Estrogen-Induced Memory Enhancements Are Blocked by Acute Bisphenol A in Adult Female Rats: Role of Dendritic Spines

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Acute effects of bisphenol (BPA), an environmental chemical, on estradiol (17α or β-E2)-dependent recognition memory and dendritic spines in the medial prefrontal cortex and hippocampus were investigated in adult female rats. Ovariectomized rats received BPA 30 min before or immediately after a sample trial (viewing objects), and retention trials were performed 4 h later. Retention trials tested discrimination between old and new objects (visual memory) or locations (place memory). When given immediately after the sample trial, BPA, 1–400 μg/kg, did not alter recognition memory, but 1 and 40 μg/kg BPA, respectively, blocked 17β-E2-dependent increases in place and visual memory. When ovariectomized rats were tested with 17α-E2, 1 μg/kg BPA blocked place memory, but up to 40 μg did not block visual memory. BPA, given to cycling rats at 40 μg/kg, blocked visual, but not place, memory during proestrus when 2 h intertrial delays were given. Spine density was assessed at times of memory consolidation (30 min) and retention (4 h) after 17β-E2 or BPA + 17β-E2. In prefrontal cortex, BPA did not alter E2-dependent increases. In the hippocampus, BPA blocked E2 increases in basal spines at 4 h and was additive with E2 at 30 min. Thus, these novel data show that doses of BPA, below the current Environmental Protection Agency safe limit of 50 μg/kg, rapidly alter neural functions dependent on E2 in adult female rats. (***Endocrinology*** **153: 3357–3367, 2012)**

Many natural and synthetic chemicals in the environment mimic or antagonize the effects of endogenous hormones. Bisphenol-A (BPA), 4,4′-isopropylidene-2-diphenol, is a synthetic non-steroidal environmental chemical with mixed estrogen agonist/antagonist, and antiandrogen properties (1). BPA is widely used in polycarbonate plastics and is found in food and beverage containers, the lining of metal cans, baby bottles, and dental sealants (2). Both heat and changes in pH cause BPA to leach out of products and spread throughout the environment (3). Although these data strongly suggest that humans are continuously exposed to low levels of this chemical, the low affinity of BPA for classic nuclear estrogen receptors (ER), 10,000-fold lower than that of 17β-estradiol (E2) (4, 5), and weak bioactivity (6), have led to the conclusion that low level exposure probably does not elicit significant estrogenic responses. Currently the U.S. Environmental Protection Agency and the U.S. Food and Drug Administration set 50 μg/kg of BPA as the reference safe daily limit (U.S. Environmental Protection Agency, 1993).

Evidence from the past 2 decades, however, indicates that BPA exerts a powerful impact on neural systems, activating membrane-associated ER systems and signaling pathways at levels below the current reference safety daily limit (7), which suggests that BPA may also alter estrogen-mediated brain functions, such as memory and learning. The majority of existing BPA studies, which have focused on behavioral consequences of developmental exposure,

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Abbreviations: BPA, Bisphenol-A; E2, 17β-estradiol; EPM, elevated plus maze; ER, estrogen receptor; GPR30, G protein-coupled receptor 30; LSD, least significant differences; OP, object placement; OR, object recognition; OVX, ovariectomized; PFC, prefrontal cortex; T1, 3-min sample trial; T2, 3-min recognition/retention trial.

show that chronic exposure is associated with impairments in spatial and nonspatial memory at adulthood (8–14). Although little is known about behavioral effects of BPA exposure in adult females, recent findings in adult male rodents indicate that chronic exposure to this chemical during adulthood results in impaired performance on the Morris water maze (15, 16) and passive avoidance tasks (16), and acute exposure compromises recognition memory (17).

In most systems, BPA is a weak estrogenic agonist when E2 is absent but blocks activity if E2 is present (18–23). For example, acute E2 increases spine synapse density in CA1 of the hippocampus within 30 min and coadministration of BPA antagonizes this effect in adult, ovariectomized (OVX) rats (21). A number of studies have demonstrated that chronic changes in circulating E2 levels are associated with changes in spine density in both the rat hippocampus and the medial prefrontal cortex (PFC) (24). Thus, it is likely that acute E2 or BPA may exert rapid effects on spine density shortly after exposure in adult OVX female rats. E2 is also known to promote learning and memory in adult and aged rats and humans (25, 26). Several studies have shown that enhancements or impairments of memory performance are associated with increases and decreases, respectively, in spines in the CA1 region of the hippocampus and the PFC (24).

The aim of the present study was to examine effects of acute BPA exposure, alone and in combination with estrogens (17β-E2 or 17α-E2), on E2-induced memory enhancement and synaptic plasticity in OVX and gonadally intact, cycling female rats. We have previously reported that a single dose of E2 rapidly facilitates spatial and nonspatial memory consolidation (27). Therefore, the current study tested effects of BPA alone, and in combination with the most effective E2 doses, on recognition memory. Furthermore, we measured dendritic spine density in the CA1 area of the hippocampus and layer II/III of the PFC. This study is the first demonstration that acute, low doses of BPA interfere with E2-dependent consolidation of memory and alter dendritic spine density in an adult female rat model.

Materials and Methods

Subjects

Female Sprague Dawley rats (83 OVX and 18 intact, 3 months of age) were obtained from Harlan, Inc. (Indianapolis, IN). Rats were double housed, maintained on a 12-h light, 12-h dark cycle, with *ad libitum* access to low phytoestrogen food (Chow 2016, 16% protein rodent diet; Harlan Teklad Global Diets, Madison, WI) and water. They were handled daily and weighed weekly. All experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Animals and the Institutional Animal Care and Use Committee of Hunter College.

Behavioral measures

Two types of hippocampal-dependent memory tasks, object placement (OP) and object recognition (OR), were adapted from methods developed by Ennaceur and colleagues (28, 29) as previously described (27, 30–32). Trials consisted of a 3-min sample trial (T1) and a 3-min recognition/retention trial (T2) separated by an intertrial delay. In T1, time spent exploring two identical objects was recorded. In T2, one of the objects was replaced with a new object (OR) or moved to a new location (OP), and the time at the old object/location and new object/location was recorded. The novel location/object and its position were counterbalanced across groups. Objects and the floor of the chamber were cleaned after each trial. All trials were videotaped with a SONY camcorder (New York, NY).

