bodies or the number of dopaminergic neuron. On the other hand, prenatal and neonatal exposure to

number of dopaminergic neuron. On the other hand, prenatal and neonatal exposure to bisphenol-A produced a dramatical increase in the levels of DAT-IR and TH-IR in the nucleus accumbens (Fig. 3-2E-H). In addition, GFAP-like immunoreactivity (GFAP-IR) was increased in the mouse ventral pallidum by prenatal and neonatal exposure to bisphenol-A (Fig. 3-2I, 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. 3-2K, L).

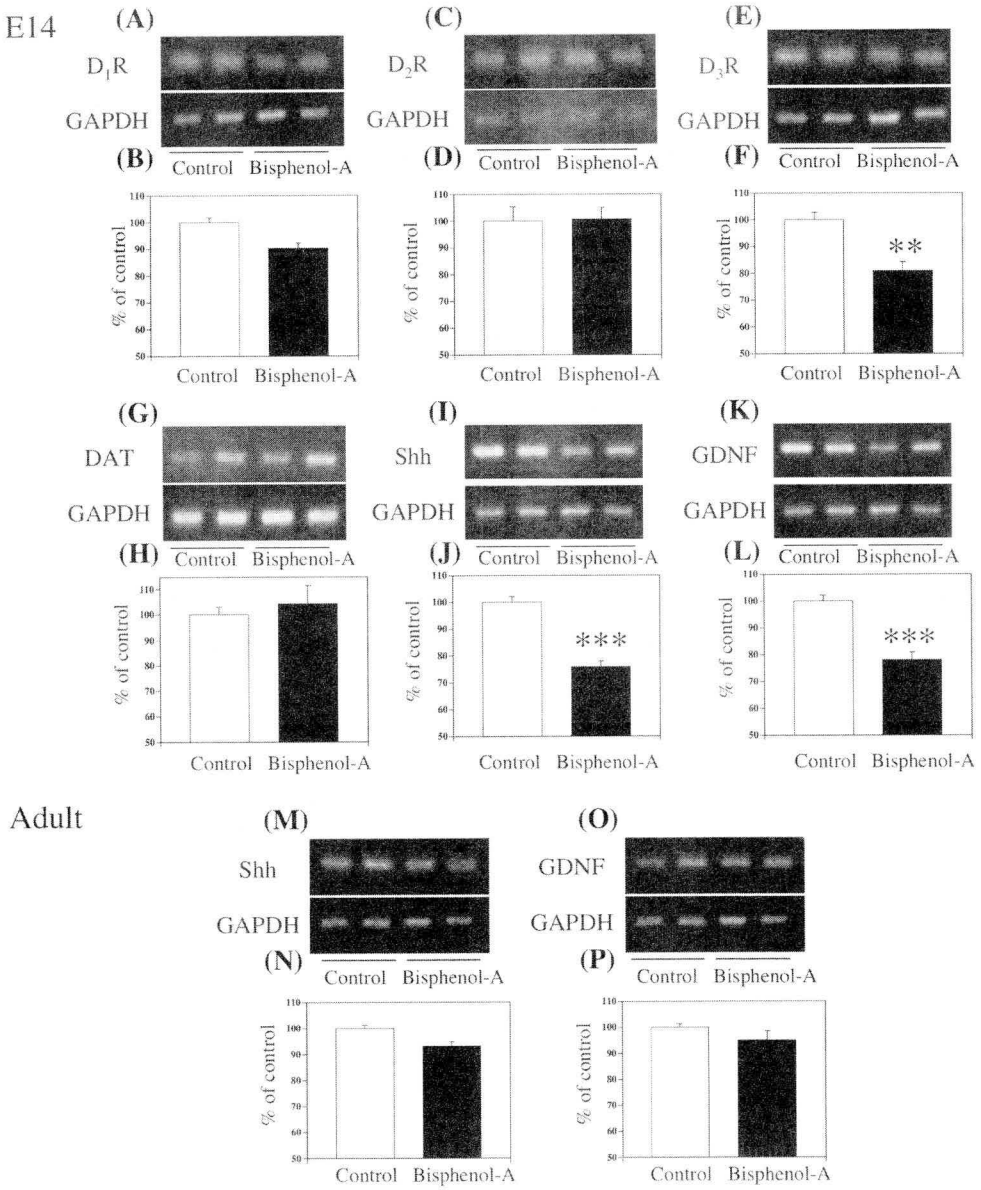


Fig. 3-1 Down-regulation of the expression of dopamine D_3 receptor, Shh and GDNF mRNAs in the whole brain obtained from embryonic mice prenatally exposed to bisphenol-A (A, C, E, G, I, K) Representative RT-PCR for the dopamine D_1 receptor (D_1R; A), dopamine D_2 receptor (D_2R; C), dopamine D_3 receptor (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 the 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) and 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) and 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. ***$p<0.001$ vs. control mice.

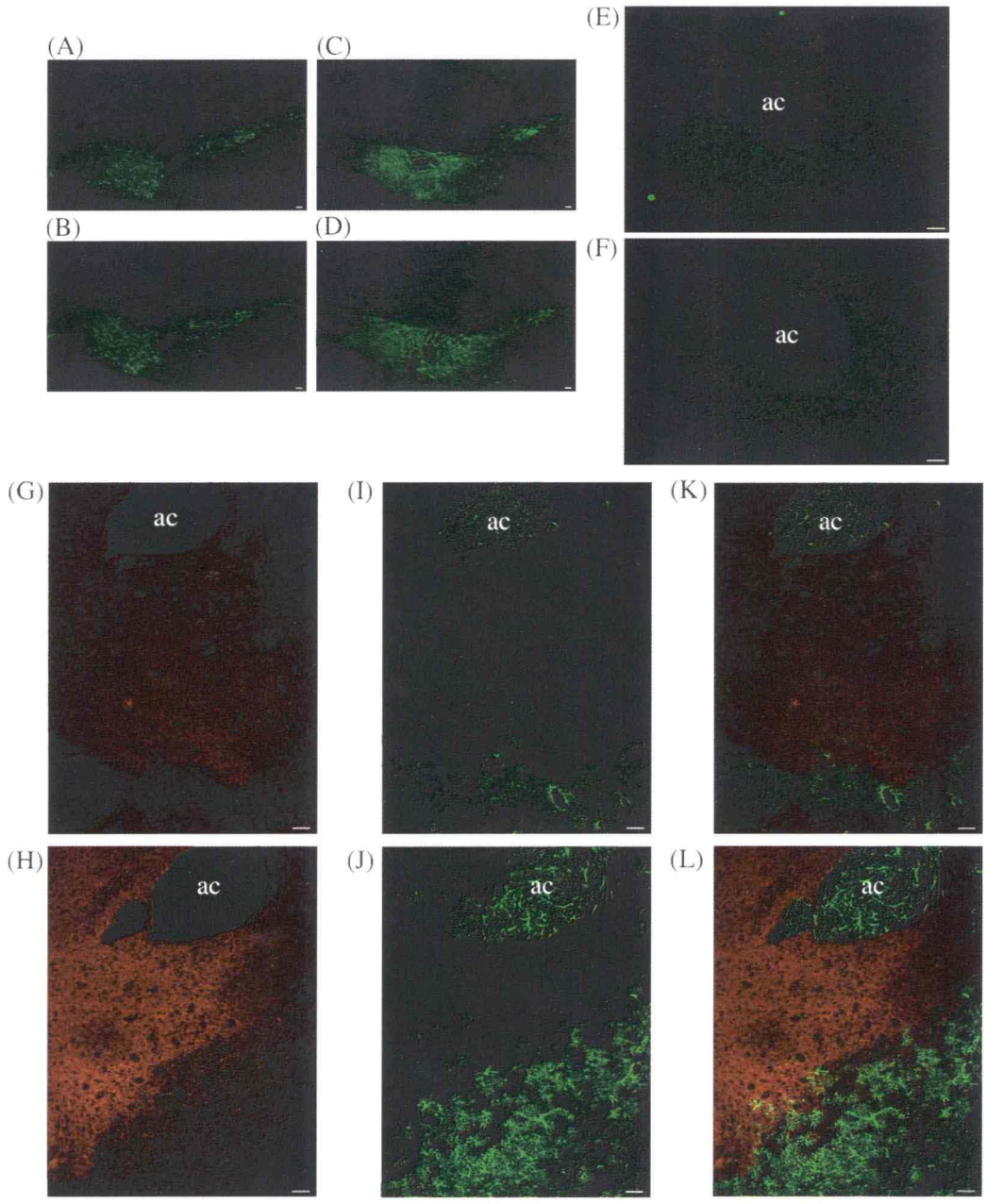


Fig. 3-2 Increase in DAT-, TH- and GFAP-IRs in the nucleus accumbens or ventral pallidum of mice prenatally and neonatally exposed to bisphenol-A (A, B) The TH-IR in the ventral tegmental area (VTA) did not change in mice prenatally and neonatally exposed to bisphenol-A (B) compared to control mice (A). (C, D) Similarly, no change of the DAT-IR in the VTA was noted in mice prenatally and neonatally exposed to bisphenol-A (D) compared to control mice (C). (E, F) On the other hand, the increased TH-IR in the nucleus accumbens was noted in mice prenatally and neonatally exposed to bisphenol-A (F) compared to control mice (E). (G, H) The increased DAT-IR in the nucleus accumbens was also observed in mice prenatally and neonatally exposed to bisphenol-A (H) compared to control mouse (G). The GFAP-IR in the ventral pallidum was dramatically increased with morphological changes in mice prenatally and neonatally exposed to bisphenol-A (J) compared to control mice (I). The green labeling for DAT and the red labeling for GFAP show no apparent colocalization in the limbic area (K, L). ac: anterior commissure. Scale bars: 50 μm.

