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Nanomolar dose of bisphenol A rapidly modulates spinogenesis in adult hippocampal neurons

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ABSTRACT

We demonstrated the rapid effects of 10 nM bisphenol A (BPA) on the spinogenesis of adult rat hippocampal slices. The density of spines was analyzed by imaging Lucifer Yellow-injected CA1 neurons in slices. Not only the total spine density but also the head diameter distribution of spine was quantitatively analyzed. Spinogenesis was significantly enhanced by BPA within 2 h. In particular, the density of middle-head spine (with head diameter of 0.4–0.5 μm) was significantly increased.

Hydroxytamoxifen, an antagonist of both estrogen-related receptor gamma (ERRγ) and estrogen receptors (ERα/ERβ), blocked the BPA-induced enhancement of the spine density. However, ICI 182,780, an antagonist of ERα/ERβ, did not suppress the BPA effects. Therefore, ERRγ is deduced to be a high affinity receptor of BPA, responsible for modulation of spinogenesis. The BPA-induced enhancement of spinogenesis was also suppressed by MAP kinase inhibitor, PD98059, and the blocker of NMDA receptors, MK-801. Washout of BPA for additional 2 h after 2 h BPA treatment abolished the BPA-induced enhancement of spinogenesis, suggesting that the BPA effect was reversible. ERRγ was localized at synapses as well as cell bodies of principal neurons. ERRγ at synapses may contribute to the observed rapid effect. The level of BPA in the hippocampal slices was determined by mass-spectrometric analysis.

1. Introduction

Low dose exposure to bisphenol A (BPA) may induce hormone-like effects on wildlife and humans. BPA is a widely used synthetic material included in polycarbonate resin used in water pipe sealant, dental prostheses, compact discs and baby bottles. Toxic effects of high dose BPA (mg/kg weight/day) have been investigated in relation to the development and functions of the reproduction systems (Fisher et al., 1999; Al-Hiyasat et al., 2002; Grote et al., 2004; Halldin et al., 2005). However, the low dose exposure to BPA (μg/kg/day or nanomolar doses) shows rather weak toxic effects on reproductive or endocrine functions in the peripheral tissues, probably due to the efficient detoxification of BPA by the liver. On the other hand, low dose exposure to BPA may significantly affect the brain function, because the detoxification of BPA in the brain is probably very weak, due to the extremely low expression of drug-metabolizing enzymes in the brain (Miksys and Tyndale, 2002; Kishimoto et al., 2004; Chinta et al., 2005).

The low dose exposure to BPA during fetal/neonatal stages has been extensively investigated. For example, fetal or neonatal exposure to BPA inhibits sexual differentiation of nonreproductive behaviors of adult animals, including maze learning behavior (Carr et al., 2003; Kubo et al., 2003; Fujimoto et al., 2006), at doses as low as 1/1000 of those required for the stimulation of uterine growth (Ashby, 2001). On the other hand, inadequate information is available for the low dose exposure to BPA in the adult stage, except some pioneer works *in vivo* (MacLusky et al., 2005; Leranth et al., 2008; Hajszan and Leranth, 2010).

The high affinity functional receptor for BPA has not been identified yet. Although ERα is one candidate of BPA receptor, the

Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BPA, bisphenol A; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DG, dentate gyrus; E2, 17β-estradiol; ER, estrogen receptor; ERRγ, estrogen-related receptor gamma; ICI, ICI182,780; MR, median raphe; MSDB, medial septum/diagonal band of Broca; NMDA, *N*-methyl-D-aspartate; OH-Tam, 4-hydroxytamoxifen; OVX, ovariectomized; PSD, postsynaptic density; PY, pyramidal neurons; SUM, supramammillary area.

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affinity of ERα for BPA is very low, in the order of 1/100–1/1000 of that for 17β-estradiol (E2) (Kuiper et al., 1997; Morohoshi et al., 2005). On the other hand, estrogen-related receptor gamma (ERRγ) is a high affinity binding protein for BPA (Takayanagi et al., 2006). However, ERRγ has not been recognized as a BPA receptor, because ERRγ shows constitutive transcriptional activity even without any ligand (Coward et al., 2001).

We here performed the investigation on the rapid modulation by nanomolar doses of BPA on the density and morphology of dendritic spines in the adult hippocampal slices, including investigations of BPA receptors. In order to observe the direct effects of BPA on hippocampal neurons, we used isolated 'acute' hippocampal slices which do not have projections of cholinergic or serotonergic neurons from outside of the hippocampus.

2. Materials and methods

2.1. Chemicals

BPA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), cycloheximide, nicardipine, *N*-methyl-D-aspartate (NMDA), PD98059, ICI 182,780 (ICI), MK-801 and Lucifer Yellow were purchased from Sigma (USA). 4-hydroxy-tamoxifen (OH-Tam) was from Calbiochem (Germany). Other chemicals used were of highest purity commercially available. Polyclonal anti-ERRγ antibody (against C-terminal of ligand binding site) was prepared by Dr. Shimohigashi at Kyushu Univ. (Tokunaga et al., 2006).

2.2. Animals

Adult male Wistar rats (12 weeks old, 340–360 g) were purchased from Saitama Experimental Animal Supply (Saitama, Japan). The experimental procedure of this research was approved by the Committee for Animal Research of the University of Tokyo.