Before testing, all rats received 8 d of habituation: each rat was first placed into the open field without objects to explore freely for 6 min. For d 2–8, rats received seven sessions with objects in progressively longer intertrial intervals. After habituation, either memory or anxiety testing began. Anxiety was tested using the elevated plus maze (EPM), which consisted of central open area and two open/enclosed arms. Rats explored for 5 min, and the number of entries and time spent in arms were recorded. Increased time or entries into open arms indicates low anxiety (33).

Treatment and experimental design

17α- and 17β-estradiol and BPA were obtained from Sigma-Aldrich Corp (St. Louis, MO). Doses of 0.4–400 μg/kg BPA, alone and in combination with the most effective doses of 17β- and 17α-E2 for OP and OR memory enhancement found in OVX rats in our previous acute E2 study were tested (27). E2 and BPA were initially dissolved in ethanol for stock solutions and diluted with corn oil (for E2) or saline (for BPA) for sc injection (final ethanol concentration $< 0.006\%$).

OVX subjects were randomly assigned into one of four groups (vehicle, E2, BPA, or BPA+E2 treated) for tests. Treatments were given sc using a two-point acute injection paradigm (Fig. 1), in which rats received vehicle or treatments 30 min before T1 sample trials (Pre-T1 injection), and vehicle or treatments immediately after T1 (Post-T1 injection), depending on experiment (see individual experiments for details). Retention and anxiety levels were tested 4 h after hormone/BPA exposure. Because OP and OR tasks can be repeatedly applied (28, 29), rats were tested every 10 d for about 2 months. For the experiments in Figs. 2 and 3, BPA doses were tested using a matched block paradigm (18–24 rats per block with approximately equal numbers of rats in each dose), and treatments were counterbalanced. Experiments were repeated until each dose group contained a sample size of at least six subjects. Ten days after the final behavioral tests, OVX rats received a pre-T1 injection, T1 sample trials, and post-T1 injections (see Fig. 6) and were killed at 30 min or 4 h later for the spine density and serum E2 level analysis. The time points were chosen so that morphological changes induced by acute E2 and BPA were examined during memory consolidation (30 min after T1) and retention (4 h after T1).

For experiment with intact, cycling rats (see Fig. 5), no Pre-T1 injections were given, but either saline or BPA (40 μg/kg) was

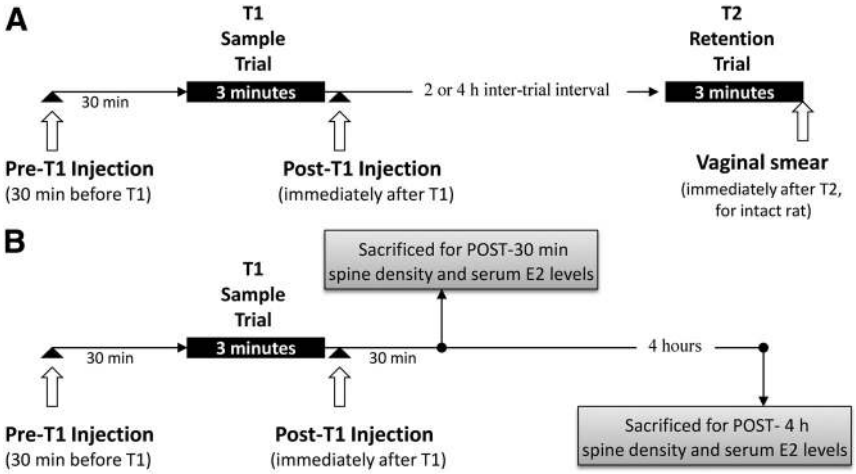

FIG. 1. Acute injection paradigms and time line for experiments. All OVX rats received two injections: one at 30 min before T1 (Pre-T1 injection) and the other immediately after T1 (Post-T1 injection). Treatment was corn oil, saline, BPA, or E2, depending on group assignment. A, For behavioral analysis with OVX rats, T2 retention was tested 4 h after post-T1 injection and anxiety was tested 4 h after injections. Intact rats received post-T1 injection only, and retention was tested 2 h later followed by vaginal smearing. B, For spine density and serum E2 measurements, all subjects performed T1 and were killed either 30 min or 4 h after T1 injection.

given immediately after T1 and retention tested 2 h later (Fig. 1A). This intertrial delay interval was chosen because intact rats performed the tasks at chance level when retention was tested 4 h later in our preliminary experiments. Vaginal smears were collected immediately after T2 and estrous cycle stage determined under a light microscope. Experiments were repeated approximately every 10 d, and data were grouped according to proestrus, estrus, metastrus, and diestrus.

Golgi impregnation and spine density analysis

After rapid decapitation, the brains were removed and cut into an anterior block (PFC) and a posterior block (the hippocampus). The Golgi impregnation procedure was carried out using the FD Rapid GolgiStain kit (FD Neurotechnologies, Inc., Columbia, MD) as previously described (34, 35). The apical and basal dendritic spine density of pyramidal cells in the medial PFC (layers II/III) and the CA1 region of the hippocampus were analyzed using a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) and the Spot Advanced Program for Windows (version 3.5; Diagnostic Instruments, Inc., Sterling Heights, MI) as previously described (34, 35). Counting was treatment blind for each dendrite segment, and spine density was determined as the number of spines divided by the length of the dendrite being measured.

RIA

Serum E2 was measured using a commercial RIA kit (Coat-A-Count; Diagnostic Products Corp., Los Angeles, CA) with modifications to account for the presence of rat serum binding proteins (36). Samples were counted in a Wizard 1470 automatic γ counter (PerkinElmer Life Sciences, Wellesley, MA). The E2 amount was calculated by comparison with the standard curve and expressed in picograms per milliliter.

