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Prenatal exposure to bisphenol A impacts midbrain dopamine neurons and hippocampal spine synapses in non-human primates

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Abstract

Prevalent use of bisphenol-A (BPA) in the manufacture of resins, plastics and paper products has led to frequent exposure of most people to this endocrine disruptor. Some rodent studies have suggested that BPA can exert detrimental effects on brain development. However as rodent models cannot be relied on to predict consequences of human exposure to BPA during development, it is important to investigate the effects of BPA on non-human primate brain development. Previous research suggests that BPA preferentially targets dopamine neurons in ventral mesencephalon and glutamatergic neurons in hippocampus, so the present work examined the susceptibility of these systems to low dose BPA exposure at the fetal and juvenile stages of development in non-human primates. Exposure of pregnant rhesus monkeys to relatively low levels of BPA during the final 2 months of gestation, induced abnormalities in fetal ventral mesencephalon and hippocampus. Specifically, light microscopy revealed a decrease in tyrosine hydroxylase-expressing (dopamine) neurons in the midbrain of BPA-exposed fetuses and electron microscopy identified a reduction in spine synapses in the CA1 region of hippocampus. In contrast, administration of BPA to juvenile vervet monkeys (14–18 months of age) was without effect on these indices, or on dopamine and serotonin concentrations in striatum and prefrontal cortex, or on performance of a cognitive task that tests working memory capacity. These data indicate that BPA exerts an age-dependent detrimental impact on primate brain development, at blood levels within the range measured in humans having only environmental contact with BPA.

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Conflict of interest statement

The authors declare that there are no conflicts of interest regarding this research article.

Keywords

Bisphenol-A; Dopamine; Hippocampus; Prenatal; Spine synapse; Substantia nigra

1. Introduction

The widespread use of bisphenol-A (BPA) in the manufacture of certain resins, plastics and paper products has led the majority population of the developed world to be frequently exposed to the compound (Liao and Kannan, 2011; Vandenberg et al., 2010), and the circulating levels of BPA measured in humans appear sufficient to induce detrimental effects (Vandenberg et al., 2010). The estrogenic properties of BPA have been recognized for many decades, although it is now apparent that the induced effects of BPA cannot be attributed solely to agonist actions at classical estrogen receptors (Alonso-Magdalena et al., 2012; Rubin, 2011). In fact, our studies have indicated that anti-estrogen effects form a major contribution to the *in vivo* pharmacology of BPA in rodent and primates (Hajszan and Leranth, 2010). Because of its interaction with estrogen receptors, many studies on the toxicity of BPA have focused on reproductive issues; however, we are concerned about the potential harmful effects of environmental exposure to BPA on brain development. Estrogens play an important role in brain function of both sexes, and estrogens synthesized locally from androgens in brain of both sexes appear to have a particularly prominent role in promoting brain development (Kipp et al., 2006; McCarthy, 2008; Roselli et al., 2009). During the prenatal period a critical timetable of biochemical and morphological events must proceed in order for normal brain development to occur, and any perturbation in this process jeopardizes future brain function (Levitt et al., 1998). In animals, prenatal BPA exposure elicits changes in several behaviors in offspring (Golub et al., 2010; Wolstenholme et al., 2011). The BPA-induced alterations that underlie such behavioral abnormalities have not been resolved, but several rodent studies have pointed to disturbance in the development of midbrain dopamine (DA) neurons (Masuo and Ishido, 2011) and synapse changes in hippocampus (MacLusky et al., 2005; Xu et al., 2010). There is now a need for such studies on BPA exposure during development to be extended to non-human primates, especially in light of suggestions of an involvement of BPA in neurodegenerative disorders such as Parkinson's disease, in addition to memory and cognitive disorders (Masuo and Ishido, 2011). In fact, recent evidence has associated exposure to endocrine disruptors with the occurrence of autism spectrum and attention deficit hyperactivity disorders (de Cock et al., 2012). The importance of studies of BPA on primate brain development is highlighted by different rodent-primate endocrine influences on the brain, in addition to distinct differences between these species in rates of brain development, and their maturity at the time of birth (Chellman et al., 2009; Clancy et al., 2001; Melemed and Conn, 2005; Morrow et al., 2007). Thus, the present research examines the effect of exposure to BPA during the prenatal stage of development in monkeys on integrity of the midbrain DA system and on spine synapses in hippocampus. These findings were compared with those induced by BPA in juvenile monkeys, which is the age at which the primate brain is in the final period of development (Giedd et al., 1999).