Discussion

Recently, several investigations have provided evidence that the treatment of adult animals with bisphenol-A could not affect the reproductive function and social behavior[67]. I found that an acute administration of bisphenol-A in adult mice failed to affect the dopamine-related behaviors (data not shown). On the other hand, several investigations clarified the behavioral abnormalities by prenatal and neonatal exposure to bisphenol-A[7-9,70]. These findings indicate that prenatal and neonatal exposures to bisphenol-A may cause neuronal toxicity, specifically in the developmental process. It has been reported that prenatal and neonatal exposure to bisphenol-A induces the abnormality of the dopamine receptor functions in the mouse limbic area, resulting in a supersensitivity of methamphetamine-induced pharmacological actions[7]. These findings indicate that exposure to bisphenol-A during development alters the postsynaptic regulation of dopamine neuron. In the present study, I therefore focused on the change in the dopaminergic neuron during development.

Here I 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)[22]. Maturation is orchestrated by several transcription factors, including

the orphan nuclear receptor (Nurr-1), which is widely expressed in both the adult and the developing CNS[25]. Furthermore, it was reported that Shh, FGF8, and Nurr-1 collaborate to induce dopaminergic phenotypes[72]. GDNF is also one of the most potent trophic factors for dopaminergic neurons, playing a role in development and survival[23]. Therefore the present data support the idea that prenatal and neonatal exposure to bisphenol-A may disrupt dopaminergic neuron development associated with the expression of Shh and GDNF.

It has been reported that prenatal and neonatal exposure to bisphenol-A induced the functional reduction in dopamine D_3 receptors in mice[9]. Du *et al.* reported that the pharmacological action of GDNF was regulated by the activation of dopamine D_3 receptor[73]. As described in chapter 2, exposure to bisphenol-A during organogenesis (EDs 7-14), but not implantation (EDs 0-7) and 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 my hypothesis that bisphenol-A disrupts dopaminergic neuron development.

Next, I 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, or morphological changes in

dopaminergic neuron in the VTA. On the other hand, I found that prenatal and neonatal exposures to bisphenol-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. Their activation may control the structural and functional plasticity of synapses in the CNS. Recent accumulating evidence suggests that astrocytes express a repertoire of neurotransmitter receptors mirroring that of the neighboring synapse. Such receptors are stimulated during synaptic activity and spread information by calcium signaling into the astrocyte network via gap-junction channels[74]. It has been widely accepted that the long-term exposure to drugs of abuse can induce neuronal plasticity. Recent reports suggested that the treatment of mouse cortical neuron/glia cocultures with methamphetamine or morphine causes morphological changes in astrocytes[33]. Moreover, treatment with methamphetamine increases the sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids[33]. It is interesting, as described in chapter 1, that the 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 the levels of GFAP. 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[75]. The nucleus accumbens and ventral pallidum have a pivotal role in regulating exploratory motor behaviors. The pharmacological manipulation of dopamine or enkephalin transmission in the nucleus accumbens induces motor activity. The nucleus accumbens has a prominent γ-aminobutylic acid (GABA)-ergic projection to the ventral pallidum. The motor response elicited by microinjecting the μ-opioid agonist D-Ala-Tyr-Gly-NMePhe-Gly-OH (DAMGO) or dopamine into the accumbens is blocked by stimulating $GABA_A$ receptors in the ventral pallidum with the agonist muscimol[76]. 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 (1966) first reported the metabolic fate of bisphenol-A in rats[77], showing that the major metabolite in urine was the glucuronide of bisphenol-A; considerable amounts of free bisphenol-A and hydoxylated bisphenol-A were found in feces. Many reports have shown that bisphenol-A is metabolized and excreted rapidly[78], and I found that its acute administration to adult mice did not affect their dopamine-related behaviors. In my preliminary biochemical studies, bisphenol-A did not increase or decrease [^{35}S]GTPγS bindings to brain membranes. Also, I could not make the Scatchard plot using [^{3}H]bisphenol-A in brain membranes. Taken together, it is almost impossible that bisphenol-A remaining in

the adult brain of mice directly affects dopaminergic neurotransmission.

At the present time it is very hard to say where the primary action site of bisphenol-A is. Although it has weak estrogenic activity, prenatal and neonatal exposures to 17β-estradiol failed to induce supersensitivity to morphine (as described in chapter 4). And as described in chapter 1, treatment with 17β-estradiol failed to induce astrocytic activation. Furthermore, although it is well known that bisphenol-A disrupts thyroid hormone, prenatal and neonatal exposures to propylthiouracil, a thyroid hormone inhibitor, reduced dopaminergic neuron activation. These findings indicate that a disruption of dopaminergic neuron development induced by prenatal and neonatal exposures to bisphenol-A can be mediated by the nonhormonergic 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.

Chapter 4

Prenatal and neonatal exposures to low-dose bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effects

Introduction

As mentioned in previous chapters, prenatal and neonatal exposures to high-dose bisphenol-A (2 x 10^3 μg of bisphenol-A/g of food) induced dopaminergic amplification following the disruption of the dopaminergic neuron development. However, Inoue *et al.* previously reported that the concentration of bisphenol-A was 0.32 ng/mL in normal human serum[39]. According to this report, it should be mentioned that the blood level of bisphenol-A in mice prenatally and neonatally exposed to high-dose bisphenol-A (approximately 10 ng/mL) is considered to be more than 30 times higher than the healthy human-exposure level. Regarding endocrine disruptor problems, the low-dose actions of endocrine disruptors are serious problems. However, little is known about its action on the central nervous system induced by low-dose bisphenol-A. The present study was then undertaken to investigate whether prenatal or neonatal exposure to low-dose bisphenol-A in mice could affect the rewarding effects and the locomotor-enhancing effects induced by morphine.

Because sex steroid hormones (estrogens and androgens) have been shown to exert profound effects on brain differentiation, neural plasticity, and central neurotransmission[34,35], and because bisphenol-A has an affinity for estrogen receptors, albeit 1:2000 that of 17β-estradiol[36], I also investigated the effect of 17β-estradiol on the rewarding effects induced by morphine.

Materials and Methods

Animals

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

In the experiments for comparison between bisphenol-A and 17β-estradiol, mice were orally administered either olive oil (control, 0.1 mL/kg), bisphenol-A (3 μg/kg/day or 200 mg/kg/day) dissolved in olive oil (Wako Pure Chemicals) or 17β-estradiol (3 μg/kg/day), through stomach sonde. Female mice (10 weeks old) were orally treated with these chemicals three times a day (8:00, 14:00, 20:00) from mating to weaning.

Place conditioning

Place conditioning was performed according to the method described in Chapter 2.

Measurement of locomotor activity

The locomotor activity of mice was measured following the method described in

Chapter 2.

[^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding assay was conducted as described in Chapter 2.

Statistical analysis

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

Result

Enhancement of morphine-induced rewarding effect in mice prenatally and neonatally exposed to low-dose bisphenol-A

At the dose of 1 mg/kg (s.c.), morphine produced neither place preference nor place aversion in control mice (Fig. 4-1A). However, treatment with 1 mg/kg (s.c.) of morphine produced a significant place preference in mice chronically treated with low-dose bisphenol-A from mating to weaning ($*p < 0.05$ vs. control group, Fig. 4-1A).

Enhancement of morphine-induced hyperlocomotion in mice prenatally and neonatally exposed to low-dose bisphenol-A

Treatment with 10 mg/kg (s.c.) of morphine produced a locomotor-enhancing effect in all groups (Fig. 4-1B). In mice chronically treated with low-dose bisphenol-A, the hyperlocomotion induced by morphine was dramatically potentiated as compared to that in control (Fig. 4-1B).

Dopamine-induced G-protein activation in the limbic forebrain of mice prenatally and neonatally exposed to low-dose bisphenol-A

The enhancement of the stimulation of [^{35}S]GTPγS binding induced by dopamine (10 μM) was noted in the limbic forebrain of mice chronically treated with low-dose bisphenol-A ($**p < 0.01$ vs. control group. Fig. 4-1C).