Statistical analysis

Data are expressed as mean ± SEM. For memory tests, total exploration times around two objects during the T1 sample trials and exploration ratios (time with new object/location divided by time with new + old object/location) during T2 retention trials were analyzed by one-way ANOVA followed by Fisher least significant differences (LSD) *post hoc* tests. When BPA effects were tested in combination with E2, conservative criteria were used to identify inhibitory effects of BPA on E2-induced memory enhancement with both vehicle- and E2 alone-treated groups as negative and positive controls. Thus, BPA inhibitory effects were determined as significant only if exploration ratios of BPA+E2 combinations significantly differed from those of the positive control and did not differ from those of the negative control in *post hoc* comparison. Estrous phase data were tested by two-way ANOVA (treatment × estrous cycle), and if significant main effects and/or interactions were found, the data were further tested by one-way ANOVA within each treatment group followed by a *post hoc* Fisher LSD and *t* tests for treatment differences in each cycle phase. Data for EPM were tested by one-way ANOVA. For spine density, one-way ANOVA with Newman-Keuls *post hoc* tests were used. Differences in serum E2 levels at 30 min and 4 h after treatment were tested by two-way-ANOVA (time × group). All differences were considered significant at $P < 0.05$.

Results

BPA effects in OVX rats: OP and OR memory consolidation

Effects of BPA on spatial (OP) and nonspatial (OR) memory consolidation were examined with 1, 4, 40, 120, 240, and 400 μg/kg BPA (n = 6–8) given after the T1 in adult OVX rats. During the T1, rats spent similar amounts of time exploring the two identical objects for both OP (26–31 sec) and OR (25–30 sec) sessions. For the T2 retention trials, group differences in OP exploration ratios were not significant (Fig. 2A), suggesting BPA alone, at these doses, did not affect OP memory performance. Although OR exploration ratios were somewhat higher after BPA (Fig. 2B), the differences did not reach significance ($F_{7,50} = 2.078$, $P < 0.068$). Thus, at the doses tested, BPA did not influence OP or OR memory consolidation in OVX rats.

BPA + 17β-E2: effects on OP memory consolidation

Acute BPA effects on 17β-E2 induced OP memory enhancement were tested with BPA (0.4, 1, 4, 40, 120, 240,

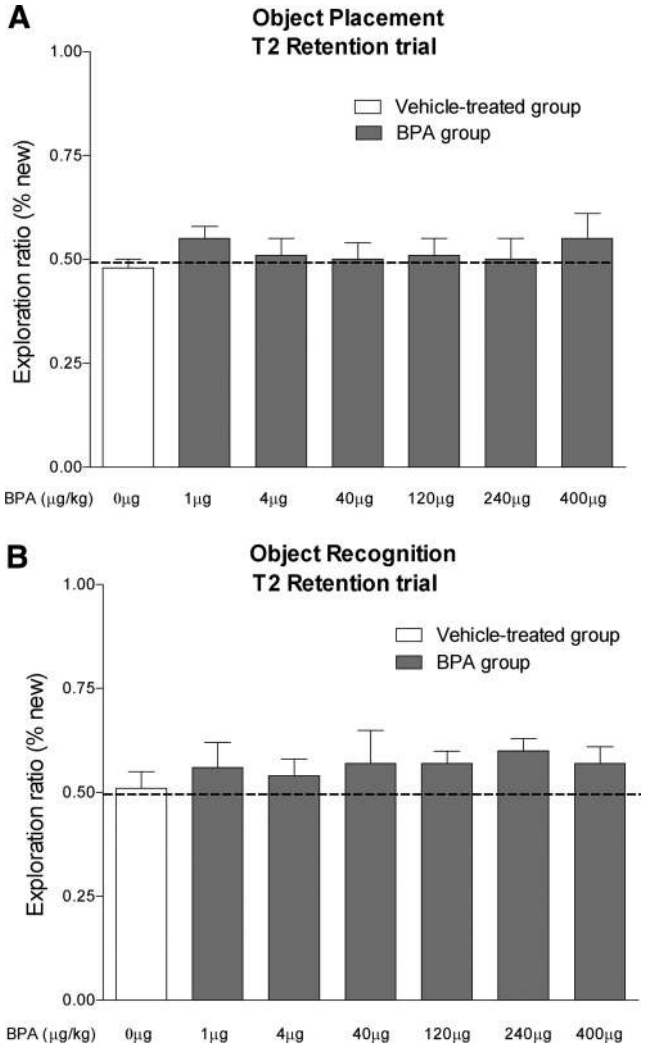

FIG. 2. Acute effects of BPA on OP and OR. A, Exploration ratios for OP retention trial. B, Exploration ratios for OR retention trial. Exploration ratios are plotted as means ± SEM. The *dashed line* at 0.5 indicates chance performance (rats spent equal amount of time around old and new locations/objects). Data were analyzed by one-way ANOVA, and no significant differences were found.

400 μg/kg) or vehicle given before the T1 and 17β-E2 (20 μg/kg) given immediately after the T1 (n = 10–12). During the T1, no group differences in the exploration times (26–31 sec) were observed, but a significant difference in T2 exploration ratios among groups ($F_{8, 102} = 2.178$, $P < 0.035$) was noted (Fig. 3A). Thus, *post hoc* tests were conducted based on the exploration ratios of the negative (vehicle treated) and positive (E2 treated) control. First, we confirmed that 20 μg/kg of E2 (ratio 0.63, $P < 0.002$) significantly enhanced OP memory compared with vehicle-treated control (ratio 0.48). *Post hoc* tests revealed that the exploration ratio for the 0.4 μg/kg of BPA+E2 group (ratio 0.62) was significantly higher than the vehicle-treated control ($P < 0.015$), whereas the exploration ratios for 1, 4, 40, 120, 240, and 400 μg/kg of BPA+E2 combinations (ratios 0.56, 0.49, 0.52, 0.48, 0.51, and 0.51, respectively) were not. These results show that 0.4 μg/kg BPA did not inhibit E2-induced memory enhancement, but all doses above 1 μg/kg reduced E2 memory-enhancing effects (marked O in Fig. 3A). When the same data were analyzed based on comparison with the positive control (E2 group), the exploration ratios of E2 combined with BPA doses at 4 μg/kg and above were significantly lower than the E2 alone group (marked Δ in Fig. 3A). Although this result suggests possible inhibitory effects of 1 μg/kg BPA on E2-induced OP memory enhancement, only BPA doses at or above 4 μg/kg met both criteria for inhibition. Thus, we found that the lowest dose of BPA that blocked OP memory enhancement, induced by 20 μg/kg of 17β-E2, was 4 μg/kg, which is far below the current reference safety dose of 50 μg/kg·d.

