Bisphenol A (BPA) is an estrogenic chemical that is widely used in the manufacture of plastics and epoxy resins. Because BPA leaches out of plastic food and drink containers, as well as the BPA-containing plastics used in dental prostheses and sealants, considerable potential exists for human exposure to this compound. In this article we show that treatment of ovariectomized rats with BPA dose-dependently inhibits the estrogen-induced formation of dendritic spine synapses on pyramidal neurons in the CA1 area of the hippocampus. Significant inhibitory effects of BPA were observed at a dose of only 40 µg/kg, below the current U.S. Environmental Protection Agency reference daily limit for human exposure. Because synaptic remodeling has been postulated to contribute to the rapid effects of estrogen on hippocampus-dependent memory, these data suggest that environmental BPA exposure may interfere with the development and expression of normal sex differences in cognitive function, via inhibition of estrogen-dependent hippocampal synapse formation. It may also exacerbate the impairment of hippocampal function observed during normal aging, as endogenous estrogen production declines. **Key words:** bisphenol A, CA1, estradiol, hippocampus, spine synapse density. *Environ Health Perspect* 113:675–679 (2005). doi:10.1289/ehp.7633 available via http://dx.doi.org/ [Online 24 February 2005]

Natural and man-made chemicals in the environment can exert hormone mimetic or antagonist activity. Bisphenol A (BPA), a widely used chemical with mixed estrogenic properties, is employed in the manufacture of plastics and dental prostheses as well as nonhuman primates (Leranth et al. 2002), estradiol induces a rapid increase in hippocampal spine density (PSSD), a response that has been pos-

Sexual differentiation of the brain is believed to involve neurotrophic effects of estrogens, mediated at least in part via activation of kinase-dependent signaling cascades (Toran-Allerand et al. 1999). Kinase-associated neuroplastic responses to estrogen are also expressed in adulthood, in the cornu ammonis (CA) pyramidal neurons of the hippocampus (Bi et al. 2001; McEwen 2002). In adult female rats (Woolley and McEwen 1992) as well as nonhuman primates (Leranth et al. 2002), estradiol induces a rapid increase in CA1 pyramidal cell dendritic spine synapse density (PSSD), a response that has been postulated to involve intermediate activation of the mitogen-activated protein (MAP) kinase cascade (Bi et al. 2001). We reasoned that if BPA inhibits sexual differentiation of the rodent brain, there might also be significant interactions between estradiol and BPA with respect to the regulation of hippocampal PSSD in adult ovariectomized (OVX) rats. Our results indicate that BPA does indeed have potent effects on the regulation of CA1 PSSD. However, the data demonstrate that, rather than inducing estrogen-like responses, BPA antagonizes the rapid inductive effects of estrogen on hippocampal PSSD.

**Materials and Methods**

**Animals.** Experimental protocols were approved by the Institutional Animal Care and Use Committee of Yale University, where all studies using animals were performed. Adult female Sprague-Dawley rats (250–300 g; Charles River Laboratories, Wilmington, MA, USA) were used. The rats were ovariectomized under ketamine/xylazine/acepromazine anesthesia (3 mL/kg intramuscular injection of a cocktail containing 25 mg ketamine, 1.2 mg xylazine, and 0.03 mg acepromazine in 1 mL saline).

**Morphologic studies.** One week after ovariectomy, animals were treated with estrogen, using groups of three animals per treatment condition. In the first PSSD study, 15 rats (five groups of three rats) were injected subcutaneously with either 17β-estradiol (60 µg/kg; 12 rats) or the sesame oil vehicle (200 µL; three rats). Nine of the 12 estradiol-treated animals were treated simultaneously with increasing doses (40, 120, and 400 µg/kg) of BPA (> 99% purity; Sigma-Aldrich, St. Louis, MO, USA). In the second PSSD experiment, 12 rats were injected subcutaneously (three rats per treatment) with 17α-estradiol (45 µg/kg), BPA (300 µg/kg), a combination of 17α-estradiol (45 µg/kg) plus BPA (300 µg/kg), or the sesame oil vehicle (200 µL) alone. Thirty minutes after injection, animals were sacrificed under deep ether anesthesia by transcardial perfusion of heparinized saline followed by a

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fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.35). The brains were removed and postfixed overnight in the same fixative without glutaraldehyde. The hippocampi were then dissected out, and 100 µm vibratome sections were cut perpendicular to the longitudinal axis of the hippocampus. The approximately 90 vibratome sections were divided into 10 subgroups using systematic random sampling and were flat-embedded in Araldite (Electron Microscopy Sciences, Fort Washington, PA, USA).