Enhancement of morphine-induced rewarding effect in mice prenatally and neonatally exposed to low and high doses bisphenol-A but not 17β-estradiol

At the dose of 1 mg/kg (s.c.), morphine produced neither place preference nor place aversion in control mice (Fig. 4-2). On the other hand, treatment with 1 mg/kg of morphine produced a significant place preference in mice prenatally and neonatally exposed to low and high doses bisphenol-A (*$p < 0.05$ vs. control group, Fig. 4-2). In contrast, treatment with morphine at 1 mg/kg failed to produce a significant place preference in offspring of mothers that had been chronically treated with 17β-estradiol (Fig. 4-2).

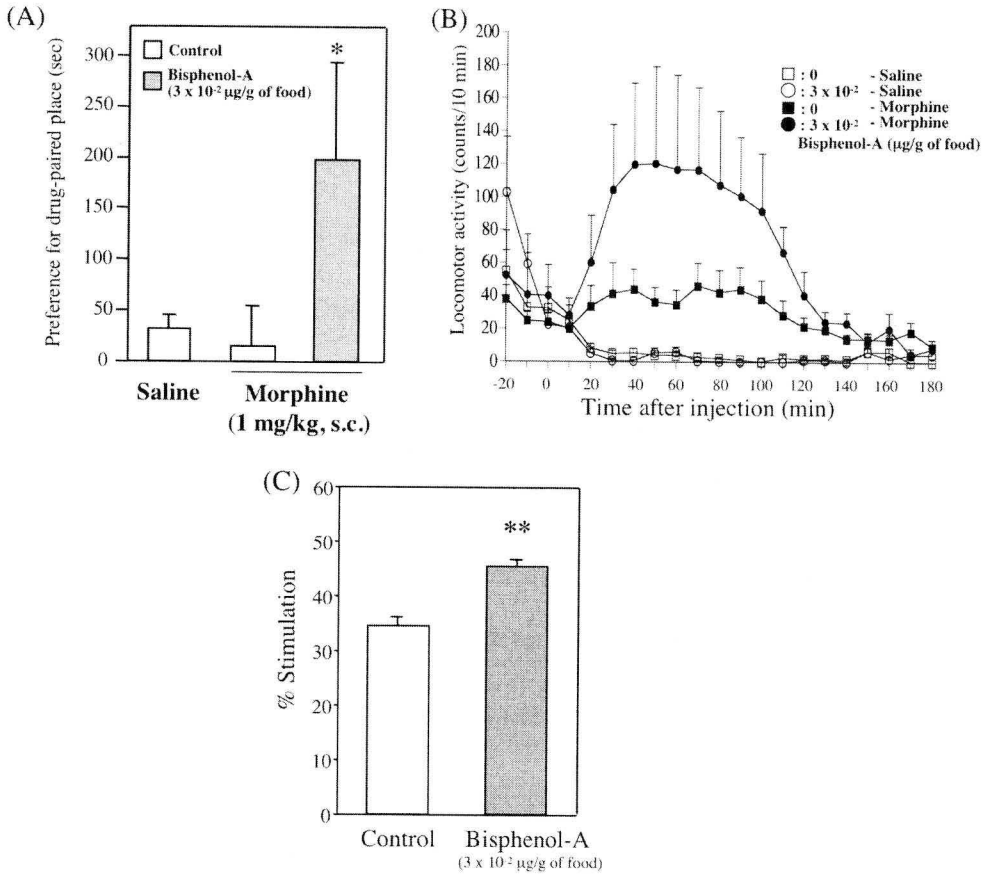


Fig. 4-1 Effect of prenatal and neonatal exposures to low-dose bisphenol-A on the morphine-induced rewarding effect and hyperlocomotion in mice. (A) Prenatal and neonatal exposures to low-dose bisphenol-A (3 x 10^{-2} μg/g of food) enhanced the morphine (1 mg/kg, s.c.) -induced rewarding effect in mice. Each column represents the mean conditioning score with SEM. *$p<0.05$ vs. morphine-treated control group. (B) In addition, prenatal and neonatal exposures to low-dose bisphenol-A also enhanced the morphine (10 mg/kg, s.c.) -induced hyperlocomotion in mice. Each point represents the mean activity counts for 10 min with SEM. (C) In these conditions, the enhancement of the stimulation of [^{35}S]GTPγS binding induced by dopamine (10 μM) was noted in the limbic forebrain of mice chronically treated with low-dose bisphenol-A. Membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with dopamine. The data are shown as the percentage of basal [^{35}S]GTPγS binding measured in the presence of GDP and absence of dopamine. Each column represents the mean with SEM of 3 samples. **$p<0.01$ vs. control group.

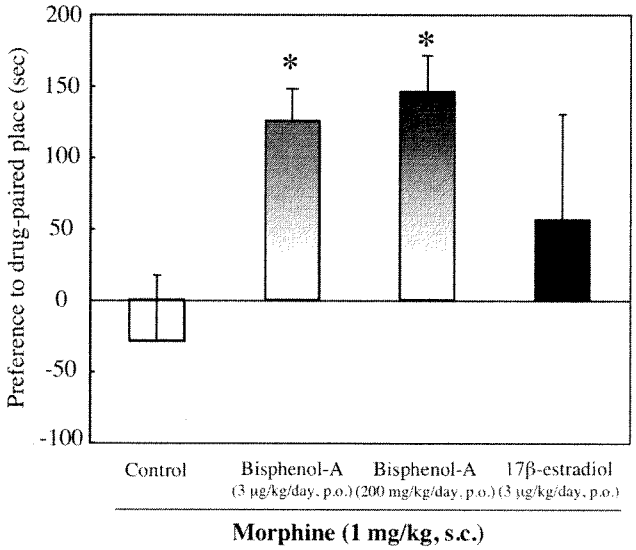


Fig. 4-2 Enhancement of the morphine-induced rewarding effect in mice that were prenatally and neonatally exposed to bisphenol-A (BPA), but not 17β-estradiol. The control group did not show any place preference or place aversion with morphine (1 mg/kg, s.c.). The bisphenol-A (3 μg or 200 mg/kg/day)-treated group showed a significant place preference induced by morphine (*$p < 0.05$ vs. control group). The 17β-estradiol (3 μg/kg/day)-treated group did not show any place preference or place aversion with morphine. Each column represents the mean place preference score ± SEM.

Discussion

As described in previous chapters, the chronic treatment of high-dose bisphenol-A induced the supersensitivity of pharmacological action of morphine associated with the disruption of dopaminergic neuron development. These phenomena were observed in mice chronically treated with bisphenol-A-admixed powder food containing 2 x 10^3 μg bisphenol-A/g of food during the developmental period. Under these conditions, the blood level of bisphenol-A in their pups was approximately 10 ng/mL, which is considered to be more than 30 times higher than the level for healthy human exposure[7]. On the other hand, vom Saal *et al.* estimated that humans are exposed to bisphenol-A at a dose of 2 to 20 μg/kg/day[79]. Based on these reports, I here ascertained the effects of low-dose exposure to bisphenol-A. Adult female mice were chronically treated with bisphenol-A-admixed powder food containing zero (control) or 3 x 10^{-2} μg bisphenol-A/g of food from mating to weaning. Here I found that prenatal and neonatal exposures to low-dose bisphenol-A also enhanced the pharmacological actions of morphine associated with functional potentiation of dopamine receptor in the limbic forebrain.

Bisphenol-A and alkylphenols have been reported to have estrogenic activity[80]. Recent molecular studies have suggested the transcriptional activation of the human dopamine D_1 receptor gene by estrogen[81]. Therefore the supersensitivity of morphine-induced pharmacological actions caused by prenatal and neonatal exposures to low-dose bisphenol-A may be mediated through estrogen receptors. I next investigated whether prenatal and neonatal exposures to 17β-estradiol induce the potentiation of morphine-

induced rewarding effect. I found that prenatal and neonatal exposures to low and high doses bisphenol-A (3 μg or 200 mg/kg/day administered by oral administration) clearly enhanced the rewarding effects of morphine in mice. In contrast, treatment with 17β-estradiol (3 μg/kg/day, administered orally) had no such effects. These findings indicate that the potentiation of morphine-induced rewarding effects induced by prenatal and neonatal exposure to bisphenol-A can be mediated by the nonestrogenic actions of bisphenol-A. It has been reported that equal doses bisphenol-A and 17β-estradiol could activate CREB via nonclassical estrogen receptors, resulting in the transcriptional activation of CREB-responsive genes[48]. On the other hand, obvious differences between bisphenol-A and 17β-estradiol have also been reported. For example, 17β-estradiol at 10 nM reduced the duration of Ca^{2+} oscillations in mouse oocytes, whereas concentrations of bisphenol-A as high as 100 μM were necessary for similar inhibition[49]. It has been reported that 17β-estradiol inhibits the astrocytic uptake of glutamate, which is the most important excitatory neurotransmitter in the CNS, whereas bisphenol-A has no such effect[50]. Taken together, these observations suggest that bisphenol-A and 17β-estradiol may be coupled to different signaling cascades in the CNS. As mentioned in chapter 1, *in vitro* experiments indicate that the enhancement of Ca^{2+} responses to dopamine induced by bisphenol-A could lead to an increase in the excitability of central dopaminergic neurotransmissions in both neurons and astrocytes mediated by nonhormonergic actions of bisphenol-A. As mentioned above, bisphenol-A has only weak estrogenic activities. Therefore it is difficult to explain whether the enhancement of dopaminergic transmission in mice prenatally and neonatally exposed to low-dose

bisphenol-A could be induced by estrogenic activities of bisphenol-A. Taken together, these results indicate that the enhancement of dopaminergic transmission in neurons and astrocytes induced by nonhormonergic actions of bisphenol-A may, at least in part, lead to the aggravation of the development of psychological dependence on morphine.