BPA+17β-E2: effects on OR memory consolidation

Next, we tested the effects of BPA given before the T1 at 1, 4, 40, 120, 240, and 400 μg/kg on OR-induced memory consolidation by 17β-E2 (5 μg/kg) given immediately after the T1 (Fig. 3B, n = 7–8 in each group). Exploration times around objects during the T1 (20–26 sec) were not significantly different between the groups. In T2, a significant group difference was found ($F_{7, 54} = 2.844$, $P < 0.013$). *Post hoc* tests showed that exploration ratios of vehicle-treated controls (ratio 0.52) were significantly lower than the E2-alone- (ratio 0.68, $P < 0.010$), 1 μg/kg BPA+E2- (ratio 0.64, $P < 0.039$), and 4 μg/kg BPA+E2 (ratio 0.64, $P < 0.045$)-treated groups, whereas the 40 (ratio 0.49), 120 (ratio 0.53), 240 (ratio 0.54), and 400 (ratio 0.54) μg/kg BPA+E2 groups had similar exploration ratios as the vehicle-treated controls (marked 0 in Fig. 3B). These results suggest that doses of BPA from 40–400 μg/kg BPA suppressed E2-induced OR memory enhancement. Similar to this result, the exploration ratio of the E2-alone-treated group was significantly higher than the 40 ($P < 0.002$), 120 ($P < 0.015$), 240 ($P < 0.016$), and 400 ($P < 0.019$) μg/kg BPA+E2 combinations (marked Δ in Fig. 3B) but not the 1 and 4 μg/kg BPA+E2-treated groups. Therefore, *post hoc* testing based on both negative and positive controls was consistent and indicated that the lowest dose of BPA that blocks 17β-E2-induced OR memory enhancement is 40 μg/kg, which is also below the current reference safety dose.

BPA+17α-E2: effects on OP and OR memory consolidation

Because 17α-E2 has stronger binding affinities to membrane-associated ER (37, 38) and can elicit greater estrogenic responses than 17β-E2 (31, 39, 40), we tested BPA action on 17α-E2-induced enhancement of recognition memory (Fig. 3, C and D). BPA+17β-E2 experiments

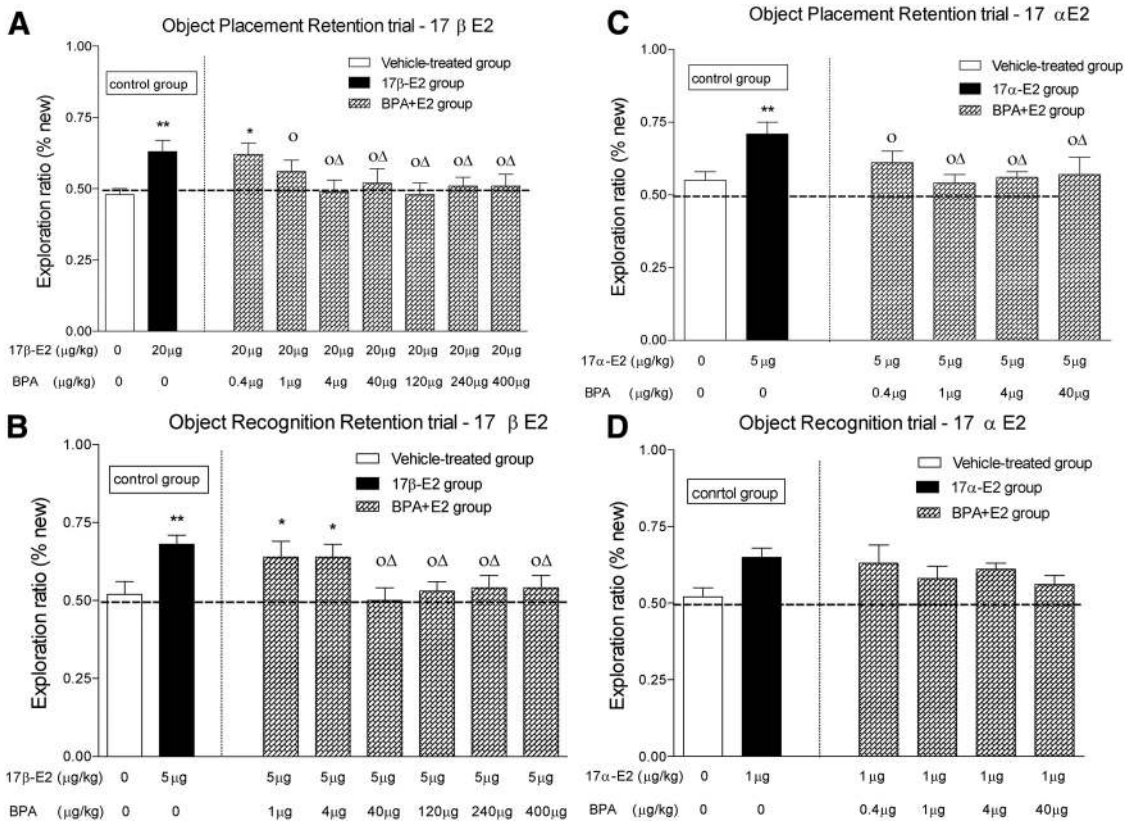

FIG. 3. Effects of BPA on 17β-E2 and 17α-E2 induced OP and OR memory enhancement. The *white bars* represent vehicle-treated controls, the *black bars* indicate E2-alone-treated controls, and *stippled bars* represent BPA and estrogen treatments. Data analyzed by one-way ANOVA with *post hoc* tests (Fisher LSD). BPA-inhibitory effects were identified if the exploration ratios of BPA+E2 were significantly lower than E2-alone-treated control (shown Δ) and not significantly different from vehicle-treated control (shown o). Thus, BPA doses that suppressed E2 effects are marked with both Δ and o. A, 17β-E2+BPA effects in OP retention trial. B, 17β-E2+BPA effects in OR retention trial. C, 17α -E2+BPA effects in OP retention trial. D, 17α -E2+BPA effects in OR retention trial. Entries are means $\pm$ SEM. *, $P < 0.05$, **, $P < 0.01$, and o, $P > 0.05$ *vs.* vehicle group; Δ, $P < 0.05$ *vs.* E2 group.