2. Methods

2.1. Animals

2.1.1. Prenatal exposure studies—Adult female rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center (CNPRC). For the duration of the study, animals were caged individually and fed a diet of Purina Monkey Diet #5045, supplemented with seasonal fruits, seed and cereals, with water *ad libitum*. Cages

were made of stainless steel, and water was delivered to each cage through unplasticized “rigid” polyvinyl chloride pipes and a “Lixit” device (Lixit Corporation, Napa, CA), which do not contain BPA. Serum BPA in all animals was not detectable prior to treatment. Only females with a history of normal menstrual cycles were selected for this study. Females ranged in age from 6 to 13 years. All females for this study were naturally mated according to standard CNPRC procedures. Pregnancy was detected by ultrasound examination and the estimated day of conception (gestation day, GD 0) was assigned. At approximately GD 40, sex of all fetuses was determined by ultrasound and only those with female fetuses were continued in this study, as an additional purpose of the study was to investigate the effects of BPA on oogenesis. In primates, oocytes are beginning to be formed into follicles at the beginning of the third trimester (about GD100), so prenatal BPA treatments were started at this time (see below). Protocols were reviewed and approved in advance by the Animal Care and Use Committee of the University of California, Davis; all studies were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1.2. Animals: postnatal exposure study—Juvenile male and female St. Kitts African green monkeys (*Chlorocebus aethiops sabaeus*; 14–18 months old year of age; 2.5–3 kg) were born and housed at St. Kitts Biomedical Research Foundation facilities. This age is within the pre-pubertal period for this species, with sexual maturity occurring at about 4 years (Bolter and Zihlman, 2003). Animals were initially housed in social group enclosures, then 2 per cage (as required for animals of this age) for the duration of the study. Monkeys were fed Harlan Teklad New Iberia Primate Diet (#8773) supplemented with seasonal local fruits. Water was constantly available, and delivered through pipes that do not contain BPA. Protocols were reviewed and approved in advance by the Animal Care and Use Committees of both Yale University and St. Kitts Biomedical Research Facility; all studies were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Bisphenol A treatments

Deuterium-labeled BPA (d-BPA, CDN Isotopes, Quebec, Canada) was used in these studies because it can be clearly distinguished from BPA by liquid chromatography–mass spectrometry (LC–MS), thus eliminating concern about potential BPA contamination from materials used in the preparation, handling or shipment of samples (Taylor et al., 2011).

2.2.1. Prenatal exposure to oral bisphenol—In the first prenatal phase of the study, d-BPA was administered to pregnant monkeys by the oral route. BPA is known to pass the placental barrier (Balakrishnan et al., 2010). Animals were given small pieces of fruit to eat daily containing either 400-μg/kg body weight of d-BPA (5 animals) or vehicle control (5 animals). Treatment was from GD 100 until the day of tissue collection. Pregnancy ended with spontaneous vaginal delivery; gestation is approximately 165 days. Maternal blood samples were obtained within two weeks of birth to quantify the on-going exposure to d-BPA. Neonates were euthanized by pentobarbital overdose at 1 to 3 days after birth and brains fixed for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, then transferred to 0.1 M phosphate buffer containing sodium azide. The number of DA neurons in each ventral mesencephalon was subsequently determined using tyrosine hydroxylase (TH) immunohistochemistry.