In regard to endocrine disruptor problems, the low-dose actions are serious problems. Besides the description in the present study, it was recently reported that there were effects caused by exposure to low-dose bisphenol-A on the rate of growth and sexual maturation, hormone levels in blood, reproductive organ function, fertility, immune function, enzyme activity, and brain structure, brain chemistry, and behavior[82]. Therefore the present findings warn that prenatal and postnatal exposures to not only high-dose, but also low-dose bisphenol-A may dramatically change the neuronal transmission, including dopaminergic transmission in the adult brain. This phenomenon could explain the aggravation of the development of dependence on drugs of abuse.

Chapter 5

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

Introduction

As mentioned in previous chapters, chronic exposure to bisphenol-A during a development period alters dopaminergic neurotransmission in the central nervous system (CNS), which results in the enhancement of psychological dependence on drugs of abuse.

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 described in chapter 4. 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. 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, my 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 abnormalities such as anxiogenic behavior, motor learning behavior, and memory.

On the endocrine disruptor problems, the low-dose actions of the endocrine-disrupting chemicals are serious problems, as described in chapter 4. However, little is known about action on the CNS induced by low-dose bisphenol-A. I also investigated whether prenatal and neonatal exposures to low-dose bisphenol-A in mice could induce behavioral abnormalities.

Materials and Methods

Animals

All experiments were performed using 7-11 weeks 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), 3 x 10^{-2}, 2 x 10^{3} μg bisphenol-A/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. During the treatment with bisphenol-A, animals did not show body weight loss.

Light-dark test

To investigate changes in anxiogenic-like effects, mice were tested using the light–dark paradigm [83,84]. I used a box consisting of a small (18 x 13 x 18 cm) dimly lit compartment with black walls and a black floor, connected by a small opening (5 x 5 cm) to a large (18 x 18 x 18 cm) intensely lit (500 lux) compartment with a white walls and a white floor. Each animal was placed in the dark compartment at the beginning of the observation session. Compartment entry and exit were defined as all four paws into and out, respectively. The time spent in the lit compartment was recorded for 10 min.

Elevated plus-maze

As another mesurement of anxiety, the mice prenatally and neonatally exosured to bisphenol-A were evaluated by the elevated plus-maze paradigm. The elevated plus-maze consists of two opposing open arms (30 x 6 x 0.3 cm) and two opposing enclosed arms (30 x 6 x 15 cm) that are connected by a central platform (9 x 9 cm, 100 lux), thus forming the shape of a plus sign. Each animal was tested using the elevated plus-maze in each experiment. The time spent in open or enclosed arms and entry into open or enclosed arms were recorded for 5 min. 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.

Rota-rod performance procedure

To investigate possible changes in motor learning impairment with prenatal and neonatal exposure to bisphenol-A, 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 non-slippery surface. The rod with 30 cm length was placed at a height of 15 cm from the base. The rod was divided into 5 equal sections by 6 disks. Thus, mice were tested simultaneously on the apparatus, with rod-rotating speed of 10 rpm. A rota-rod test during which each animal was placed on a rotating rod and had to move forward; if the mouse stayed on the rotating rod for 5 min, the rota-rod test was stopped; each animal was tested for 5 min. The mouse was replaced on the rotating rod immediately after the mouse fell from the rotating rod. I measured the time

until falling first time and the numbers of falling during 5 min as the indicators of motor impairment twice a day. The score was the mean of latencies or numbers of falling in two trials.

Step-through passive avoidance test

The influence of prenatal and neonatal exposure to bisphenol-A on memory processes in mice was evaluated by the step-through passive avoidance test. The experimental apparatus for the step-through passive avoidance test is a shuttle-box that is divided into an illuminated small compartment (12 x 5 x 14 cm) and a dark/large compartment (25 x 25 x 20 cm) by a wall with a guillotine door. At the first day (conditioning day), each animal was placed in the illuminated compartment. After 90 sec, the door was opened and the mouse moved into the dark compartment freely. 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 hr from conditioning day, retention test was started. The retention test was performed in the similar manner 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.

Immunohistochemistry

Immunohistochemistry was performed according to the method described in Chapter 2. 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 sections were blocked in 3% normal horse serum with 0.2% Triton X-100 in 0.01M PBS for 1 hr at room temperature. The primary antibody of choline acetyltransferase (ChAT, 1: 100 Chemicon International Inc., CA, USA) was diluted in 0.01 M PBS containing 3% NHS with 0.2% Triton X-100 and incubated for 2 days at 4°C. The samples were then rinsed and incubated with the appropriate secondary antibody conjugated with Alexa 488 for 2 hr at room temperature. Fluorescence immunolabeling was observed with a light microscope and photographed with a digital camera as described in Chapter 2. 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.

Statistical analysis

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

Result

Lack of anxiogenic effect by prenatal and neonatal exposure to bisphenol-A

In the present study, prenatal and neonatal exposure to bisphenol-A failed to induce anxiogenic-like effect using the light-dark paradigm (Fig. 5-1-A). 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 were shown in Fig. 5-1-B. The prenatal and neonatal exposure to bisphenol-A failed to affect the percentage of time spent in the open arms.

Lack of motor learning impairment by prenatal and neonatal exposure to bisphenol-A

To investigate possible changes in motor learning impairment with prenatal and neonatal exposure to bisphenol-A, mice were tested using the rota-rod test. In the present study, the mice prenatally and neonatally exposed to low or high doses bisphenol-A progressively improved their skill in the rota-rod test as like control mice (Fig. 5-1-C, D). The improvement of latency to fall and number of falling in the rota-rod test were indistinguishable between control and bisphenol-A treated mice (Fig. 5-1-C, D). These results suggest that prenatal and neonatal exposure to bisphenol-A has no direct effect on motor skill learning.

Memory impairment induced by prenatal and neonatal exposure to bisphenol-A

The influence of prenatal and neonatal exposure to bisphnol-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 bisphenol-A was similar to that of control mice (Fig. 5-1-E). The mice prenatally and neonatally exposed to bisphenol-A dramatically decrease latencies to step into the dark compartment as compared with control mice, although all groups increased the latency to step into the dark compartment compared with the latency shown at conditioning (Low; $F_{(1,9)}=6.246$, $p<0.05$ vs. control, High; $F_{(1,10)}=9.167$, $p<0.05$ vs. control, Fig. 5-1-F).

Dramatical reduction in ChAT-like immunoreactivitiy in the hippocampus of mice prenatally and neonatally exposed to low and high doses bisphenol-A

Immunohistochemical study showed that prenatal and neonatal exposures to low- and high-doses bisphenol-A dramatically decreased the level of choline acetyltransferase-like immunoreactivity (ChAT-IR) in the widespread regions of the hippocampus compared to control (Fig. 5-2-Ai-iii). Especially, as shown in high magnification images, cholinergic fiber was dramatically decreased in mice prenatally and neonatally exposed to low- and high-doses bisphenol-A compared to control (Fig. 5-2-Bi-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 bisphenol-A produced a significant decrease in the level of ChAT-

IR in the hippocampus (Low: 64.3 ± 5.6 % of control, ***$p < 0.001$ vs. control mice; High: 50.0 ± 3.8 % of control, ***$p < 0.001$ vs. control mice, 5-2-C).