showed that less than 40 μg/kg BPA was sufficient to inhibit E2-induced memory enhancement; therefore, only the lower doses of BPA (0.4, 1, 4, 40 μg/kg, n = 7–8 in each group) were tested in combination with 5 μg/kg 17α-E2 for OP and 1 μg/kg 17α-E2 for OR [doses of 17α-E2, which enhance recognition memory (27)]. For OP memory, there were no significant differences in the exploration times (25–31 sec) during T1, but significant differences were found in T2 exploration ratios ($F_{5,\,48} = 2.50$, $P < 0.043$). *Post hoc* tests revealed a higher ratio in the E2-alone-treated group (ratio 0.71) compared with the vehicle-treated group (ratio 0.55), but the exploration ratios of the BPA+E2-treated groups were not different from the vehicle-treated group (0.4 μg/kg, ratio 0.6; 1 μg/kg, ratio 0.54; 4 μg/kg, ratio 0.56; and 40 μg/kg, ratio 0.57, marked o in Fig. 3C). When the same data were compared based on the exploration ratio of the E2-alone-treated group, we found significant lowering in the 1 μg/kg ($P < 0.035$), 4 μg/kg ($P < 0.008$), and 40 μg/kg ($P < 0.014$) BPA+E2 groups (marked Δ in Fig. 3C), but not in the 0.4 μg/kg BPA+E2 ($P < 0.06$) group. Thus, based on overall criteria for BPA inhibitory effects, we concluded that the lowest dose of BPA that inhibits 17α-E2-induced OP memory enhancements was1 μg/kg.

Finally, the effects of BPA on 17α-E2 induced OR memory enhancement (n = 8 in each group) was tested (Fig. 3D). There were no significant group differences in exploration times (22–27 sec) around the objects during T1. For T2 retention trials, group differences in exploration ratios showed a trend but were not statistically significant ($F_{5,\,57} = 2.254$, $P < 0.064$). Thus, the tested BPA doses did not block 17α-E2-induced OR memory enhancement.

E2 and BPA effects on the elevated plus maze

Whether BPA may indirectly impair recognition memory by increasing anxiety levels of the subjects was tested by treating OVX rats with 20 μg/kg 17β-E2, which enhanced OP memory, and 40 μg/kg BPA, which did not affect memory but antagonized E2. Greater percentage entries into open arms in E2- (38%), BPA- (28%), and BPA+E2 (34%)-treated rats compared with vehicle-treated subjects (27%) were found, but these differences

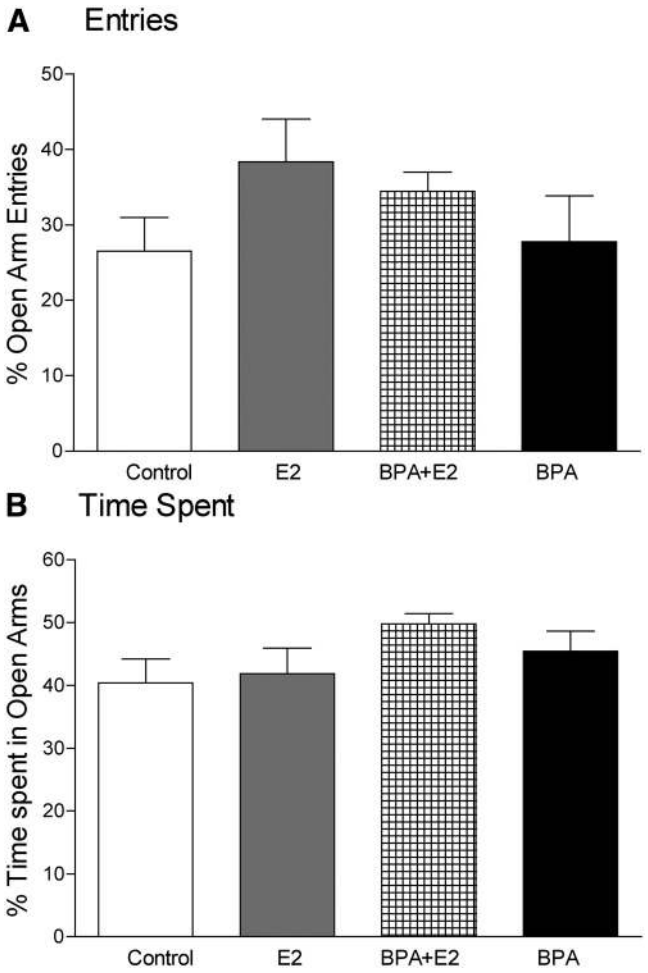

FIG. 4. Effect of E2 and BPA, alone and in combination, on EPM. A, Percentage of open arm entries ± SEM (number of open arm entries/number of open + closed arm entries). There were no significant differences between groups. B, Percentage of time spent in open arms ± SEM (time spent in open arms/time spent in open + closed arms) is shown. Data were analyzed by one-way ANOVA, and no significant differences were found.

were not significant (Fig. 4A). Consistent with this result, all groups spent a similar percentage of time in the open arms (Fig. 4B). Therefore, neither this dose of E2 (20 μg/kg) nor BPA (40 μg/kg), alone or in combination, influenced anxiety levels.

BPA effects on OR and OP memory in cycling females

Effects of 40 μg/kg of BPA were examined over the estrous cycle. Based on BPA's generally weak effect on memory (Fig. 2) but its ability to block E2-enhanced recognition memory (Fig 3, A–C), we hypothesized that memory would be impaired in treated rats on proestrus when E2 levels are highest but may not be affected on other days of the cycle. For OP memory performance, there were no significant main effects of estrous cycle, treatment, or interactions in T2 exploration ratios, indicating that acute

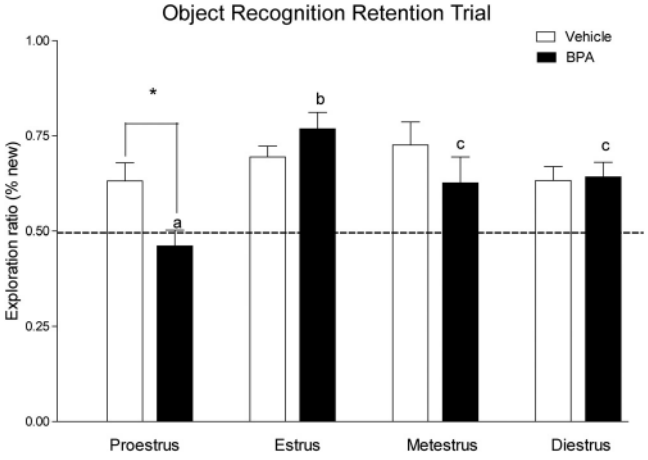

FIG. 5. Effects of BPA on OR memory consolidation across the estrous cycle. The *white bars* are vehicle-treated subjects, and the *black bars* are BPA (40 μg/kg)-treated subjects. No differences in vehicle-treated rats across the cycle were found, but significant differences were found in the BPA groups (*letters* a, b, and c). *Post hoc* tests showed that BPA suppressed OR memory performance at proestrus (*, $P < 0.02$). Entries are means ± SEM.