2.2.2. Prenatal exposure to subcutaneous implants of bisphenol A—In the second phase of the prenatal study, pregnant animals received d-BPA doses delivered from silastic tubing implants, so that the animals were continually exposed to BPA. Implants were prepared using three pieces of 3-in. long silastic tubing 0.132 ID × 0.183-in OD (Dow

Corning Corp., cat. # 508–011). d-BPA was suspended at 50 mg/ml in tocopherol-stripped corn oil, and a total volume of 2.5 ml d-BPA suspension (6 animals) or vehicle (3 animals) was distributed among the three tubing pieces for each animal. The implants were placed subcutaneously in the upper back region while animals were anesthetized. Exposure was from GD 100 to 155; implants were removed and replaced with freshly prepared implants after about 25 days of treatment (half-way through the dosing period) to assure that d-BPA levels remained near the maximum release rate. Maternal blood samples were obtained at GD155 to determine serum levels of d-BPA. Fetuses were removed by hysterotomy at GD155, and brains were fixed for 24 h in 4% paraformaldehyde with 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, then transferred to 0.1 M phosphate buffer containing sodium azide. The total number of spine synapses in the stratum radiatum of the CA1 subfield of the hippocampus and layers II/III of the dorsolateral prefrontal cortex (DLPFC) was determined in these brains by electron microscopy.

2.2.3. Juvenile exposure to subcutaneous implants of bisphenol A—Juvenile monkeys were implanted as above (2.2.2), except that each monkey received 2 pieces of silastic tubing that each contained 75 mg d-BPA (4 males and 4 females) or vehicle (4 males and 4 females). After 30 days of d-BPA exposure, animals were euthanized. Brains were perfused with cold saline, and coronal slabs were rapidly cut using a brain mold. Sections were kept cold on a refrigerated surface, and tissue punches removed for biochemical analysis by HPLC. Sections containing the midbrain, hippocampus and prefrontal cortex were post-fixed in paraformaldehyde and glutaraldehyde solutions as before (Elsworth et al., 2012), and then transferred to 0.1 M phosphate buffer containing sodium azide. We have shown that changes in number of spine synapses can be identified in this post-fixed tissue as effectively and accurately as perfusion fixed tissue (Elsworth et al., 2012). The number of DA neurons in each midbrain was determined using immunohistochemistry, and the total number of spine synapses in the CA1 stratum radiatum of the hippocampus and layers II/III of the prefrontal cortex was determined by electron microscopy.

2.3. BPA levels

Serum was prepared from maternal blood, and unconjugated (free) d-BPA concentration was measured by LC–MS analysis as described elsewhere (Taylor et al., 2011). Briefly, this entailed addition of internal standard (^{13}C -BPA), followed by extraction twice in methyl tertiary butyl ether. The extract was evaporated to dryness under nitrogen, and the residue reconstituted in 60% methanol. An aliquot was separated by reverse phase HPLC, with d-BPA and ^{13}C -BPA detected by selected ion monitoring of the mass-charge ratios 239 and 233 respectively. Quantification was achieved by reference to a standard curve of known concentrations of d-BPA and ^{13}C -BPA. Plasma d-BPA levels in juvenile monkeys were determined before implant, and again at 4 and 20 days after implantation, and a final time at euthanasia (30 days after implantation). Quantification was again accomplished by LC–MS, but the extraction procedure was simplified for the plasma samples from juveniles. Thus, proteins were precipitated by acetonitrile and methanol (2:1), and after centrifugation, the clear supernatant reduced to dryness under vacuum. The residue was reconstituted by mixing with 60% methanol, and an aliquot of this supernatant was analyzed by LC–MS. As a solvent extraction step was not included, an internal standard was not used and quantification was made by reference to a concentration range of 10 external standards of d-BPA.

2.4. Number of DA neurons in ventral mesencephalon

Serial vibratome sections (40 μm) were cut throughout the region, and every 10th section was immunostained for TH, a marker for DA neurons. Staining in ventral mesencephalon (substantia nigra and ventral tegmental area) was performed as described before (Leranth et

al., 1998). Briefly, vibratome sections were incubated in monoclonal mouse anti-TH monoclonal antibody (MAB318 at 1:1000, Chemicon, Temecula, CA), then after washing, sections were further processed according to the avidin-biotin-peroxidase complex (ABC) method using the Vectastain Elite Kit (Vector Laboratories, Burlingame, Calif., USA). Tissue-bound antibody was visualized by exposure to biotinylated horse anti-mouse IgG (BA-2000 at 1:200, Vector Laboratories), followed by incubation with avidin: biotinylated enzyme complex, and finally reaction with nickel-intensified diaminobenzidine as chromogen. Quantification comprised unbiased stereological counts of tyrosine hydroxylase immunoreactive cells in the regions of interest (Leranth et al., 2000). These measures were made using StereoInvestigator 7 software (MicroBrightField Inc.).