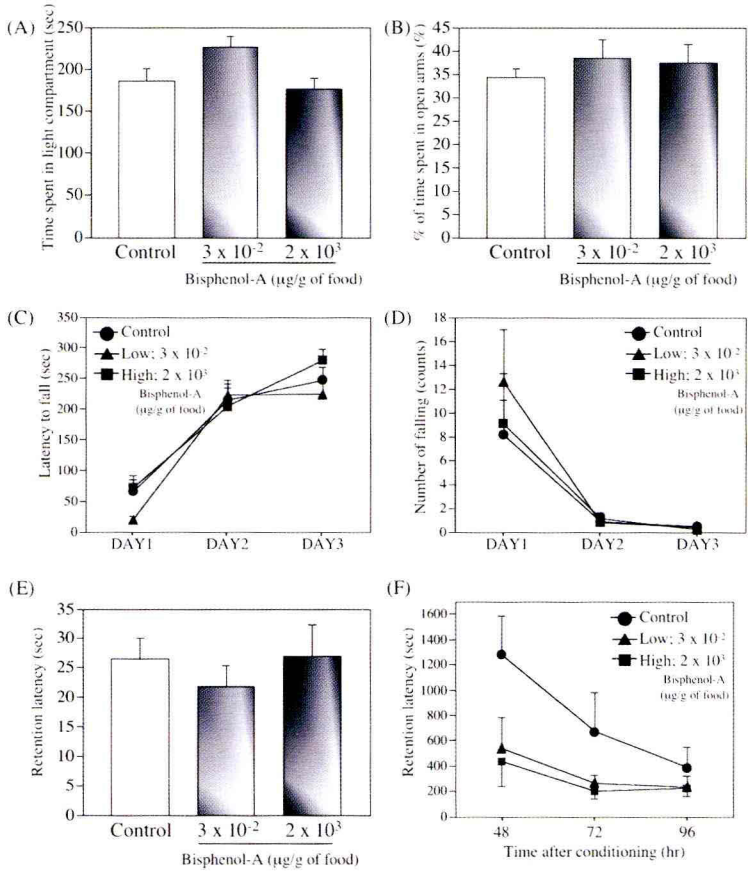


Fig. 5-1 Behavioral analysis in adult mice prenatally and neonatally exposed to bisphenol-A (A) Lack of anxiogenic effect by prenatal and neonatal exposures to bisphenol-A using the light-dark test procedure. Prenatal and neonatal exposures to bisphenol-A failed to change the time spent in light compartment. Each column represents the mean with SEM 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 faild to change the percentage of time spent in the open arms. Each column represent the mean with SEM of 5-7 mice/group. (C, 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 sec was allowed for each animal per trial. The score was the mean of latencies in two trials. Each point represents the mean with SEM of 6-7 mice/group. (D) The numbers that the animal fell from a rotating rod to the ground during 300 sec were counted twice a day. The score was the mean of numbers of falling in two trials. Each point represents the mean with SEM of 6-7 mice/group. (E, F) Effect of prenatal and neonatal exposures to bisphenol-A on performance in a step-through passive avoidance procedure. (E) At conditioning, mice were placed into the light 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 control mice. (F) Prenatal and neonatal exposures to low- and high-doses bisphenol-A induced a significant memory impairment (Low; $F_{(1,9)}=6.246$, $p<0.05$ vs. control, High; $F_{(1,10)}=9.167$, $p<0.05$ vs. control). Each point represents the mean with SEM of 5-7 mice/group.

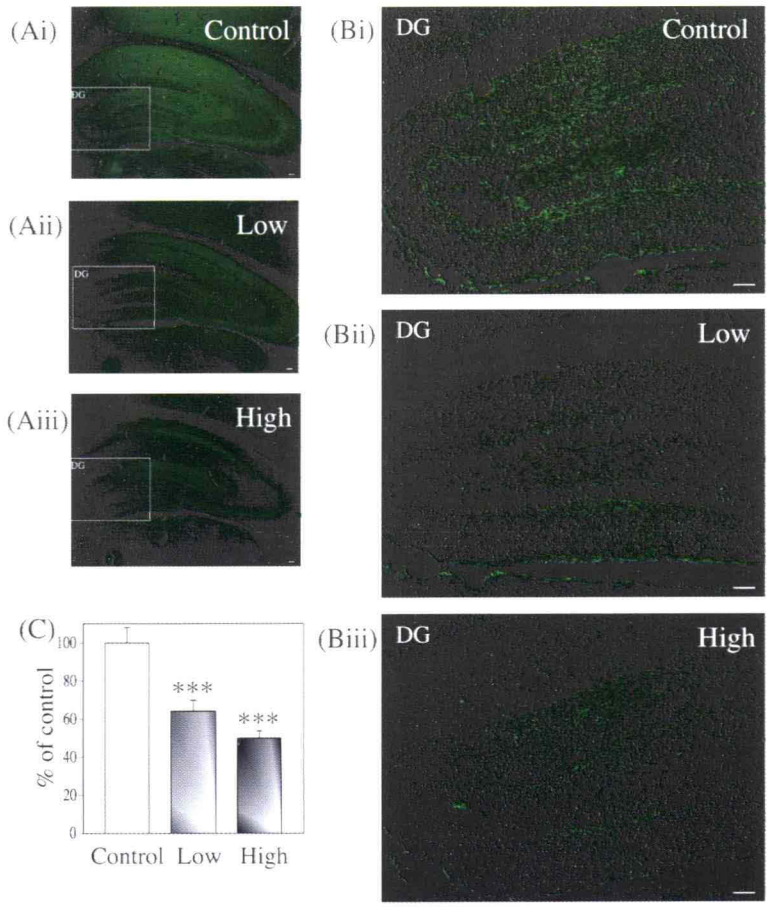


Fig. 5-2 Dramatical reduction in ChAT-like immunoreactivitiy in the hippocampus of mice prenatally and neonatally exposed to low- and high-doses bisphenol-A (Ai-iii) Prenatal and neonatal exposures to low (Aii)- and high-doses (Aiii) bisphenol-A dramatically decreased the level of ChAT-IR in the hippocampus compared to control (Ai). (Bi-iii) High magnification images showed 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) Semi-quantitative analysis of ChAT-IR was performed using Image J (Low: 64.3 ± 5.6 % of control, ***$p < 0.001$ vs. control mice; High: 50.0 ± 3.8 % of control, ***$p < 0.001$ vs. control mice). Each column represents the mean ± SEM of three independent samples. DG: dentate gyrus. Scale bars: 50 μM.

Discussion

In the present study, prenatal and neonatal exposures to bisphenol-A, using the light-dark paradigm and the elevated plus-maze paradigm, failed to induce anxiogenic-like effects. These results suggest that these exposures induced no anxiogenic-like behaviors. Furthermore, to investigate possible changes in motor learning impairment by prenatal and neonatal exposures to bisphenol-A, the mice were tested by means of the rota-rod test. I found that the mice prenatally and neonatally exposed to low-dose or high-dose bisphenol-A progressively improved their skills in the rota-rod test as the control mice did. Furthermore, the improvement of latency to fall and the number falling in the rota-rod test were indistinguishable between the control and bisphenol-A treated mice. These results suggest that prenatal and neonatal exposures to bisphenol-A has no direct effect on motor skill learning.

On the other hand, 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 mice prenatally and neonatally exposed to low and high doses bisphenol-A was similar to that of control mice. The mice prenatally and neonatally exposed to bisphenol-A dramatically decrease latencies to step into the dark compartment in comparison with the control mice, though all groups increased the latency to step into the dark compartment compared with the latency shown at conditioning. These results strongly suggest that chronic treatment with low and high doses bisphenol-A induced the memory impairment. The contextual

fear conditioning is hippocampal-dependent memory. Therefore I next investigated the morphological and/or functional changes in the hippocampus of mice prenatally and neonatally exposed to low and high doses bisphenol-A.

Immunohistochemical study showed that prenatal and neonatal exposures to low and high doses bisphenol-A dramatically decreased the level of ChAT-IR in the widespread regions of the hippocampus compared to control. It is widely accepted that the cholinergic function in the hippocampus is important for the learning and memory processes[85-87]. 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 my findings that the memory impairment corresponded well to the dysfunction of cholinergic neurons in the hippocampus of mice prenatally and neonatally exposed to bisphenol-A.

As described in previous chapters, it is widely accepted that one of the most common endocrine disruptors, bisphenol-A, has weak estrogenic activity. Moreover, an endogenous estrogen, 17β-estradiol, plays the critical role in the neurotransmission of the hippocampus associated with spinogenesis or neuroprotection[88,89]. Furthermore, many cholinergic neurons also express the growth-associated protein GAP-43, which may be taken as a marker of neurite outgrowth[90]. Estrogens, among other factors, up-regulate the expression of GAP-43 in the developing and adult brain[91]. On the other hand, bisphenol-A also disrupts the thyroid hormone. Thyroid hormone deficiency during brain development disrupts on the activities of enzymes of central acetylcholine

metabolism, the activities of ChAT and acetylcholinesterase in the hippocampus[92]. 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[93,94]. According to these reports, I 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 previous chapters, I described that prenatal and neonatal exposures to bisphenol-A potentiated the dopaminergic neurotransmission. As mentioned above, this phenomenon was induced by the nonhormonergic effect of bisphenol-A. In the present study, I 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 bisphenol-A, but also to low-dose. Although it is very difficult to explain where the primary action site of bisphenol-A is, I must therefore consider its mechanism through nonhormonergic effect.