BPA did not influence OP memory performance at any phase of the estrous cycle (data not shown). For OR memory, however, there was a significant main effect of the estrous cycle ($F_{3, 57} = 7.57$, $P < 0.001$) and an estrous cycle × treatment interaction ($F_{3, 57} = 3.65$, $P < 0.02$). Thus, we conducted separate ANOVA in the vehicle- and BPA-treated groups. In vehicle-treated animals, T2 exploration ratios across the estrous cycle (proestrus = 0.64, estrus = 0.69, metastrus = 0.72, diestrus = 0.63, Fig. 5, *white bars*) were not significantly different. In contrast, for the BPA-treated groups, there were significant differences in exploration ratios ($F_{3, 22} = 8.39$, $P < 0.001$) across the cycle (proestrus = 0.46, estrus = 0.77, metastrus = 0.64, diestrus = 0.63, Fig. 5, *black bars*). *Post hoc* tests revealed that exploration ratios of BPA-treated proestrus subjects were significantly lower than those of other days of the cycle and that exploration ratios of BPA-treated estrus subjects were significantly higher than those of the other days of the cycle (Fig. 5, *letters a, b, and c*). Finally, we compared exploration ratios between vehicle- and BPA-treated subjects in each estrous phase using *t* tests. The results showed that BPA significantly reduced exploration ratios of the proestrus group ($t = 2.72$, $P < 0.018$) but not the other groups. Overall, these results indicate that BPA inhibits OR memory performance on proestrus when endogenous E2 levels are the highest.

Effects of E2 and BPA on spine density in CA1 and the PFC of OVX rats

Spine density of the pyramidal neurons in the PFC and CA1 were quantified in OVX vehicle-treated subjects and in groups after Post-T1 treatment with 17β-E2 (20 μg/kg)

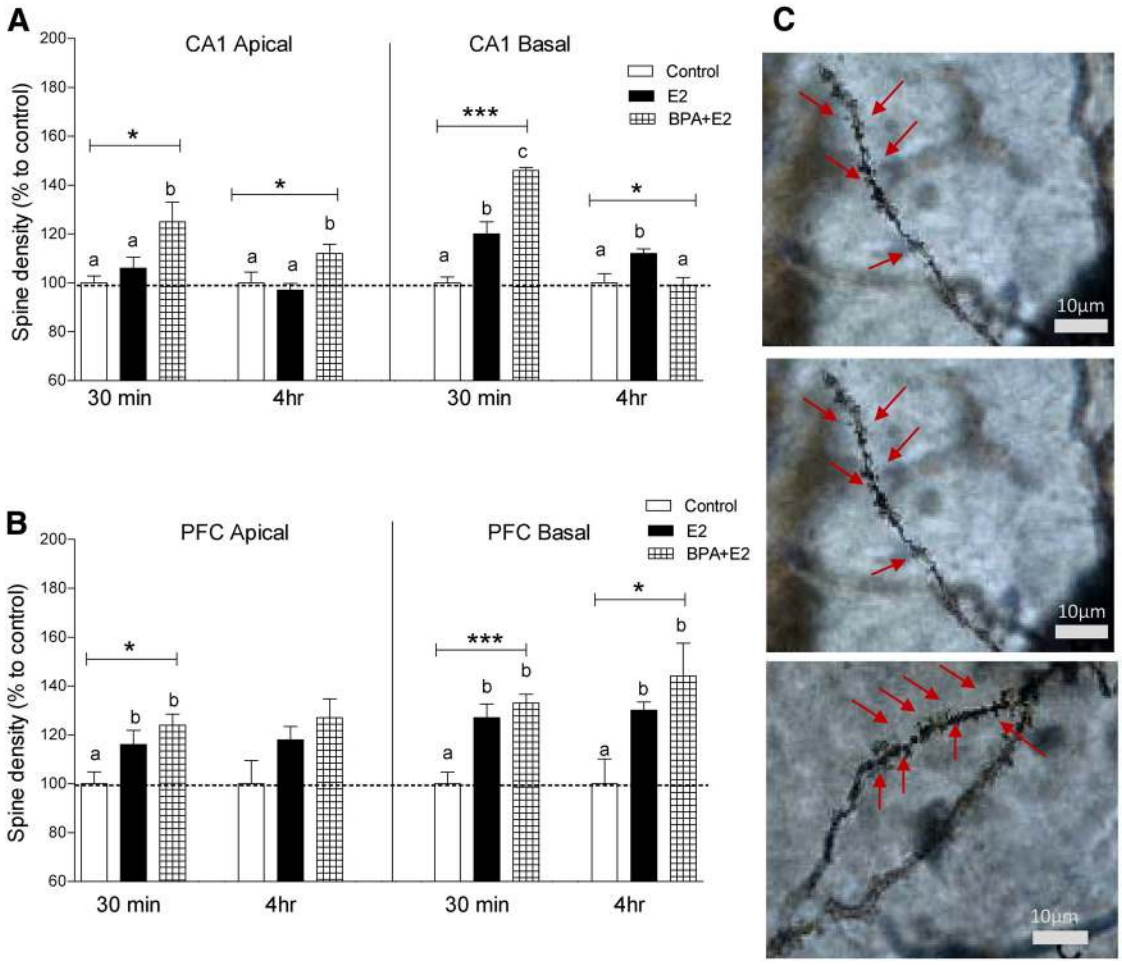

FIG. 6. Spine density in apical and basal PFC and CA1 pyramidal neurons at 30 min and 4 h after estradiol treatment. Data are presented as percent control spine density ± SEM (controls ranged from approximately six to 12 spines/10 μm, depending on area). Spine density in each area was analyzed by one-way ANOVA. *Post hoc* results (Newman-Keuls multiple comparisons) are shown by *letters* a, b, and c in which *groups with different letters* are significantly different from each other ($P < 0.05$). *White bars* and *dashed lines* represent vehicle-treated control. A, Normalized apical (*left panel*) and basal (*right panel*) spine density in CA1. B, Normalized apical (*left panel*) and basal (*right panel*) spine density in PFC. C, Representative photomicrographs from pyramidal neurons of CA1 hippocampus 30 min after acute vehicle (*top panel*), E2 (*middle panel*), or BPA+E2 (*bottom panel*) treatment. *Arrows* indicate spines on the secondary basal dendrites at ×100.