2.5. Number of spine synapses in hippocampus and prefrontal cortex

The number of asymmetric (excitatory) spine synapses in CA1 stratum radiatum and layers II/III of Walker's area 46 of DLPFC was calculated as published previously (Elsworth et al., 2011; Miettinen et al., 2012). Layers II/III of DLPFC were examined because pyramidal neurons in these layers send collaterals to associational cortical regions (Hoftman and Lewis, 2011) and asymmetric spine density in these layers has been correlated with working memory performance in primates (Peters et al., 2008). In addition in primates there is a relatively dense DA innervation of layers II/III in DLPFC (Kroner et al., 2007), which appears to regulate asymmetric spine synapse number (Elsworth et al., 2012). Furthermore, asymmetric spine number in layers II/III of DLPFC in adult monkeys is known to be susceptible to BPA (Hajszan and Leranth, 2010). The CA1 subfield of hippocampus was studied as it is known that this region is critical for memory formation (Bird and Burgess, 2008) and that asymmetric spine synapse number in this region in adult monkeys is particularly sensitive to BPA and to changes in gonadal hormone levels (Hajszan and Leranth, 2010; Leranth et al., 2002). Serial coronal sections (200 μ m) were made through hippocampus and DLPFC and systemically sorted to 10 groups. One randomly selected set was post-fixed in 1% osmium tetroxide, dehydrated in 70% alcohol containing 1% uranyl acetate, then flat-embedded in Durcupan (Electron Microscopy Sciences, Hatfield, PA). Boundaries of the hippocampal sampling region were readily identified by the highly organized cytoarchitecture of the region, and the boundaries of DLPFC were defined as described in (Tang et al., 2004). Twenty sampling sites for electron microscopic analysis were localized in both hippocampus CA1 region and layers II/III in DLPFC using a systematic-random approach. Ultrathin sections (75 nm) were cut at each identified sampling site, and digitized electron micrographs acquired at a magnification of 11,000 $\times$. Asymmetric spine synapses were counted according to the rules of the disector technique (MacLusky et al., 2006) within an unbiased counting frame superimposed onto each electron micrograph. Spine synapses were identified by the presence of postsynaptic densities, as well as by the absence of mitochondria, microtubules, and synaptic vesicles.

2.6. Concentrations of DA, serotonin and metabolites

Concentrations DA, homovanillic acid (HVA), 5-hydroxytryptamine (serotonin, 5HT) and 5-hydroxyindoleacetic acid (5HIAA) were measured in tissue dissected from the dorsal caudate nucleus and ventral bank of DLPFC of the juvenile green monkeys, using the method described previously (Morrow et al., 2011). Extracts were separated by reverse-phase isocratic HPLC, detected electrochemically, and quantified with respect to internal and external standards. The protein content of the centrifuged tissue pellet was measured using the Lowry method, and concentrations of DA, HVA, 5HT and 5HIAA were expressed as ng per mg protein.

2.7. Working memory performance

Juvenile subjects were tested for their ability to perform a 2-well spatial delayed response task in a custom-designed Wisconsin General Test apparatus, which fitted directly onto the cage (James et al., 2007). Training continued until performance was at 80% accuracy in at least two consecutive training sessions (chance performance in this 2-choice task is 50%). The “baseline” test was carried out before exposure to d-BPA, followed by 2 more testing days at 1 week and 2 weeks after the implants of d-BPA were inserted subcutaneously. Monkeys encountered 10 trials at each of 3 different delays (0, 2 and 4 s). Delays were counterbalanced and randomized across trials on each testing day, with 20 s elapsing between each trial. As preliminary analyses indicated that there was no effect of d-BPA exposure on the choice accuracy under the tested conditions, a final test was given during the week before euthanasia that employed 2 longer delays (6 and 12 s) with 20 trials at each delay and an inter-trial interval of 10 s. The conditions for this test were altered in order to increase the cognitive load.