To correct for processing-induced changes in the volume of the tissue, we calculated a correction factor assuming that the treatments did not alter the total number of pyramidal cells. In all hippocampi, we examined six or seven disector pairs (pairs of adjacent 2-µm semi-thin sections mounted on slides and stained with toluidine blue). We calculated a pyramidal cell density value (D) using the formula

\[ D = N/sT, \]

where \( N \) is the mean disector score across all sampling windows, \( s \) is the thickness of the sections (2 µm), and \( T \) is the length of the window. Based on these values, a dimensionless volume correction factor \( k_v \) was introduced:

\[ k_v = D/D_1, \]

where \( D_1 \) is the mean cell density across the groups of hippocampi (Rusu et al. 1997).

To exclude the possibility that alterations in PSSD might be a consequence of changes in the volume of reference, we used a subset of the vibratome sections for volume estimation of the stratum radiatum of CA1, using the Cavalieri’s principle (Gundersen and Jensen 1987). Areas of CA1 stratum radiatum were measured in each section using Scion Image software (Scion Corp., Frederick, MD, USA), and the total volume of CA1 stratum radiatum in each rat was estimated as

\[ V = T \times \sum_{90} A(\text{CA1}_90), \]

where \( T \) is the distance between the top of one sampled section and the top of the next section, and \( A(\text{CA1}_90) \) is the measured area of CA1 stratum radiatum for each section.

Thereafter, serial ultrathin sections were cut from randomly sampled vibratome sections and collected on formvar-coated single-slot grids. Disector pairs of digitized electron micrographs (“reference” and “look-up”) were taken from adjacent ultrasections at a magnification of 11,000× in a Tecnai 12 transmission electron microscope (FEI Company, Hillsboro, OR, USA) furnished with an AMT Advantage 4.00 HR/HR-B CCD camera system (Hamamatsu Photonics, Hamamatsu, Japan), from an area located between the upper and middle third of the CA1 stratum radiatum (300–500 µm from the pyramidal cell layer; Leranth et al. 2004). Identical regions in adjacent sections were identified using landmarks such as myelinated fibers, large dendrites, or blood vessels that did not change significantly between neighboring sections. The investigator taking the electron micrographs was blinded to the treatment of individual animals. Areas occupied by potentially interfering structures such as blood vessels, large dendrites, or glial cells were subtracted from the measured fields. The digitized electron micrographs were printed out using a laser printer and coded. The code was not broken until the analysis was completed. Synapses were counted using a two-dimensional unbiased counting frame with an area of 79 µm² superimposed on the electron microscopic prints. Only those spine synapses were counted that were present in the reference micrograph but not in the look-up micrograph, and vice versa. At least 10 disector pairs were photographed on each electron microscopic grid. With at least three grids (containing two adjacent ultrathin sections) prepared from each vibratome section (cut from three different regions of the hippocampus along its longitudinal septotemporal axis), each animal provided at least \( 3 \times 3 \times 10 = 180 \) neuropil fields for evaluation. The density of spine synapses in each animal was calculated as

\[ N_v(syn) = \frac{\Sigma Q(syn)}{2 \times \Sigma 4 \times t} = \frac{\Sigma Q(syn)}{2 \times 90 \times 79 \times t}, \]

where \( \Sigma Q(syn) \) is the total number of synapses sampled by the disector; \( 2 \times 90 = 180 \) is the number of evaluated electron micrographs per animal; the section thickness \( t \) was measured.

Figure 1. BPA inhibits the effect of 17β-E2 on CA1 PSSD. (A) At 30 min after 17β-E2 injection, PSSD increased by almost 100%. (B) The PSSD response to 17β-E2 is inhibited in a dose-dependent manner by coadministration of BPA. Data in all cases represent mean ± SD of independent observations from three rats at each dose level. In the case of the 120-µg/kg dose, the bars indicating SD are so close to the mean that they are partially obscured by the symbol. The line through the points represents the four-parameter logistic best-fit regression analysis of the data. The ED50 for BPA inhibition of the 17β-estradiol–induced increase in PSSD, calculated from the four-parameter curve fit, is 117 µg/kg.