and after Pre-T1 treatment with BPA (40 μg/kg) followed by Post-T1 E2 treatment (20 μg/kg) (n = 6 in each group) at 30 min (Post-30 min: time for memory consolidation) or 4 h (Post-4 h: time for retention testing) after a T1 sample trial. Data are shown as the percent change in spine density relative to control for each time point (Fig. 6, A and B), and representative Golgi impregnation images from CA1 are shown in Fig. 6 C.

In CA1, the group differences in Post-30 min spine density were significant in apical ($F_{2,15} = 5.493$, $P < 0.016$) and basal ($F_{2,15} = 40.523$, $P < 0.0001$) dendrites. *Post hoc* tests indicated that acute E2 significantly increased basal (29% above control, $P < 0.001$) but not apical spine density; however, the combination of BPA+E2 increased both apical (25% above control, $P < 0.006$) and basal (48% above control, $P < 0.001$) spines significantly. Moreover, the BPA+E2-treated groups were significantly higher than the E2 group (apical, 18% above E2, $P < 0.02$; basal,13% above E2 group, $P < 0.002$) branches, indicating that coadministration of BPA further increased E2-induced spine density in CA1. In the PFC, there were significant group differences in Post-30 min spine density for both apical ($F_{2,15} = 5.817$, $P < 0.013$) and basal dendrites ($F_{2,15} = 13.92$, $P < 0.001$). *Post hoc* tests indicated that E2 significantly increased both apical (16% above control, $P < 0.038$) and basal (27% above control, $P < 0.001$) spine density. Coadministration of BPA with E2 also significantly increased apical (24% above control, $P < 0.004$) and basal (33% above control, $P < 0.001$) spine density compared with vehicle-treated subjects, but differences in between E2- and BPA+E2-treated subjects were not significant. Thus, acute BPA does not affect E2-induced spine density in the PFC but further elevates spine density in CA1 at 30 min after T1.

At 4 h after T1, CA1 spine density showed significant group differences for both apical ($F_{2,18} = 4.81$, $P < 0.02$) and basal ($F_{2,18} = 5.14$, $P < 0.02$) dendrites. E2 did not affect apical spine density, but BPA+E2 significantly increased it (12% above control, $P < 0.006$). In contrast, basal spine density was significantly increased by E2 (12% above control, $P < 0.01$), but there were no differences in spine density between vehicle-treated and BPA+E2-treated subjects. Thus, at 4 h after treatment, BPA suppressed E2-induced increases in spine density in CA1 basal dendrites, but it further enhanced the E2-induced increases in spine density in the apical dendrites. In the PFC, ANOVA showed significant group differences in basal ($F_{2,18} = 5.059$, $P < 0.02$) but not apical spine density. *Post hoc* tests revealed that basal spine density in the PFC was significantly increased by both E2 (33% above control, $P < 0.05$) and BPA+E2 (44% above control, $P < 0.006$), but the groups were not different from each other.

Serum E2 levels

Circulating E2 levels in the vehicle, E2, and BPA+E2 groups were measured in the OVX subjects used for the spine density analyses. A two-way ANOVA (group × time) revealed a significant main effect of group ($F = 19.13$, $P < 0.0001$) but not time. One-way ANOVA with Tukey honestly significant difference were performed as *post hoc* tests for each time point, and significant group differences in serum E2 levels at both 30 min ($F_{2,18} = 6.72$, $P < 0.006$) and 4 h ($F_{2,18} = 24.50$, $P < 0.0001$) after treatment were found. Acute E2 (20 μg/kg) treatment rapidly elevated levels at 30 min (from 48 pg/ml in control to 657 pg/ml, $P < 0.004$), and levels remained elevated at 4 h (711 pg/ml, $P < 0.0001$). Similarly, BPA+E2 increased serum E2 levels at 30 min (from 49 to 1082 pg/ml, $P < 0.0001$) and 4 h (901 pg/ml, $P < 0.0001$). E2 levels in the cotreatment groups were not different from E2 treatments alone.

Discussion

The current study is the first to demonstrate that acute exposure to BPA alters E2-induced enhancements of spatial (OP) and nonspatial (OR) memory consolidation and spine density in adult female rats. The results indicate that acute BPA blocks enhancement of memory consolidation by both 17α- and β-E2 in a task- and dose-specific manner in OVX females and suppresses OR memory during proestrus. In contrast, BPA, in the absence of E2, did not affect memory performance. Moreover, within 30 min E2 increases dendritic spine density in basal dendrites of CA1 and in both dendritic fields of the PFC, areas known to be important for cognitive function. Coadministration of BPA, at 40 μg/kg, alters the E2 effects in the hippocampus but not in the PFC. These results provide compelling evidence that acute BPA interacts with exogenous and endogenous E2 in the mature brain and rapidly alters behavioral and neuronal responses to E2 in adult female rats. The rapid onset of BPA action and the poor affinity of BPA for nuclear ER suggest that the observed effects are mediated through membrane-associated ER.

Previous behavioral studies show that developmental exposure to BPA interrupts normal development and alters nonreproductive behavior at adulthood (1) including spatial and nonspatial memory performance, anxiety levels, and exploratory behavior (8–14). In the present study, we demonstrate rapid effects of BPA, at doses lower than the Environmental Protection Agency safe limit, on memory function in adult females, which were not accompanied by changes in anxiety levels or motor activity levels. Although BPA alone did not affect memory consolidation in OVX females, it did impair OR when given to females in proestrus when E2 levels are high. Blocking at proestrus is consistent with a recent study from our laboratory showing that the same dose of BPA (40 μg/kg) impairs recognition memory in gonadally intact adult male rats (17) and suggests that BPA may interfere with memory processes in the presence of circulating gonadal hormones. The lack of inhibition of OP in intact females by BPA may be due to the poor performance of spatial memory tasks in general by intact females (41); use of shorter intertrial delays might reveal BPA effects. Chronic BPA regimens also impair memory. Jain *et al.* (16) found that 28 d of 2 or 20 μg/kg of BPA impaired spatial memory on the Morris water maze and passive avoidance learning, and Kim *et al.* (15) reported that a higher dose, 20 mg/kg, impaired Morris water maze performance in mice. Thus, increasing evidence suggests that BPA affects memory in adult as well as developmental animal models.