2.8. Statistics

Variability within groups was calculated as $\pm$ standard error of the mean value. Group means were compared by parametric statistics, and details of individual results are provided in the appropriate sections of Section 3. Significance was achieved when $p < 0.05$, and lower probabilities were regarded as not significant (NS). Preliminary statistical comparison of data from males and females in the juvenile datasets revealed no difference for any of the dependent measures (plasma d-BPA level, accuracy on behavioral test, spine synapse number, DA neurons, and DA, HVA, 5HT, 5HIAA levels), so sex was not included as a separate factor in the final analyses.

3. Results

3.1. Oral prenatal exposure

Daily treatment of 5 monkeys with oral d-BPA for approximately the last 10 weeks of pregnancy produced a mean serum level of 0.68 ± 0.28 ng/ml unconjugated d-BPA. The number of DA neurons in the midbrain of each of the neonatal offspring of these pregnancies was counted and compared with 5 neonates that were treated prenatally with vehicle, using TH immunoreactivity to identify DA neurons. Fig 1 shows that exposure to d-BPA resulted in a significant loss of TH immunoreactivity in the substantia nigra and ventral tegmental area [2-tailed unpaired t -test, $t(8) = 5.4$, $p < 0.001$].

3.2. Subcutaneous prenatal exposure

Exposure of 6 pregnant monkeys for 50 days to d-BPA released from subcutaneously implanted silastic tubing resulted in a mean serum level of 0.91 ± 0.13 ng/ml unconjugated d-BPA. The number of spine synapses in sub-regions of hippocampus and prefrontal cortex of the offspring were counted, using electron microscopy (Fig. 2). The data were analyzed by 2-way ANOVA and Fig. 3 presents the data for these measurements. Significant effects of region [$F(1,11) = 186.5$, $p < 0.0001$], treatment [$F(1,11) = 7.3$, $p < 0.05$] and interaction [$F(1,11) = 6.0$, $p < 0.05$] between the factors were identified. Further analysis revealed that, compared with 3 control subjects, d-BPA treatment was associated with a significant loss of spine synapses in the CA1 region of hippocampus (6 samples, $p < 0.01$), but not in DLPFC (3 samples).

3.3. Juvenile exposure

Exposure of 8 juvenile monkeys to d-BPA delivered subcutaneously from silastic tubing resulted in a mean plasma level of 14.6 ± 0.9 ng/ml at 4 days after implantation, 16.8 ± 1.0

at 10 days after implantation and 13.1 ± 1.4 ng/ml at 30 days after implantation (time of euthanasia). Treatment of juveniles with d-BPA did not produce a change in the number of TH immunoreactive neurons in ventral mesencephalon compared with control monkeys [unpaired 2-tailed *t*-test, $t(6) = 0.68$, NS], see Fig. 1. More TH-immunoreactive neurons were detected in control ventral mesencephalon samples from juveniles compared with controls from the fetal exposure study, which may due to either the increase in number of ventral mesencephalon TH-expressing neurons that occurs during development (Morrow et al., 2005), the different Old World monkey species used in the studies (rhesus and green monkeys) or the slightly different fixative used in the studies. However as each study was internally controlled, the data on the effect of BPA on the number ventral mesencephalon TH-immunoreactive neurons is valid. No significant change was detected among the dopaminergic (Table 1) or serotonergic (Table 2) measures made in tissue sampled from caudate nucleus or DLPFC of juvenile monkeys treated with either d-BPA or vehicle. MANOVA was used to assess the effect of d-BPA on DA, HVA and HVA/DA ratio in the regions; caudate nucleus [$F(3,12) = 1.02$, NS] and DLPFC [$F(3,12) = 0.95$, NS]. HVA is the major metabolite of DA in the primate, and HVA/DA ratio provided an index of DA turnover. Similarly, MANOVA was used to assess the effect of d-BPA on 5HT, 5HIAA and 5HIAA/5HT ratio in the regions; caudate nucleus [$F(3,12) = 0.50$, NS] and DLPFC [$F(3,12) = 0.40$, NS]. 5HIAA is the major metabolite of 5HT, and 5HIAA/5HT provided a biochemical index of 5HT turnover.