2.3. Preparation of 'acute' hippocampal slices

Rats were deeply anesthetized with ethyl ether and decapitated. The brains from adult rats were removed and placed at 4 °C in artificial cerebrospinal fluid (ACSF) consisted of (mM): 124 NaCl, 5.0 KCl, 1.25 NaH_2PO_4, 2.0 $MgSO_4$, 2.0 $CaCl_2$, 22 $NaHCO_3$, 10 glucose and was equilibrated with 95% O_2/5% CO_2. The hippocampus was dissected and 300 μm-thick transverse dorsal 'fresh' slices to the long axis were prepared with a vibratome (Dosaka EM, Kyoto, Japan). 'Acute' slices were prepared from these 'freshly prepared' slices by 2 h recovery incubation at 25 °C in ACSF.

2.4. Imaging and analysis of spinogenesis

Experimental details are described in elsewhere (Mukai et al., 2007).

2.4.1. Current injection of Lucifer Yellow

'Acute' slices were further incubated with BPA in the presence or absence of other drugs such as OH-Tam or PD98059. Drug-treated slices were then prefixed with 4% paraformaldehyde in PBS (0.1 M phosphate buffer and 0.14 M NaCl, pH 7.3) at 4 °C for 2–4 h.

Neurons within slices were visualized by an injection of Lucifer Yellow under a Nikon E600FN microscope (Japan) equipped with a C2400–79H infrared camera (Hamamatsu Photonics, Japan) and with a 40× water immersion lens (Nikon). A glass electrode was filled with 5% Lucifer Yellow, which was then injected for 15 min using Axopatch 200B (Axon Instruments, USA). Approximately five neurons within a 100–200 μm depth from the surface of a slice were injected (Duan et al., 2002). After injection, slices were fixed again with 4% paraformaldehyde at 4 °C overnight.

2.4.2. Confocal laser microscopy and morphological analysis

The imaging was performed from sequential *z*-series scans with LSM5 PASCAL confocal microscope (Zeiss, Germany). For analysis of spines, three-dimensional images were constructed from approximately 40 sequential *z*-series sections of neurons scanned every 0.45 μm with a 63× water immersion lens, NA 1.2 (Zeiss). For Lucifer Yellow, the excitation and emission wavelengths were 488 nm and 515 nm, respectively. The applied zoom factor (3.0) yielded 23 pixels per 1 μm. The *z*-axis resolution was approximately 0.71 μm. The confocal lateral resolution was approximately 0.26 μm. Confocal images were then deconvoluted using AUTODEBLUR software (AutoQuant, USA). The density of spine as well as the head diameter was analyzed with Spiso-3D (automated software mathematically calculating geometrical parameters of spines) developed by Bioinformatics Project of Kawato's group (Mukai et al., 2011). Results obtained by Spiso-3D are almost identical to those by Neurolucida (manual-based analysis software) (MicroBrightField, USA) within assessment difference of 2%, and Spiso-3D considerably reduces human errors and experimental labor of manual software. We analyzed the secondary dendrites in the stratum radiatum, lying between 100 and 250 μm from the soma. The spine density was calculated from the number of spines on dendrites having a total length of 50–80 μm. In total, we investigated 3–4 rats, 6–8 slices, 12–16 neurons, 24–32 dendrites and 1200–2000 spines. Spine shapes were classified into three categories as follows. (1) A small-head spine whose head diameter is 0.2–0.4 μm. (2) A middle-head spine whose head diameter is 0.4–0.5 μm. (3) A large-head spine whose head diameter is 0.5–1.0 μm. These three categories were useful to distinguish different responses upon inhibitor application. Because the majority of spines (>95%) had a distinct head and neck, and stubby type and filopodium type spines did not contribute much to overall changes, we analyzed spines having a distinct head.

All protrusions from the dendrites were treated as 'spines', although with confocal microscopy, it was not possible to determine whether they formed synapses, or whether some of them were filopodia protrusions which did not form synapses (Sorra and Harris, 2000). While counting the spines in the reconstructed images, the position and verification of spines were aided by rotation of three-dimensional reconstructions and by observation of the images in consecutive single planes.

2.5. Immunohistochemical staining of hippocampal slices

Immunohistochemical staining of hippocampal slices was performed as described in elsewhere (Kimoto et al., 2001; Kawato et al., 2002) and Supplementary material. Staining of ERRγ was performed using the avidin–biotin peroxidase complex technique. After application of anti-ERRγ antibody (1/250), the slices were incubated for 24 h at 4 °C, in the presence of 0.5% Triton X-100 and 3% skim milk with gentle shaking.

For preabsorption of anti-ERRγ antibody with antigen, excess amount of antigen was preincubated with anti-ERRγ antibody for 15 h at 4 °C. Characterization of anti-ERRγ antibody is described in Supplementary material with Fig. S1.

2.6. Preparation of synaptic, cytoplasmic and nuclear fractions

Fractionation of the homogenates obtained from hippocampal slices was performed by a combination of centrifugations at 4 °C (Cohen et al., 1977). Detailed procedures to obtain the raft fraction, nuclear fraction, postsynaptic density (PSD) fraction, low density membrane fraction (presynaptic membrane-enriched fraction),