Knaak and Sullivan (1966) first reported the metabolic fate of bisphenol-A in rats, showing that the major metabolite in urine was the glucuronide of bisphenol-A; sizable amounts of free bisphenol-A and hydroxylated bisphenol-A were found in feces[77]. Many reports have shown that bisphenol-A is metabolized and excreted rapidly[95-97]. Taken together, it is almost impossible that the bisphenol-A that remained in the adult brain of mice directly affects the CNS. Therefore prenatal and neonatal exposures to bisphenol-A disrupts the neuron development, resulting in behavioral abnormalities in 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 bisphenol-A, dramatically decrease the cholinergic transmission in the adult brain, and this results in learning and memory deficits.

General Conclusion

These findings lead to the following conclusions.

In Chapter 1:

In the present study, I demonstrated for the first time that *in vitro* treatment with low-dose bisphenol-A could lead to astrocytic activation according without the steroid-hormonergic effects of bisphenol-A.

In Chapter 2:

The present findings provide that the organogenesis and lactation are the most important periods to cause a disruption of dopaminergic neuron development by bisphenol-A exposure in the mouse.

In Chapter 3:

The present data provide direct evidence that prenatal and neonatal exposures to bisphenol-A disrupts the dopaminergic neurotransmission in the process of dopaminergic neuron development associated with changes in dopaminergic neuron developmental factors.

In Chapter 4:

Based on the present study, I can warn that prenatal and postnatal exposures not only to high-dose, but also to low-dose bisphenol-A, may dramatically change the neuronal transmission, including dopaminergic transmission, in the adult brain.

In Chapter 5:

The present findings provide direct evidence that prenatal and neonatal exposures to low and high doses bisphenol-A-induced memory impairment associated with a dysfunction of the hippocampal cholinergic system.

Final conclusion:

For several years, concern has been growing over changes in the health and fecundity of humans and wildlife that may be associated with the disruption of hormonal systems by environmental chemicals. The issue of environmental endocrine disruptors has become a focus of considerable media attention worldwide and is now on the agenda of many expert groups, panels, and steering committees of governmental organizations, industry, and academia in Europe, the United States, and Japan. Recently, several reports have suggested that endocrine disruptors also affect the central nervous systems.

In the present study, I investigated the relationship between prenatal and neonatal exposures to bisphenol-A and the dopaminergic neuron development. I found that prenatal and neonatal exposures to bisphenol-A could disrupt dopaminergic neuron development associated with astrocytic activation, and it may, at least in part, contribute

to the potentiation of the development of psychological dependence on drugs of abuse. On the endocrine disruptor problems, the low-dose actions of the endocrine disruptor are serious problems. I found that changes in the dopaminergic neurotransmissions were induced by prenatal and neonatal exposures to bisphenol-A at a dosage lower than the "Lowest Observed Adverse Effect Level."

On the other hand, I found that prenatal and neonatal exposures to low and high doses bisphenol-A also induced memory impairment associated with a dysfunction of the hippocampal cholinergic system.

Taken together, prenatal and neonatal exposures to bisphenol-A could disrupt the various neuron developments, resulting in long-lasting abnormalities in various neurotransmissions.

It is ironic that the chemicals made for a convenient life could be harmful to our health. I hope this research will be a warning about the problems of endocrine disruptors.

List of Publications

This dissertation is based on the following original publications:

1, Mayumi Miyatake, Kazuya Miyagawa, Keisuke Mizuo, Minoru Narita and Tsutomu Suzuki: Dynamic Changes in Dopaminergic Neurotransmission Induced by a Low Concentration of Bisphenol-A in Neurones and Astrocytes. *J Neuroendocrinol* **18**, 434-444 (2006): **Chapter 1 and 4**

2, Minoru Narita, Kazuya Miyagawa, Keisuke Mizuo, Takuya Yoshida, Tsutomu Suzuki: Changes in central dopaminergic systems and morphine reward by prenatal and neonatal exposure to bisphenol-A in mice: Evidence for the importance of exposure period. *Addict Biol* (in press): **Chapter 2**

3, Kazuya Miyagawa, Minoru Narita, Michiko Narita, Keiichi Niikura, Hisahiko Akama, Yuri Tsurukawa and Tsutomu Suzuki: Changes in central dopaminergic systems with the expression of Shh or GDNF in mice perinatally exposed to bisphenol-A. *Jpn J Neuropsychopharmacol* (in press): **Chapter 3**

4, Minoru Narita, Kazuya Miyagawa, Keisuke Mizuo, Takuya Yoshida and Tsutomu Suzuki: Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect. *Neurosci Lett* **402**, 249-252

(2006): **Chapter 4**

5, Kazuya Miyagawa, Minoru Narita, Michiko Narita, Hisahiko Akama and Tsutomu Suzuki: Memory impairment associated with a dysfunction of the hippocampal cholinergic system induced by prenatal and neonatal exposures to bisphenol-A. *Neurosci Lett* (in press): **Chapter 5**

Acknowledgements

This research will never be materialized without the help of the following people and organizations:

First, I would like to express my gratitude and appreciation to Professor Tsutomu Suzuki (Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences) and Associate Professor Minoru Narita (Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences) for their encouragement, helpful guidance in my research work and preparing this dissertation, and for giving a chance of this research work.

I would like to thank Mrs. Michiko Narita for her excellent technical assistance and helpful guidance in my research work.

Further, I would like to thank Dr. Masami Suzuki (Research Assistant, Department of Toxicology) and Dr. Yoshinori Yajima for helpful suggestions and valuable advice. Also I would like to thank Dr. Keisuke Mizuo and Dr. Mayumi Miyatake for their great technical assistance and helpful suggestion in my research work.

I wish to thank Mr. Keiichi Niikura, Mr. Takuya Yoshida and Mr. Hisahiko Akama for their technical assistance in my research work. Also, I wish to thank Ms. Kana Nanjyo, Ms. Sayaka Enomoto, Mr. Mamoru Sakata, Ms. Mariko Tomita, Ms. Nao Wako, Ms. Mayumi Nakajima, Ms. Saori Aoki and Ms. Yuri Tsurukawa for their technical assistance in my research work. Further, I wish to thank Mr. Satoshi Imai and Dr. Hideaki Kato for their stimulating discussions and kindly guidance in my research work.

Also, I wish to thank all of graduate and undergraduate students of Department of Toxicology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, especially Ms. Naoko Kuzumaki, Mr. Yasuyuki Nagumo and Mr. Kan Miyoshi for helpful guidance in my research work.

Finally, I would like to express my gratitude to my parents, grandparents, brother and friends for their assistance in my life.

References

1) Brotons J. A., Olea-Serrano M. F., Villalobos M., Pedraza V. and Olea N. Xenoestrogens released from lacquer coatings in food cans. *Environ Health Perspect* **103**, 608-12 (1995).

2) Howdeshell K. L., Peterman P. H., Judy B. M., Taylor J. A., Orazio C. E., Ruhlen R. L., Vom Saal F. S. and Welshons W. V. Bisphenol A is released from used polycarbonate animal cages into water at room temperature. *Environ Health Perspect* **111**, 1180-7 (2003).

3) Kang J. H., Kito K. and Kondo F. Factors influencing the migration of bisphenol A from cans. *J Food Prot* **66**, 1444-7 (2003).

4) Kang J. H. and Kondo F. Determination of bisphenol A in milk and dairy products by high-performance liquid chromatography with fluorescence detection. *J Food Prot* **66**, 1439-43 (2003).

5) Howdeshell K. L., Hotchkiss A. K., Thayer K. A., Vandenbergh J. G. and vom Saal F. S. Exposure to bisphenol A advances puberty. *Nature* **401**, 763-4 (1999).

6) Negishi T., Kawasaki K., Suzaki S., Maeda H., Ishii Y., Kyuwa S., Kuroda Y. and Yoshikawa Y. Behavioral alterations in response to fear-provoking stimuli and tranylcypromine induced by perinatal exposure to bisphenol A and nonylphenol in male rats. *Environ Health Perspect* **112**, 1159-64 (2004).

7) Suzuki T., Mizuo K., Nakazawa H., Funae Y., Fushiki S., Fukushima S., Shirai T. and Narita M. Prenatal and neonatal exposure to bisphenol-A enhances the

central dopamine D1 receptor-mediated action in mice: enhancement of the methamphetamine-induced abuse state. *Neuroscience* **117**, 639-44 (2003).

8) Mizuo K., Narita M., Miyagawa K., Okuno E. and Suzuki T. Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice. *Neurosci Lett* **356**, 95-8 (2004).

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

10) Bardo M. T. Neuropharmacological mechanisms of drug reward: beyond dopamine in the nucleus accumbens. *Crit Rev Neurobiol* **12**, 37-67 (1998).