In contrast to the data on prenatal exposure to d-BPA, treatment of 8 juveniles with d-BPA did not alter the number of spine synapses in the CA1 region of hippocampus or the DLPFC compared with 8 controls (Fig. 3). Thus, 2-way ANOVA did identify a significant effect of region [$F(1,28) = 331$, $p < 0.0001$], but no effect of treatment [$F(1,28) = 0.45$, NS] or interaction between the factors [$F(1,28) = 0.01$, NS].

Statistical analysis of the behavioral data indicated that exposure to d-BPA for 30 days did not affect performance of juvenile monkeys on the 2-well spatial delayed response task (Fig. 4). A repeated measures ANOVA (factors: 1-between, 2-within) identified a significant interaction between the 3 testing days (each using 0, 2 and 4 s delays) and treatment [$F(2,56) = 5.8$, $p < 0.01$], yet examination of the individual days by repeated measures ANOVA (factors: 1-between, 1-within) failed to locate a significant effect of d-BPA on performance accuracy on any test day. A separate and final test was given to the monkeys during the week before euthanasia, using conditions that are cognitively more demanding. If there was a borderline effect of d-BPA on performance accuracy under the original conditions then the more stringent parameters should have unmasked the effect; however, no effect of treatment [$F(1,28) = 0.04$, NS] was found.

4. Discussion

Exposure of non-human primate fetuses to relatively low levels of the endocrine disruptor, BPA, during the final 2 months of gestation induced a decrease in the number TH-expressing (DA) neurons in the ventral mesencephalon together with a reduction in the number of spine synapses in the CA1 region of hippocampus. In contrast, administration of BPA to juvenile monkeys in doses that achieved relatively high plasma levels was without effect on the number of TH-immunoreactive neurons in ventral mesencephalon or number of spine synapses in hippocampus. In addition, BPA treatment of juvenile monkeys did not alter performance on a cognitive task that tests working memory capacity, and which is dependent on the function of hippocampus and midbrain DA neurons. Taken together, these data strongly indicate that prenatal BPA exposure exerts a detrimental impact on primate brain development, at blood levels within the range measured in humans having only environmental contact with BPA.

4.1. BPA levels

The relevance of these findings has to be interpreted within the framework of documented levels of incidental oral exposure to BPA in humans. A large proportion of orally ingested BPA is metabolized by conjugation and has no relevant estrogenic potency (Taylor et al., 2011). Several studies have shown that the serum level of unconjugated BPA is typically in the range of 0.3–4.4 ng/ml in the general population (Vandenberg et al., 2010), and in the current study the mean BPA serum levels in the pregnant monkeys was less than 1 ng/ml. Pregnant monkeys were treated with either oral or subcutaneous BPA, and while both routes yielded similar mean serum levels of BPA, the pharmacokinetics of these 2 modes is different (Taylor et al., 2011). Thus, the oral route models a single daily exposure to BPA with a peak circulating level occurring at about an hour after ingestion and a terminal half-life of about 7–9 h. Subcutaneous treatment models more continuous exposure to BPA, with circulating levels that do not reach the extremes attained following oral BPA. Both the oral and subcutaneous deliveries used in the prenatal exposure studies reported here produced blood levels well within the range measured in human adults, and so the signs of brain abnormalities observed in non-human primate offspring appear to be a valid signal of possible impending neurological or psychiatric consequences of gestational exposure to these levels of BPA in humans.

4.2. BPA and DA function

A marked loss of TH-immunoreactivity in the ventral mesencephalon was observed following exposure to BPA at the prenatal, but not the juvenile, stage of development. These data suggest that BPA has a detrimental impact on midbrain DA neurons during fetal development but not at the juvenile stage of life, and this conclusion is supported by the normal DA and HVA concentrations measured in terminal regions of midbrain DA neurons in juvenile monkeys that were treated with BPA. If prenatal exposure to BPA were to lead to a permanent loss of some DA neurons then there could be detrimental consequences for functions mediated by DA signaling, such as executive function (including working memory), control of movement, and regulation of reward pathways. Even if BPA exposure only produced a temporary down-regulation of DA neuron signaling during fetal development, then other secondary effects would be expected, as DA acts as a morphogen or trophic factor in the developing brain, affecting neurite outgrowth, target selection and synapse formation (Herlenius and Lagercrantz, 2001; van Kesteren and Spencer, 2003).