Dose-response patterns between acute E2 treatment and memory performance are inverted U for both isomers of E2 (27, 42, 43). Estrogen and/or BPA effects on neural systems, such as Ca^{2+} influx via L-type channels (44) and dopamine efflux through membrane-associated receptors (45), also have nonmonotonic dose response relationships. However, the current results show that BPA did not affect behavior, although it is possible that the tested doses were not broad enough. Two recognition tasks assessed memory, and both are working memory tests, which require an intact hippocampus. However, OP is a spatial task that, although primarily dependent on an intact hippocampus (46) and/or fornix (28), also requires PFC input (29). On the other hand, OR is less dependent on the hippocampus, is not considered a spatial memory task, and

requires PFC activity (29). For example, damage to 30–50% of the dorsal hippocampus impairs spatial memory, whereas lesions of 75–100% are necessary to impair object memory (46). The cognitive load for spatial memory is also considered greater because objects can be encoded and discriminated through multiple sensory modalities (visual or tactile), whereas the discrimination of location involves abstract categorizations and use of cognitive maps. These differences between the two recognition tasks may be important for understanding why different doses of estrogens and BPA are required to enhance or impair consolidation in the two different recognition tasks.

E2 or specific ER agonists, given acutely or chronically enhance performance of OR and OP (27, 31, 47–49). The current data demonstrate that low doses of BPA block estrogen enhancements of both tasks; however, it should be noted that responses to both E2 and BPA have different dose-response relationships for the tasks. A dose of 4 μg/kg of BPA suppresses 17α- or 17β-E2-induced OP memory enhancement, whereas a higher dose, 40 μg/kg, is required to inhibit 17β-E2-induced OR memory enhancement. In addition, BPA failed to suppress 17α-E2-induced OR memory enhancement at any dose used. These results suggest specific interactions between memory tasks and circulating E2 and BPA and that the interactions with each isomer of E2 may rely on different mechanisms or receptors. In support of these findings, *in vitro* studies show that BPA effects are highly cell type or target tissue specific and agonistic/antagonistic effects depend on ER subtypes in the target cells (49). BPA and E2 interact differently within the ligand-binding domain at ER-α (50, 51), which may induce different molecular effects via membrane-associated ER. In addition, BPA may exert agonistic activity via ER-β but mixed agonist/antagonist activity via ER-α (49). Moreover, studies in rats (47, 52) or ER-β knockout and ER-α knockout mice indicate that beneficial effects of E2 on cognition and hippocampal synaptic plasticity may be mediated by ER-β, whereas inhibitory effects of large doses of E2 may be mediated by ER-α (53). Rissman (53) suggested that low-intermediate levels of E2 or ER-β agonists bind to ER-β, which produces better spatial memory performance, but large doses of E2 or ER-α agonists bind to ER-α, which results in poor spatial memory performance.

Although estradiol and BPA binding to G protein-coupled receptor 30 (GPR30) receptors might also mediate effects on memory, the observation that 17α-estradiol shows little competitive binding to GPR30 in the micromolar range (54) but activates OR memory at 5 μg/kg suggests that GPR30 is not involved. Another possible, although unlikely alternative, is that interactions of estradiol and BPA might be through ER-independent mechanisms. Nongenomic actions could induce rapid, large CA^{2+} influxes (55, 56) and increase CA1 excitability (57) without activating ER. These actions might directly affect neural signaling systems and hence result in memory impairment.

Dendritic spines were examined as a mechanism for the memory effects because chronic changes in E2 alter both recognition memory and spine density in CA1 and the PFC (24). More pertinent to the current study is that synaptic changes occur within minutes to hours (39) after E2, spine density has been shown to increase 4 h after E2 in the arcuate nucleus (58), and BPA rapidly inhibits E2-dependent increases in hippocampal spine synapse density (20). However, whether alterations in spines underlie changes in memory seen after E2 and/or BPA remains unclear. Estradiol administration increased spine density in the medial PFC and hippocampus within 30 min, a time course concomitant with enhanced recognition memory consolidation. In addition, BPA impaired E2-dependent recognition memory and altered expression of spines by estrogen. The acute effects on spines were nonetheless complex with both the dose and duration of treatments critical for the outcome of the responses. BPA did block estrogen dependent increases in spine density in basal CA1 dendrites 4 h later. Because CA1 is critical for memory (48, 59), this block may contribute to BPA's impairment of memory consolidation. In support of this relationship, BPA rapidly decreases recognition memory, CA1 spine density, and hippocampal post synaptic density protein 95 (PSD95) in male rats (17). In contrast to the hippocampal changes, BPA did not alter estrogen-dependent decreases in PFC spines in our females. Compromised recognition memory and decreased PFC spine density is found in chronic models of estrogen deprivation (60, 61). Thus, it is possible that spine density in the PFC may contribute to only chronic, but not acute, changes in memory function by estrogens or antagonists like BPA. Also pertinent is that in some cases dendritic spine density is greater 30 min after estradiol administration than at 4 h. These high levels may represent increased filipodial or immature processes that mature by 4 h. *In vitro* studies show that BPA alone increases filopodia outgrowth but inhibits outgrowth in the presence of E2 (62). Thus, more detailed measurements assessing possible changes in spine subtypes over time after estradiol and BPA administration might clarify the role of spine plasticity in memory consolidation processes.

In summary, the present experiments demonstrate that low doses of BPA rapidly interact with E2 to alter cognitive and neural responses in adult rats. About 6.5 billion pounds of BPA are produced per year, and humans and animals are exposed to this chemical on a daily basis. Whether this exposure is hazardous remains under debate,

but the current results raise additional concerns about BPA because low level exposure rapidly and powerfully impacts estrogen-mediated cognitive function in an adult rodent model.

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