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A multi-generational study on low-dose BPA exposure in Wistar rats: Effects on maternal behavior, flavor intake and development☆

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ABSTRACT

Bisphenol A (BPA) is a common endocrine disruptor found as an environmental and food contaminant. It exerts both developmental and behavioral effects, mainly when exposure occurs in early life. The aim of this study was to determine the multi-generational effects of chronic, human-relevant low-dose exposure to BPA on development, maternal behavior and flavor preference in Wistar rats.

BPA was orally administered at a daily dose of 5 μg/kg body weight to F0 pregnant dams from the first day of gestation (GD 1) until the last day of lactation (LD 21), and then to F1 offspring from weaning (PND 21) to adulthood (PND 100). F2 offspring were not exposed. Development and clinical signs of toxicity were assessed daily. Maternal behavior was evaluated by observing nursing and pup-caring actions, as well as "non-maternal" behaviors in F0 and F1 dams from parturition until LD 8. The flavor preferences of F1 and F2 offspring were evaluated based on the intake of sweet, salt and fat solutions using the two-bottle choice test on PND 21–34 and PND 86–99.

BPA exposure: 1) decreased maternal behavior in F1 dams, 2) caused developmental defects in both F1 and F2 offspring, with a noticeable decrease in anogenital distance in male rats, and 3) did not affect flavored solution intake in F1, but induced changes in sweet preference in F2 juveniles and in salt and fat solution intakes in F2 adults, and 4) induced a body weight increase in the F2 generation only, whereas food intake and water consumption did not change.

Taken as a whole, our findings showed that both gestational (F0) and lifelong (F1) exposures to a human-relevant dose of BPA could induce multi-generational effects on both development and behavior. These results suggest possible selective neuroendocrine defects and/or epigenetic changes caused by BPA exposure.

1. Introduction

BPA is a man-made compound that is used to make polycarbonate plastics and epoxy resins. It is found in human blood and urine due to its ubiquitous use in the production of water bottles, plastic food containers, linings of food and beverage cans and medical products, such as syringes and dental sealants. It is used as an additive in plastics such as PVC. Halogenated derivatives of BPA are also widely used as flame retardants (http://www.bisphenol-a.org).

The United States (U.S.) Environmental Protection Agency (EPA) reference dose for BPA (50 μg/kg BW/day) was calculated by applying a safety factor of 1000 to the lowest observed adverse effect level (LOAEL) derived from high-dose toxicological studies (http://www.epa.gov/iris/subst/0356.htm). However, several *in vivo* studies have reported developmental and behavioral effects of low-dose BPA exposure depending on non-monotonic dose responses that contradict the logic of "the dose makes the poison" (Della Seta et al., 2005; Melzer et al., 2010; Ryan et al., 2010; Xu et al., 2011b).

In humans, recent reports have revealed a link between the endocrine disruptor levels in biological fluids and the prevalence of certain diseases, including heart disease, obesity and diabetes (Melzer et al., 2010; Lang et al., 2008; Shankar and Teppala, 2011). Based on the widespread use of BPA, it has been reported as a factor involved in the emergence of these diseases (Arnich et al., 2011).

As evidenced by *in vivo* studies, adverse reproductive and developmental effects of BPA can result from early exposure to very low doses, down to the no observable adverse effect level (NOAEL = 5 mg/kg BW/day) (Salian et al., 2009; Rubin et al., 2001; Salian et al., 2011; Braniste et al., 2010; Cabaton et al., 2011). Similarly, prenatal

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daily exposure to BPA in the range of [1–50] μg/kg BW is associated with several behavioral disorders, including altered sexual, social, learning and maternal behaviors (Farabollini et al., 2002; Palanza and Gioiosa, 2008). The prenatal period is not the only determinant exposure window during which maternal behavior disruption can be initiated, since the administration of BPA at [10–40 μg/kg BW] daily low doses later in adulthood causes similar adverse effects (Della Seta et al., 2005; Palanza et al., 2002). Likewise, similar daily exposure during puberty affects behavioral sex differences by masculinizing the social and emotional behaviors of females (Yu et al., 2011). These findings indicate that exposure to BPA can have adverse effects at various ages for doses below the acceptable daily intake referenced as a "safe" dose for humans by the US Food and Drug Administration (FDA).

Moreover, it is suspected that the adipogenic effect, induced by exposures to low doses of BPA early in life, could be related to neuronal disruptions of the brain areas involved in the selection of flavor intake and food choice (Rubin et al., 2001; Somm et al., 2009; Miyawaki et al., 2007; Ryan et al., 2010). Sexual dimorphism in taste preferences has been well established, and the influence of estrogen exposure during the neonatal period has been clearly shown on both salt (Krecek et al., 1972) and sweet intakes (Curtis et al., 2005). Thus, due to the fact that BPA can produce estrogen- or anti-estrogen-like effects that modify the sexual dimorphism of the brain (Palanza and Gioiosa, 2008), one important question was whether BPA exposure alters the manifestation of the sexual dimorphism in sweet and in salt solution intake. Only one study has shown that perinatal exposure to BPA low dose (<10 μg/kg BW) caused changes in body weight and in sweet preference in both male and female offspring at adulthood (Xu et al., 2011b). However, knowledge about the impact of a continuous exposure on taste dimorphism remains to be developed.