4.3. BPA and spine synapses

A significant loss of spine synapses in hippocampus, but not prefrontal cortex, was observed following exposure in utero to BPA. In contrast, no effect of BPA on spine synapses was detected in hippocampus or DLPFC in juvenile monkeys. Unlike the hippocampus, the primate prefrontal cortex is undergoing significant developmental reorganization until early adulthood (Eckenhoff and Rakic, 1984; Giedd et al., 1999; Petanjek et al., 2011), so it is possible that the relative immaturity of the region enabled the fetal DLPFC to escape from a BPA-induced synaptic effect. The demonstrated marked effect of BPA on hippocampus spine synapses during development suggests a potential impact of BPA on mnemonic processes in the exposed offspring.

4.4. Effects of BPA on juveniles

In contrast to its effect on the fetus, BPA exposure at the juvenile stage of development did not appear to undermine DA neuron function or spine synapse formation despite these animals being exposed to relatively high circulating levels of BPA. In addition, juveniles treated with BPA did not suffer any measureable decrease in cognitive performance. However, the juvenile resistance to the effects of BPA is not retained in the adult monkey

(Hajszan and Leranath, 2010; Leranath et al., 2008). Even though no impact of BPA was detected on any of our endpoint measures in juvenile monkeys, it remains possible that exposure to BPA at this stage of life induces latent effects that have repercussion later in life.

4.5. Mechanisms responsible for the age-dependent impact of BPA

As there were significant effects of BPA exposure during prenatal development but not at the juvenile stage, it is important to consider factors that may explain this distinction. Many studies on BPA have attributed its biological properties to an agonist interaction with estrogen receptors. For example in vitro studies with hippocampal neurons have shown that BPA, like estradiol, enhances synapse and spine formation (Tanabe et al., 2012; Xu et al., 2010). However, the current data with fetal exposure to BPA, and our previous studies involving treatment of adult monkeys with BPA (Hajszan and Leranath, 2010), have demonstrated a that an anti-estrogen-like, rather than an estrogen-like, property of BPA being responsible for loss of synapses. The effects of BPA at estrogen receptors are complicated by its affinity for multiple subtypes of estrogen receptors, where it may initiate stimulatory or inhibitory effects on downstream events (Alonso-Magdalena et al., 2012; Marino et al., 2012; Zsarnovszky et al., 2005). Interference of estrogen activity by BPA in the developing brain would be expected to have especially marked and lasting detrimental consequences, as high levels of aromatase are expressed in the developing primate brain, and locally synthesized estrogens have a critical role in prenatal development, such as preventing cell death and stimulating the extension of neuronal processes (Roselli et al., 2009). In fact, a marked reduction of TH occurs in the midbrain of mice lacking estrogen receptors and also in normal mice in which aromatase activity is blocked during fetal development (Ivanova and Beyer, 2003; Kupperts et al., 2008). Thus, in the present study it seems quite feasible that BPA interfered with DA neuron development by disrupting the normal interaction of estrogens with receptors located on DA neurons. It is important to realize that BPA possesses diverse and complex pharmacological properties that appear to reach beyond its effects at estrogen receptors and which may well contribute to the observed developmental effects in the current study. It is particularly noteworthy that there is an interaction of BPA with thyroid hormones (Moriyama et al., 2002; Zoeller et al., 2005), as interruption of thyroid hormone function during the prenatal stage has more severe and lasting effects on brain structure and function than thyroid insufficiency later in life (Gilbert, 2011; Koibuchi and Iwasaki, 2006). Furthermore the regulation of some genes requires concerted action of both estradiol and thyroid hormone, and crosstalk between these hormones is probably necessary for normal neuronal development (Vasudevan and Pfaff, 2005). Thus, interference of the synergy between the estradiol and thyroid hormones is potentially a potent mechanism by which BPA could adversely impact brain development. As juvenile monkeys were exposed to higher levels of BPA than during the fetal development study, another seemingly remote possibility (but see Zsarnovszky et al., 2005) to account for the different effects of BPA is that higher doses of BPA have less apparent impact on our measures than lower doses. Although each of the above possible reasons for the lack of effect of BPA in the juvenile monkeys needs to be considered, a particularly plausible explanation for the age-dependent difference in response relies on the lower circulating estradiol concentration and lower level of centrally-derived estradiol at the juvenile stage compared with that in the fetus (Grumbach, 2002; Kupperts et al., 2001; McCarthy, 2008; McEwen, 1987). In the prenatal brain, high levels of circulating estradiol as well as locally synthesized estradiol mediate crucial developmental events and these effects may synergize with thyroid hormone actions. In such an environment, BPA acting in an anti-estrogen and anti-thyroid manner has great potential to inflict a significant and lasting impact on many aspects of brain development. On the other hand, in the juvenile brain, when levels of estradiol are relatively low, the consequences of BPA acting as in anti-

