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CORTICOSTERONE-REGULATED ACTIONS IN THE RAT BRAIN ARE AFFECTED BY PERINATAL EXPOSURE TO LOW DOSE OF BISPHENOL A

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Abstract—The estrogen-mimicking endocrine disrupter bisphenol A (BPA) which is used in the manufacture of plastic and epoxy resins, is one of the world's most heavily produced synthetic chemicals. BPA is detected in animal tissues, and its bio-accumulation has shown to be higher in the fetus than the mother. Exposure to doses below the daily safe limit has been reported to affect the sexual differentiation of the brain and modify the behavior of the exposed rodent offspring. The aim of the present study was to investigate in the rat the possible organizational effects of low BPA exposure on glucocorticoid-regulated responses. Female breeders were exposed to 40 μg/kg b.w. BPA daily throughout pregnancy and lactation. Plasma corticosterone levels and the two types of hippocampal corticosteroid receptors (GR and MR) were determined in mid-adolescent offspring under basal conditions and following a Y-maze task. BPA treated females had higher corticosterone levels than control females and BPA males and lower GR levels than BPA males, under basal conditions. Following the mildly stressful experience of Y-maze, corticosterone levels were increased in BPA-treated animals of both sexes, compared to the controls. GR levels were also increased in BPA-treated females compared to males. No effect of BPA was observed on MR levels, whereas the Y-maze experience significantly decreased receptors' levels in both female groups. The animals' performance in the task was also evaluated. BPA exposure significantly impaired the spatial recognition memory in both sexes, and modified the behavioural coping in a sex-dependent manner. Female BPA-treated offspring exhibited increased "anxiety-like" behaviour and dramatic loss of exploration attitude during the task, in comparison to males. This study provides for the first time evidence that corticosterone and its actions in the brain are sensitive to the programming effects of BPA at a dose below the currently acceptable daily intake.

Key words: glucocorticoid receptors, hippocampus, endocrine disrupters, Y-maze.

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Abbreviations: AD, anogenital distance; ANOVA, analysis of variance; BPA, bisphenol A; CBG, corticosterone binding globulin; GAPDH, glyceraldehyde-phosphate dehydrogenase; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; MR, mineralocorticoid receptor; OD, optical density; RIA, radioimmunoassay; SEM, standard error of mean; TDI, tolerable daily intake.

Bisphenol A (BPA) is one of the highest volume chemicals produced worldwide, with over 6 billion lbs. produced every year. It is a plastic monomer, used for production of polycarbonate plastics, epoxy resins, dental composite resins and many other everyday life products. In these products, BPA is gradually being hydrolyzed and leaches after use, leading to chronic organisms' exposure (Tsai, 2006). BPA has been detected in human and animal tissues and its retention has shown to be higher in the fetus than the mother (Vandenberg et al., 2009).

Initial toxicological studies in rodents determined the dose of 50 mg/kg/day as the non effect dose (Morrissey et al., 1987), based mainly on the absence of effects in the reproductive system. However, in the last years the biological harmlessness of low BPA doses has been questioned by several investigators, as even exposure below the "safe" dose of 50 μg/kg/day has been shown to affect the physiology of non-reproductive systems, particularly the brain function in rodents (vom Saal and Hughes, 2005).

A substantial body of evidence suggests that exposure of rodents to low doses of BPA during critical developmental periods, leads to persistent changes in brain structure and physiology (Richter et al., 2007). BPA administered perinatally in rats or mice can disrupt the development of sexually dimorphic brain areas (Kubo et al., 2003; Funabashi et al., 2004) and modify behavior (Farabollini et al., 1999; Kubo et al., 2001, 2003; Kawai et al., 2003; Rubin et al., 2006) or cognitive skills (Carr et al., 2003; Xu et al., 2007; Miyagawa et al., 2007). However, less is known on the molecular changes induced in the nervous system of offspring exposed to low dose BPA during the fetal and suckling period. Increased levels of estrogen receptors have been detected in the preoptic area and the pituitary of BPA-exposed male rats (Khurana et al., 2000; Ramos et al., 2003) and in the dorsal raphe nucleus of male mice (Kawai et al., 2007). In addition, 1-week old male rats exposed perinatally to BPA are known to exhibit increased levels of the steroid receptor co-activator 1 in the hippocampus (Xu et al., 2007).

During development, many neuronal circuits, including these of social behavior, stress response and cognition, mature under the organizational actions of sex steroids (Goy and McEwen, 1980). Previous studies suggest that the hypothalamic–pituitary–adrenal (HPA) axis and the hippocampus are potential targets for estrogen-mediated organizational events (O'Keefe and Handa, 1990; Handa et al., 1994). The limbic-HPA axis circuit is involved in the central regulation of stress response and in addition hippocampus is critically implicated in corticosterone-regulated spatial learning and memory (de Kloet et al., 2005a).

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Sex hormones stimulate and maintain hippocampal synaptic plasticity in a gender-specific way (Spencer et al., 2008). This structure hosts both types of corticosterone receptors (high affinity mineralocorticoid receptors, MR, and lower affinity glucocorticoid receptors, GR), as well as estrogen and androgen receptors and an interplay between glucocorticoids and gonadal steroids is responsible for the fine-tuning of hippocampal function in both sexes (Ahima et al., 1992; Patchev et al., 1999). Since maturation in the HPA axis and the hippocampus is not complete before adolescence in rodents (McCormick and Mathews, 2007) their circuits could be particularly vulnerable in the programming effects of endocrine disrupters.