11) Welzl H., Kuhn G. and Huston J. P. Self-administration of small amounts of morphine through glass micropipettes into the ventral tegmental area of the rat. *Neuropharmacology* **28**, 1017-23 (1989).

12) Phillips A. G. and LePiane F. G. Reinforcing effects of morphine microinjection into the ventral tegmental area. *Pharmacol Biochem Behav* **12**, 965-8 (1980).

13) Self D. W. and Stein L. Pertussis toxin attenuates intracranial morphine self-administration. *Pharmacol Biochem Behav* **46**, 689-95 (1993).

14) Maldonado R., Saiardi A., Valverde O., Samad T. A., Roques B. P. and Borrelli E. Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature* **388**, 586-9 (1997).

15) Ujike H., Onoue T., Akiyama K., Hamamura T. and Otsuki S. Effects of selective D-1 and D-2 dopamine antagonists on methamphetamine-induced

behavioral sensitization. *Seishin Shinkeigaku Zasshi* **90**, 636-41 (1988).

16) Riddle R. and Pollock J. D. Making connections: the development of mesencephalic dopaminergic neurons. *Brain Res Dev Brain Res* **147**, 3-21 (2003).

17) Smidt M. P., Smits S. M. and Burbach J. P. Molecular mechanisms underlying midbrain dopamine neuron development and function. *Eur J Pharmacol* **480**, 75-88 (2003).

18) Wallen A., Zetterstrom R. H., Solomin L., Arvidsson M., Olson L. and Perlmann T. Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice. *Exp Cell Res* **253**, 737-46 (1999).

19) Marti J., Wills K. V., Ghetti B. and Bayer S. A. A combined immunohistochemical and autoradiographic method to detect midbrain dopaminergic neurons and determine their time of origin. *Brain Res Brain Res Protoc* **9**, 197-205 (2002).

20) Bayer S. A., Wills K. V., Triarhou L. C. and Ghetti B. Time of neuron origin and gradients of neurogenesis in midbrain dopaminergic neurons in the mouse. *Exp Brain Res* **105**, 191-9 (1995).

21) Voorn P., Kalsbeek A., Jorritsma-Byham B. and Groenewegen H. J. The pre- and postnatal development of the dopaminergic cell groups in the ventral mesencephalon and the dopaminergic innervation of the striatum of the rat. *Neuroscience* **25**, 857-87 (1988).

22) Lee S. H., Lumelsky N., Studer L., Auerbach J. M. and McKay R. D. Efficient

generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nat Biotechnol* **18**, 675-9 (2000).

23) Lin L. F., Doherty D. H., Lile J. D., Bektesh S. and Collins F. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130-2 (1993).

24) Prakash N. and Wurst W. Development of dopaminergic neurons in the mammalian brain. *Cell Mol Life Sci* **63**, 187-206 (2006).

25) Zetterstrom R. H., Solomin L., Mitsiadis T., Olson L. and Perlmann T. Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1. *Mol Endocrinol* **10**, 1656-66 (1996).

26) Parpura V., Basarsky T. A., Liu F., Jeftinija K., Jeftinija S. and Haydon P. G. Glutamate-mediated astrocyte-neuron signalling. *Nature* **369**, 744-7 (1994).

27) Li A., Guo H., Luo X., Sheng J., Yang S., Yin Y. and Zhou J. Apomorphine-induced activation of dopamine receptors modulates FGF-2 expression in astrocytic cultures and promotes survival of dopaminergic neurons. *Faseb J* **20**, 1263-5 (2006).

28) Johansson S. and Stromberg I. Guidance of dopaminergic neuritic growth by immature astrocytes in organotypic cultures of rat fetal ventral mesencephalon. *J Comp Neurol* **443**, 237-49 (2002).

29) Little A. R. and O'Callagha J. P. Astrogliosis in the adult and developing CNS: is there a role for proinflammatory cytokines? *Neurotoxicology* **22**, 607-18

(2001).

30) Raivich G., Bohatschek M., Kloss C. U., Werner A., Jones L. L. and Kreutzberg G. W. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res Brain Res Rev* **30**, 77-105 (1999).

31) Nestler E. J. Molecular neurobiology of addiction. *Am J Addict* **10**, 201-17 (2001).

32) Hyman S. E. and Malenka R. C. Addiction and the brain: the neurobiology of compulsion and its persistence. *Nat Rev Neurosci* **2**, 695-703 (2001).

33) Narita M., Miyatake M., Shibasaki M., Tsuda M., Koizumi S., Yajima Y., Inoue K. and Suzuki T. Long-lasting change in brain dynamics induced by methamphetamine: enhancement of protein kinase C-dependent astrocytic response and behavioral sensitization. *J Neurochem* **93**, 1383-92 (2005).

34) Cyr M., Calon F., Morissette M. and Di Paolo T. Estrogenic modulation of brain activity: implications for schizophrenia and Parkinson's disease. *J Psychiatry Neurosci* **27**, 12-27 (2002).

35) McEwen B. S. and Alves S. E. Estrogen actions in the central nervous system. *Endocr Rev* **20**, 279-307 (1999).

36) Krishnan A. V., Stathis P., Permuth S. F., Tokes L. and Feldman D. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**, 2279-86 (1993).

37) Fellin T. and Carmignoto G. Neurone-to-astrocyte signalling in the brain

represents a distinct multifunctional unit. *J Physiol* **559**, 3-15 (2004).

38) Haydon P. G. GLIA: listening and talking to the synapse. *Nat Rev Neurosci* **2**, 185-93 (2001).

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

40) Pacheco M. A. and Jope R. S. Comparison of [3H]phosphatidylinositol and [3H]phosphatidylinositol 4,5-bisphosphate hydrolysis in postmortem human brain membranes and characterization of stimulation by dopamine D1 receptors. *J Neurochem* **69**, 639-44 (1997).

41) Jin L. Q., Goswami S., Cai G., Zhen X. and Friedman E. SKF83959 selectively regulates phosphatidylinositol-linked D1 dopamine receptors in rat brain. *J Neurochem* **85**, 378-86 (2003).

42) Zhu W. H., Conforti L. and Millhorn D. E. Expression of dopamine D2 receptor in PC-12 cells and regulation of membrane conductances by dopamine. *Am J Physiol* **273**, C1143-50 (1997).

43) Takeuchi Y., Fukunaga K. and Miyamoto E. Activation of nuclear Ca(2+)/calmodulin-dependent protein kinase II and brain-derived neurotrophic factor gene expression by stimulation of dopamine D2 receptor in transfected NG108-15 cells. *J Neurochem* **82**, 316-28 (2002).

44) Surmeier D. J., Eberwine J., Wilson C. J., Cao Y., Stefani A. and Kitai S. T.

Dopamine receptor subtypes colocalize in rat striatonigral neurons. *Proc Natl Acad Sci U S A* **89**, 10178-82 (1992).

45) Mizuo K., Narita M., Miyatake M. and Suzuki T. Enhancement of dopamine-induced signaling responses in the forebrain of mice lacking dopamine D3 receptor. *Neurosci Lett* **358**, 13-6 (2004).

46) Petersen D. N., Tkalcevic G. T., Koza-Taylor P. H., Turi T. G. and Brown T. A. Identification of estrogen receptor beta2, a functional variant of estrogen receptor beta expressed in normal rat tissues. *Endocrinology* **139**, 1082-92 (1998).

47) Nagel S. C., vom Saal F. S., Thayer K. A., Dhar M. G., Boechler M. and Welshons W. V. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ Health Perspect* **105**, 70-6 (1997).

48) Quesada I., Fuentes E., Viso-Leon M. C., Soria B., Ripoll C. and Nadal A. Low doses of the endocrine disruptor bisphenol-A and the native hormone 17beta-estradiol rapidly activate transcription factor CREB. *Faseb J* **16**, 1671-3 (2002).

49) Mohri T. and Yoshida S. Estrogen and bisphenol A disrupt spontaneous [Ca(2+)](i) oscillations in mouse oocytes. *Biochem Biophys Res Commun* **326**, 166-73 (2005).

50) Sato K., Matsuki N., Ohno Y. and Nakazawa K. Estrogens inhibit l-glutamate uptake activity of astrocytes via membrane estrogen receptor alpha. *J Neurochem* **86**, 1498-505 (2003).

51) Chaban V. V., Lakhter A. J. and Micevych P. A membrane estrogen receptor mediates intracellular calcium release in astrocytes. *Endocrinology* **145**, 3788-95 (2004).

52) Hosli E., Jurasin K., Ruhl W., Luthy R. and Hosli L. Colocalization of androgen, estrogen and cholinergic receptors on cultured astrocytes of rat central nervous system. *Int J Dev Neurosci* **19**, 11-9 (2001).