*Significantly different from oil-injected controls (p < 0.001, Student t-test). **Significantly different from PSSD in animals treated with 17β-E2 alone (Bonferroni-Dunn post hoc test, p < 0.05).

Figure 2. High-power electron micrographs taken from the CA1 stratum radiatum of rats treated with either (A) 60 µg/kg 17β-E2 or (B) 60 µg/kg 17β-E2 + 400 µg/kg BPA. Arrows indicate spine synapses. Bar = 500 nm.
by the method of Small’s smallest fold (Weibel 1979; average 0.075 µm); and 79 is the area of the counting frame in square micrometers. PSSD for each animal was calculated by dividing \( N_s \) (syn) by the volume correction factor \( k_v \).

**Rat uterine weight assay.** To assess uterotropic responses, a separate group of 12 rats (four groups of 3) was treated 1 week after ovariectomy with subcutaneous injections of 17\( \beta \)-E\(_2\) (60 µg/kg), BPA (400 µg/kg), or the combination of BPA (400 µg/kg) and 17\( \beta \)-E\(_2\) (60 µg/kg), daily for 3 days. Control animals received the sesame oil vehicle (200 µL/day) alone. Six hours after the last injection, the animals were sacrificed; their uteri were dissected free of adhering fat and connective tissue, drained of intraluminal fluid, and weighed.

**Data analysis.** Results, in all cases, are presented as mean ± SD of observations from three animals per treatment group. We have verified that the use of three animals per treatment group provides sufficient statistical power to detect effects of the magnitude typically observed after steroid replacement, because of the precision obtained by analyzing large numbers of sections per animal. SDs for counting CA1 synapses in this laboratory are usually < 5% of mean PSSD. With an SD of 5% and sample sizes of three per group, a 15% change in mean PSSD can be detected with \( \alpha = 0.05 \) and 80% power. In the present studies, SDs for measurement of PSSD were in most instances considerably < 5% of mean.

Statistical analysis. Results, in all cases, are presented as mean ± SD of observations from three animals per treatment group. We have verified that the use of three animals per treatment group provides sufficient statistical power to detect effects of the magnitude typically observed after steroid replacement, because of the precision obtained by analyzing large numbers of sections per animal. SDs for counting CA1 synapses in this laboratory are usually < 5% of mean PSSD. With an SD of 5% and sample sizes of three per group, a 15% change in mean PSSD can be detected with \( \alpha = 0.05 \) and 80% power. In the present studies, SDs for measurement of PSSD were in most instances considerably < 5% of mean.

Data were analyzed statistically using Statview (SAS Institute, Cary, NC, USA) or SPSS for Windows (Systat Inc., Chicago, IL, USA). We used Bartlett’s test to verify homogeneity of variance. One- and two-way analysis of variance (ANOVA) and the Bonferroni-Dunn test were used for comparison of individual treatment groups. When only control versus treatment comparisons were considered, we used the Student t-test. Four-parameter least-squares regression analysis of the BPA dose-response data was performed using ALLFIT (DeLean et al. 1978).

**Results**

Treatment of OVX rats with 17\( \beta \)-E\(_2\) (60 µg/kg body weight) increased CA1 PSSD almost 2-fold (Figure 1A). This dose of 17\( \beta \)-E\(_2\) has previously been shown to induce a maximal PSSD response (MacLusky et al. 2005). Treatment with BPA did not further enhance hippocampal synapse formation but dose-dependently inhibited the effect of 17\( \beta \)-E\(_2\).

![Figure 3. BPA administration only slightly impairs the uterotropic response to 17\( \beta \)-E\(_2\). Two-way ANOVA: 17\( \beta \)-E\(_2\) effect, \( F = 301.2 \), df 1, 8, \( p < 0.0001 \); BPA effect, \( F = 11.1 \), df 1, 8, \( p = 0.01 \); 17\( \beta \)-E\(_2\) × BPA interaction, \( F = 0.29 \), df 1, 8, \( p > 0.6 \).](image)