Based on the above, we sought to investigate whether the organizational actions of BPA, at concentrations below safe dose, could affect components of the stress response and spatial memory system. We have thus determined the levels of plasma corticosterone and hippocampal corticosteroid receptors (GR and MR) in adolescent rats, exposed perinatally to BPA. The above parameters were evaluated under basal conditions and following a hippocampus-dependent spatial task, considered a mild stress for the rat (Conrad et al., 2004). As a functional endpoint, the spatial recognition memory of the animals was also recorded. Our results provide for the first time evidence that corticosterone and its brain actions are potential targets for BPA-induced organizational effects.

EXPERIMENTAL PROCEDURES

Animals and experimental design

The study was conducted in the offspring of rats treated with BPA during pregnancy and lactation (i.e. for a total period of 42 days). Eight female and four male Wistar rats (obtained at 6 weeks of age from Hellenic Pasteur Institute, Athens, Greece) were used for breeding. Breeders were housed under a 12:12 h light/dark cycle (lights on at 7:00 AM), temperature 22±2 °C and relative humidity of 60±10%, having free access to normal chow and tap water. After a habituation period of 2 weeks, two females were put in a cage with one male rat. Pregnancy was determined by vaginal smears obtained in the next morning. Pregnant dams were transferred to individual cages and were randomly assigned in two experimental groups of four animals each. Dams of the experimental group were administered orally BPA (40 μg/kg/day, Sigma-Aldrich Co, USA) during the entire period of pregnancy and lactation. Body weight of dams was determined daily. BPA was dissolved in ethanol, further diluted in water (1% in ethanol) and 1 μl/10 g b.w. of this solution was pipetted onto one cornflake that was allowed to dry completely before use. Fresh solution and flakes were prepared daily and administered between 10.00 and 11.00 AM. Female breeders of the control group were offered dried cornflakes carrying the vehicle alone.

The day of birth was defined as postnatal day 0. In total, 34 offspring were born in the experimental group (13 male and 21 female) and 36 offspring (17 male and 19 female) in the control group. Anogenital distance was measured at postnatal day 1. All pups were weaned at postnatal day 22, earmarked and transferred into new cages according to sex and treatment. At mid-adolescence (postnatal day 46; McCormick and Mathews, 2007), 18 offspring from the BPA group and 18 of the control group were sacrificed by decapitation under basal conditions. The rest 16 BPA offspring (seven male and nine female) and 18 control offspring (nine male and nine female) were tested in the Y-maze task and sacrificed immediately afterwards. Trunk blood was collected from all animals in heparinized vials and plasma was separated by centrifugation and stored at −20 °C until use. Hippocampi were dissected on ice, immediately frozen in liquid nitrogen and stored at −80 °C for Western blot analysis.

All animal treatments were carried out in agreement with ethical recommendation of the European Communities Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Behavioral testing

Animals' ability for spatial recognition memory was assessed at postnatal day 46 by using the Y-maze test (Dellu et al., 1992; Conrad et al., 1996). In brief, a black, wooden Y-maze apparatus, having three identical arms (50 long×16 wide×32 cm high) was used. Extra-maze visual cues (posters and laboratory equipment) were placed asymmetrically around the maze enabling spatial orientation. The investigator was not visible by the rat. Rats were tested during the light phase of their cycle (between 9:00 and 13:00 h). The testing room was next to animals' room. The floor of the maze was covered with sawdust bedding that was mixed for each animal of the same gender and completely renewed for animals of the other sex, to reduce the possibility of using odors as a cue. The task consisted of two trials having a 4 h interval. The same arm was always designated as the "start" arm, while the other two arms were alternating as "novel" and "known" arm between rats to reduce arm-bias effects. In trial 1, novel arm was blocked with a black wooden guillotine. The animal was introduced in the start arm facing the wall of the arm and allowed to explore the unblocked arms for 15 min. At the end of trial 1, the rat was returned to its home cage. In trial 2, all arms were accessible and the rat was allowed to explore them for 5 min. The animals were recorded with a video-camera adjusted on the ceiling of the room. The videos were analyzed blindly by two independent observers. The dependent variables measured were: (a) the total number of entries in all arms (for the 5 min of trial 1 and for the 1st and 5 min period of trial 2), (b) the % entries in each arm for the 1st and 5 min period of trial 2 and (c) the duration the animal stayed in each arm and in the center of the apparatus during trial 2. An arm entry was counted when the head and two front paws were inside the arm, and duration of an arm visit was ended when the head and two front paws were outside the arm again.

Hormonal determinations

Plasma corticosterone levels were determined by radioimmunoassay using a RIA kit for small rodents (MP Biomedicals, Orangeburg, NY, USA). The inter- and intra-assay coefficients of variation were both 8%. As an index of gonadal status plasma levels of testosterone and progesterone were determined at sacrifice. Progesterone levels were determined by electrochemiluminescence immunoassay (ECLIA) using a human progesterone kit (Roche Diagnostics GmbH, Mannheim, Germany; analytical sensitivity 0.03 ng/ml). Testosterone levels were determined by radioimmunoassay (Immunotech SAS, Marseille, France). The analytical sensitivity of the kit is 0.025 ng/ml. The inter- and intra-assay coefficients of variation were 15% and 14.8% respectively.

Western blot analysis

Frozen hippocampi were homogenized using a hand-held motorized homogenizer in NP-40 lysis buffer (137 mM NaCl, 20 mM Tris–HCl pH 8.0, 1% NP-40 (v/v), 10% glycerol (v/v), 48 mM NaF, 2 mM Na_3VO_4, and protease inhibitor mix). The homogenates were incubated on ice for 30 min and centrifuged for 20 min at 16000×g at 4 °C. The supernatants were removed and their protein content was measured. Samples of 50 μg total protein were loaded onto 4%–12% polyacrylamide gels (Invitrogen Co.,