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

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

55) Suzuki T., Funada M., Narita M., Misawa M. and Nagase H. Pertussis toxin abolishes mu- and delta-opioid agonist-induced place preference. *Eur J Pharmacol* **205**, 85-8 (1991).

56) Narita M., Funada M. and Suzuki T. Regulations of opioid dependence by opioid receptor types. *Pharmacol Ther* **89**, 1-15 (2001).

57) Stinus L., Nadaud D., Jauregui J. and Kelley A. E. Chronic treatment with five different neuroleptics elicits behavioral supersensitivity to opiate infusion into the nucleus accumbens. *Biol Psychiatry* **21**, 34-48 (1986).

58) Koob G. F. Drugs of abuse: anatomy, pharmacology and function of reward

pathways. *Trends Pharmacol Sci* **13**, 177-84 (1992).

59) Wise R. A. and Rompre P. P. Brain dopamine and reward. *Annu Rev Psychol* **40**, 191-225 (1989).

60) Phillips A. G., LePiane F. G. and Fibiger H. C. Dopaminergic mediation of reward produced by direct injection of enkephalin into the ventral tegmental area of the rat. *Life Sci* **33**, 2505-11 (1983).

61) Shippenberg T. S., Bals-Kubik R. and Herz A. Examination of the neurochemical substrates mediating the motivational effects of opioids: role of the mesolimbic dopamine system and D-1 vs. D-2 dopamine receptors. *J Pharmacol Exp Ther* **265**, 53-9 (1993).

62) Funada M., Suzuki T., Sugano Y., Tsubai M., Misawa M., Ueda H. and Misu Y. Role of beta-adrenoceptors in the expression of morphine withdrawal signs. *Life Sci* **54**, PL113-8 (1994).

63) Maldonado R., Dauge V., Feger J. and Roques B. P. Chronic blockade of D2 but not D1 dopamine receptors facilitates behavioural responses to endogenous enkephalins, protected by kelatorphan, administered in the accumbens in rats. *Neuropharmacology* **29**, 215-23 (1990).

64) Sokoloff P., Giros B., Martres M. P., Bouthenet M. L. and Schwartz J. C. Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. *Nature* **347**, 146-51 (1990).

65) Suzuki T., Maeda J., Funada M. and Misawa M. The D3-receptor agonist (+/-)-7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OH-DPAT) attenuates morphine-

induced hyperlocomotion in mice. *Neurosci Lett* **187**, 45-8 (1995).

66) De Boer P., Enrico P., Wright J., Wise L. D., Timmerman W., Moor E., Dijkstra D., Wikstrom H. V. and Westerink B. H. Characterization of the effect of dopamine D3 receptor stimulation on locomotion and striatal dopamine levels. *Brain Res* **758**, 83-91 (1997).

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

68) Farabollini F., Porrini S., Della Seta D., Bianchi F. and Dessi-Fulgheri F. Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. *Environ Health Perspect* **110 Suppl 3**, 409-14 (2002).

69) Temple S. The development of neural stem cells. *Nature* **414**, 112-7 (2001).

70) Narita M., Miyagawa K., Mizuo K., Yoshida T. and Suzuki T. Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect. *Neurosci Lett* **402**, 249-52 (2006).

71) Narita M., Mizoguchi H., Suzuki T., Dun N. J., Imai S., Yajima Y., Nagase H. and Tseng L. F. Enhanced mu-opioid responses in the spinal cord of mice lacking protein kinase Cgamma isoform. *J Biol Chem* **276**, 15409-14 (2001).

72) Kim T. E., Lee H. S., Lee Y. B., Hong S. H., Lee Y. S., Ichinose H., Kim S. U. and Lee M. A. Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem cell. *Biochem Biophys Res*

Commun **305**, 1040-8 (2003).

73) Du F., Li R., Huang Y., Li X. and Le W. Dopamine D3 receptor-preferring agonists induce neurotrophic effects on mesencephalic dopamine neurons. *Eur J Neurosci* **22**, 2422-30 (2005).

74) Pasti L., Volterra A., Pozzan T. and Carmignoto G. Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. *J Neurosci* **17**, 7817-30 (1997).

75) Tang X. C., McFarland K., Cagle S. and Kalivas P. W. Cocaine-induced reinstatement requires endogenous stimulation of mu-opioid receptors in the ventral pallidum. *J Neurosci* **25**, 4512-20 (2005).

76) Austin M. C. and Kalivas P. W. Blockade of enkephalinergic and GABAergic mediated locomotion in the nucleus accumbens by muscimol in the ventral pallidum. *Jpn J Pharmacol* **50**, 487-90 (1989).

77) Knaak J. B. and Sullivan L. J. Metabolism of bisphenol A in the rat. *Toxicol Appl Pharmacol* **8**, 175-84 (1966).

78) Volkel W., Colnot T., Csanady G. A., Filser J. G. and Dekant W. Metabolism and kinetics of bisphenol a in humans at low doses following oral administration. *Chem Res Toxicol* **15**, 1281-7 (2002).

79) vom Saal F. S., Cooke P. S., Buchanan D. L., Palanza P., Thayer K. A., Nagel S. C., Parmigiani S. and Welshons W. V. A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicol Ind Health* **14**, 239-60

(1998).

80) Kloas W., Lutz I. and Einspanier R. Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo. *Sci Total Environ* **225**, 59-68 (1999).

81) Lee S. H. and Mouradian M. M. Up-regulation of D1A dopamine receptor gene transcription by estrogen. *Mol Cell Endocrinol* **156**, 151-7 (1999).

82) Vom Saal F. S. and Hughes C. An extensive new literature concerning low-dose effects of bisphenol a shows the need for a new risk assessment. *Environ Health Perspect* **113**, 926-33 (2005).

83) Bilkei-Gorzo A., Gyertyan I. and Levay G. mCPP-induced anxiety in the light-dark box in rats--a new method for screening anxiolytic activity. *Psychopharmacology (Berl)* **136**, 291-8 (1998).

84) Schramm N. L., McDonald M. P. and Limbird L. E. The alpha(2a)-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety. *J Neurosci* **21**, 4875-82 (2001).

85) Bartus R. T., Dean R. L., 3rd, Beer B. and Lippa A. S. The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217**, 408-14 (1982).

86) Dutar P., Bassant M. H., Senut M. C. and Lamour Y. The septohippocampal pathway: structure and function of a central cholinergic system. *Physiol Rev* **75**, 393-427 (1995).

87) Miyamoto M., Kato J., Narumi S. and Nagaoka A. Characteristics of memory impairment following lesioning of the basal forebrain and medial septal nucleus

in rats. *Brain Res* **419**, 19-31 (1987).

88) Gould E., Woolley C. S., Frankfurt M. and McEwen B. S. Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *J Neurosci* **10**, 1286-91 (1990).

89) Prange-Kiel J. and Rune G. M. Direct and indirect effects of estrogen on rat hippocampus. *Neuroscience* **138**, 765-72 (2006).

90) McKinney M. and Kent C. Differential expression of GAP-43 mRNA in adult central cholinergic neuronal populations. *Brain Res Mol Brain Res* **23**, 213-20 (1994).

91) Lustig R. H., Sudol M., Pfaff D. W. and Federoff H. J. Estrogenic regulation and sex dimorphism of growth-associated protein 43 kDa (GAP-43) messenger RNA in the rat. *Brain Res Mol Brain Res* **11**, 125-32 (1991).

92) Li Z., Yang R. and Chen Z. [Effects of iodine and thyroid hormone deficiency during brain development on activities of cholinergic neurone-related enzymes in central nervous system of rats]. *Zhonghua Yu Fang Yi Xue Za Zhi* **30**, 337-9 (1996).

93) Juarez de Ku L. M., Sharma-Stokkermans M. and Meserve L. A. Thyroxine normalizes polychlorinated biphenyl (PCB) dose-related depression of choline acetyltransferase (ChAT) activity in hippocampus and basal forebrain of 15-day-old rats. *Toxicology* **94**, 19-30 (1994).

94) Donahue D. A., Dougherty E. J. and Meserve L. A. Influence of a combination of two tetrachlorobiphenyl congeners (PCB 47; PCB 77) on thyroid status,

choline acetyltransferase (ChAT) activity, and short- and long-term memory in 30-day-old Sprague-Dawley rats. *Toxicology* **203**, 99-107 (2004).

95) Pottenger L. H., Domoradzki J. Y., Markham D. A., Hansen S. C., Cagen S. Z. and Waechter J. M., Jr. The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. *Toxicol Sci* **54**, 3-18 (2000).

96) Snyder R. W., Maness S. C., Gaido K. W., Welsch F., Sumner S. C. and Fennell T. R. Metabolism and disposition of bisphenol A in female rats. *Toxicol Appl Pharmacol* **168**, 225-34 (2000).

97) Matthews J. B., Twomey K. and Zacharewski T. R. In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta. *Chem Res Toxicol* **14**, 149-57 (2001).