![Figure 4. BPA inhibits the effects of 17\( \alpha \)-E\(_2\) on CA1 PSSD. In the absence of BPA, 17\( \alpha \)-E\(_2\) induced an increase in synapse density of 0.463 synapses/\( \mu m^2 \), a 65% increase above the mean synapse density in vehicle-injected controls. In the presence of BPA, the effect of the estrogen was reduced to an increase of 0.192 synapses/\( \mu m^2 \), 39% above the level observed in animals treated with BPA alone. Two-way ANOVA: 17\( \alpha \)-E\(_2\) effect, \( F = 237.2 \), df 1, 8, \( p < 0.0001 \); BPA effect, \( F = 292.8 \), df 1, 8, \( p < 0.0001 \); 17\( \alpha \)-E\(_2\) × BPA interaction, \( F = 40.8 \), df 1, 8, \( p = 0.0002 \). *Significantly higher PSSD than in vehicle-treated rats, without BPA (Student t-test, \( p < 0.05 \)). **Significantly inhibitory effect of BPA compared with animals given the same vehicle or 17\( \alpha \)-E\(_2\) without BPA (Bonferroni-Dunn post hoc test, \( p < 0.05 \)).](image)

**Discussion**

Our data indicate that low-dose BPA exposure inhibits the rapid hippocampal synaptogenic response to estradiol. The minimum BPA dose required to elicit this effect is within the range of dose levels believed, until now, to have little or no significant biologic impact, even under conditions of long-term BPA exposure. In the United States, the current U.S. Environmental Protection Agency (EPA) maximum acceptable “reference” dose for chronic BPA ingestion is 50 µg/kg/day, calculated as 0.1% of the lowest observed adverse effect level (LOAEL) determined from toxicity studies (U.S. EPA 1993). The corresponding
European Commission tolerable daily intake (TDI; 10 µg/kg/day) is based on the assumption of a 500-fold safety margin over the no observed effect level (NOEL) dose derived from three-generation rat reproductive toxicity trials (EC Scientific Committee on Food 2002). In OVX rats, our data indicate that a single BPA dose of 40 µg/kg, below the U.S. EPA reference dose and only 4-fold higher than the European Commission TDI safe daily limit, is sufficient to significantly impair the PSSD response to maximal 17β-E2 stimulation. Under conditions of low physiologic estrogen exposure, as is the case during prepubertal development as well as after reproductive senescence, considerably lower doses of BPA may be sufficient to interfere with the synaptogenic effects of the hormone. Circumstantial evidence supporting the view that the effects of BPA are not confined to rapid PSSD responses to estrogen administration is provided by the data for OVX rats. Even without estrogen treatment, in OVX rats BPA significantly reduced CA1 PSSD (Figure 4). Preliminary data from our laboratories indicate that the “baseline” PSSD observed in OVX rats includes a contribution from the phytoestrogens present in normal rat chow. Removal of these estrogens, by feeding with phytoestrogen-free chow, reduces CA1 PSSD to levels comparable with those observed in the present study after treatment of OVX animals with BPA (Leranth C, Hajszán T, MacLusky NJ, unpublished data).

Estradiol has important neurotrophic and neuroprotective functions in the brain, in addition to its role as a reproductive steroid (Nathan et al. 2004). A growing body of evidence indicates that estradiol is synthesized in the hippocampus (Hojo et al. 2004), providing a local source of estrogen onto which the effects of circulating levels of the hormone are superimposed. Because synapse formation in the hippocampus is believed to be involved in the mechanisms mediating the acquisition and retention of memory (Silva 2003), interference with estrogen action in the hippocampus could have serious long-term consequences. Deficiencies in gonadal steroid-induced stimulation of hippocampal synaptogenesis have been suggested to contribute to neurodegenerative disorders and age-related cognitive impairment, for which women with low bioavailable circulating estradiol concentrations appear to be at enhanced risk (Gandy 2003; Yaffe et al. 2000). The ability of BPA to block the effects of estrogen on CA1 PSSD raises the possibility that chronic environmental exposure to BPA might interfere with estrogen effects on the development and function of the brain, inhibiting normal sex differences in nonreproductive behavior (Carr et al. 2003; Farabollini et al. 2002) as well as exacerbating the negative impact on the aging brain of declining gonadal hormone levels (Gandy 2003; Yaffe et al. 2000). Although it remains to be determined whether such effects have a significant impact on the health of human and animal populations exposed to BPA, the current exposure limits clearly do not provide a wide margin of safety in terms of the acute estrogen-dependent regulation of CA1 PSSD.