Taken as a whole, the available data led us to hypothesize that exposure to BPA low-doses could simultaneously affect development and behavior, but questions arise about the impact of window and time exposure on the untreated offspring. The present study focused on the multigenerational impact of oral exposure to 5 μg BPA/kg BW/day (*i.e.*, 10 fold lower than the NOAEL) on development and behavior endpoints using an integrative approach. Our experimental model was designed to monitor the effects of BPA exposure over three generations: the F0 generation was only exposed to BPA in adulthood (pregnancy/lactation), while the F1 generation was exposed from conception until mating, and the F2 generation was not exposed. In this way, multigenerational effects on development, maternal behavior and taste preferences could be observed simultaneously.

2. Materials and methods

2.1. Chemicals

BPA, saccharin, xanthan gum and sodium chloride (≥99% purity) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Corn oil was purchased from Lesieur (Asnières-Sur-Seine, France). All of the products complied with the quality standards of the European Pharmacopoeia (http://www.edqm.eu/en/Homepage-628.html).

2.2. Animal care and experimental design

All procedures involving rats were approved by the local authorities, and adhered to the French Ministry of Agriculture's ethical guidelines for the care and use of laboratory animals. This study was carried out within the framework of a national research program (PNR-PE) involving several teams using the same pool of animals. The experimental design is presented in Fig. 1.

A total of 36 SPF (specific pathogen-free) female and 36 SPF male Wistar Han rats were purchased from Harlan France Sarl (Gannat, France) at 12 weeks of age. They were fed a low-soy diet (2018 Harlan Teklad, Gannat, France) up to their arrival. On arrival, they were acclimatized to SPF housing conditions (22 °C room temperature with 55% relative humidity and a 12-hour light/dark period (8 am–8 pm)) for 2 weeks before mating. Polypropylene cages and bottles were used to avoid any BPA or phthalate contamination. Furthermore, to avoid exposure to any endocrine-like chemical residues, aspen sawdust was used as bedding, and animals were allowed *ad libitum* access to charcoal-filtered water and a purified phytoestrogen-free diet (INRA, Jouy-en-Josas, France) containing 18% casein, 40% corn starch, 20% maltodextrin, 6% sucrose, 5% corn oil, 5% cellulose, 5% mineral mixture and 1% vitamin cocktail as previously described (Stroheker et al., 2003).

At 14 weeks of age, one female was mated with one male rat. Females were examined daily to determine the first gestational day (GD 1) by observing the presence of spermatozoids in the vaginal smears or the vaginal plug. The mating period did not exceed 7 days. At GD 1, each dam was identified by a numbered ear tag, caged individually and assigned randomly to either the control or BPA group (15–16/group). They were treated and examined daily up to parturition.

On the day of parturition, designated as postnatal day 1 (PND 1), all pups in each litter were weighed and sexed, and each litter was randomly culled to ten pups (5 males and 5 females per litter, when possible). The pups remained with their biological dam whenever possible, but some pups were cross-fostered by another dam of the same group from the day of parturition to maintain equal numbers of each sex.

At weaning (PND 21), male and female offspring were identified by introducing a microchip under the skin. They were then divided into two sets; one was used to produce the F2 generation (16 animals per group; one male and one female per litter for each group) and the other was used for the developmental and behavioral studies at the juvenile and adult stages (24 animals per group; one male and one female per litter for each group). They were housed 4 per cage with other members of the same sex and the same treatment (one pup per litter), except for the flavor testing periods (PND 21–34 and PND 86–99), during which the animals were housed in individual cages. They were fed the same diet and water *ad libitum* and received BPA until PND 100 (Fig. 1). The remaining animals were used by other teams involved in the same "PNR-PE" research program.

At PND 100, BPA treatments ended. The F1 offspring, designated to generate the F2 generation, were similarly mated by pairing one male with one female (8 pairs per group). No brother–sister mating pairs were used. The F2 generation was established in a manner similar to that used for the F1 generation, using the same husbandry conditions.

2.3. BPA exposure

BPA (5 μg/kg BW) was dissolved in corn oil and administered orally with a micropipette following the procedure of Palanza et al. (2002). A 100 μl micropipette (Microman Gilson M100) and a 250 μl micropipette (Microman Gilson M250) were used to administer BPA to rats weighing between 50 and 250 g and weighing over, respectively. Animals were weighed daily so as to adjust the BPA dose to the increasing body weight. The dams were treated from day 0 of gestation (GD 1) until the last day of lactation (LD 21). The F1 offspring were then treated every two days with the same dosing solutions from weaning (PND 21) until mating at adulthood (PND 100). The control animals received vehicle alone (doses were delivered in 0.4 ml/kg BW). The F2 offspring did not receive any BPA treatment.

To prevent photolysis and oxidation, the solutions were stored at 4 °C in aluminum-foil-wrapped vials.

2.4. Measurement of gestational end points and postnatal development of offspring

For each generation, the dams and litters were observed daily for clinical signs of developmental or behavioral toxicity. Dam body weights and food and water consumption were recorded once a week during gestation and lactation. On the parturition day (PND 1), gestation