estrogen fashion would be muted, which is consistent with the effects observed in the current study.

5. Conclusion

Exposure of juvenile monkeys to relatively high levels of BPA did not alter our measurements of working memory performance, midbrain DA function or the number of hippocampal spine synapses. In contrast, exposure of non-human primate fetuses to levels of BPA measured in humans having only environmental contact with BPA induced a loss of midbrain TH-immunoreactive neurons and loss of hippocampal spine synapses.

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Fig. 1.

Age-dependent effect of BPA exposure on TH-immunoreactive (TH+) neurons in ventral mesencephalon (VM). Decrease in number of TH+ neurons in VM of fetuses exposed to BPA compared with controls. BPA was delivered orally each day to the pregnant monkeys in this study. There were 5 samples in each group, and * indicates significant difference from the control group ($p < 0.001$). In contrast, no difference in number of TH+ neurons in the VM of juvenile monkeys treated with BPA ($n = 4$) compared with controls ($n = 4$). Juvenile monkeys received BPA from a slow-release subcutaneous implant, which induced higher circulating BPA levels than oral administration of BPA to pregnant monkeys.

Fig. 2. Electron micrograph of the CA1 region of hippocampus, illustrating counting of spine synapses. Arrows show synapses, and “D” marks a typical dendrite. Scale bar is shown (0.5 μm)

Fig. 3. Age-dependent effect of BPA exposure on spine synapses in hippocampus. Loss of spine synapses in fetuses exposed to BPA compared with controls in CA1 region of hippocampus (6 BPA-exposed and 3 control samples). No change was detected in DLPFC (3 BPA-exposed and 3 controls). *Indicates a significant difference of the BPA group compared with controls ($p < 0.01$). In contrast, no loss of spine synapses in CA1 region of hippocampus or DLPFC of juvenile monkeys ($n = 8$) compared with controls ($n = 8$)

Fig. 4.

Juvenile monkeys ($n = 8$) treated with BPA displayed no deficits in working memory on a 2-well spatial delayed response task compared with controls ($n = 8$). There was no difference in accuracy of responding on any of the tested delays (0, 2, or 4 s)

Table 1

Levels of DA, 5HT and metabolites in caudate nucleus of control and BPA-treated juvenile monkeys.

	Control	BPA
DA	112.0 ± 9.2	121.5 ± 3.6
HVA	111.5 ± 4.7	119.2 ± 7.6
HVA/DA	1.0 ± 0.1	1.0 ± 0.1
5HT	1.6 ± 0.1	1.7 ± 0.2
5HIAA	3.2 ± 0.1	3.7 ± 0.4
5HIAA/5HT	2.1 ± 0.2	2.2 ± 0.2

Levels are in ng/mg protein. $n = 8$ for each group.

Table 2

Levels of DA, 5HT and metabolites in DLPFC of control and BPA-treated juvenile monkeys.

	Control	BPA
DA	0.26 ± 0.03	0.22 ± 0.02
HVA	5.0 ± 0.6	4.3 ± 0.4
HVA/DA	21.0 ± 3.7	20.2 ± 2.8
5HT	1.1 ± 0.1	1.2 ± 0.1
5HIAA	1.6 ± 0.2	1.8 ± 0.2
5HIAA/5HT	1.6 ± 0.2	1.6 ± 0.2

Levels are in ng/mg protein. $n = 8$ for each group.