The mechanisms responsible for BPA’s effects remain unknown. BPA interacts with a number of hormone receptor systems, including androgen and thyroid receptors as well as ER-α and ER-β (Moriyama et al. 2002; Wetherell et al. 2002; Zoeller et al. 2005), providing several potential pathways through which BPA could interfere with hippocampal synaptogenesis. Recent work has demonstrated that BPA and 17β-E2 are equipotent activators of CREB phosphorylation in pancreatic islet cells, a response mediated via a “nonclassical” membrane ER (Quesada et al. 2002). Although the in vitro equilibrium binding affinities of ER-α and ER-β for BPA are low (Kuiper et al. 1998a), this does not preclude the possibility that BPA could act via these ERs as well because rapid membrane ER-mediated responses may not reflect equilibrium binding affinity. ER-α and ER-β both partially localize to the plasma membrane, where they mediate activation of kinase-dependent signaling pathways. Induction of these rapid kinase-mediated mechanisms exhibits a different pharmacologic specificity than do nuclear receptor–activated responses. Thus, activation of extracellular-signal–regulated kinase (ERK) phosphorylation in rat-2 cells transfected with ER-α or ER-β is equally sensitive to 17α-E1 and 17β-E1 (Wade et al. 2001), despite the large difference that exists between these steroids in nuclear ER-α and ER-β equilibrium binding affinity (Kuiper et al. 1998b) and urotropic activity (Lundeen et al. 1997). Studies in bone cells and ER-transfected HeLa cells suggest that rapid membrane receptor–activated responses to estrogen have a much broader ligand specificity than do slower nuclear receptor–mediated transcriptional effects because ER ligand association rates tend to have a much more relaxed structural specificity than do dissociation rates. Therefore, ligands that are incapable of forming stable nuclear ER complexes because they dissociate rapidly from the receptor may, nonetheless, modulate membrane ER-mediated effects (Kousteni et al. 2001).

Circumstantial evidence points to a role for nonclassic estrogen mechanisms in the hippocampal response to estrogen. Effects of 17β-E2 on CA1 dendritic structure are accompanied by increased ERK phosphorylation (Bi et al. 2001), as well as changes in the distribution of the phosphorylated form of the serine-threonine kinase Akt in CA1 pyramidal cell dendrites (Znamensky et al. 2003). The fact that 17α-E2 and 17β-E2 both induce an increase in PSSD is consistent with the hypothesis that membrane-associated ERs may mediate rapid estrogen activation of CA1 spine synapse formation (Wade et al. 2001). That the rapid actions of estradiol on CA1 PSSD involve nonclassical ER systems is also suggested by recent data from this laboratory demonstrating that short-term induction of CA1 spine synapses requires relatively high circulating 17β-E2 concentrations (MacLusky et al. 2005). The effects of BPA on rapid estrogen induction of CA1 PSSD may reflect interference, directly or indirectly, with this putative novel estrogen response pathway. Such a hypothesis would be consistent with recent studies in Mytilus that have demonstrated marked inhibition of p38 MAP kinase phosphorylation by low concentrations of BPA, a response diametrically opposite to that of estradiol (Canesi et al. 2004, 2005). A critical experiment for future studies will be to determine whether the effects on hippocampal PSSD of sustained physiologic circulating levels of 17β-E2 (Woolley and McEwen 1992), which may involve a greater contribution from nuclear ER-α and/or ER-β, are similarly affected by low-dose BPA exposure.

In summary, these data demonstrate that the environmental estrogen BPA inhibits estrogen–activated hippocampal spine synapse formation. Because hippocampal spine synapses are believed to be involved in the mechanisms responsible for the formation of memory (Silva 2003), these observations raise concerns regarding the potential impact of low-dose continuous BPA exposure on cognitive development and function. In addition, they further emphasize the dangers inherent in reliance on only one or a few nuclear estrogen-dependent tests as a basis for environmental estrogen risk assessment (Safe et al. 2002). There may be other compounds in the environment—natural and man-made—that, like 17α-E2 and BPA, exert potent effects on neural estrogen response mechanisms, even though their reported affinities for ER-α and ER-β are low. If so, current screening methods for the evaluation of putative estrogen-like “endocrine disruptors” (U.S. EPA 1998) may underestimate the potential risk of exposure to such compounds.

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