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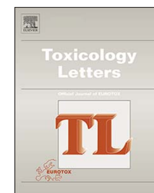

Neonatal exposure to bisphenol A alters the hypothalamic-pituitary-thyroid axis in female rats

Marina O. Fernandez[a,*], Nadia S. Bourguignon[a], Paula Arocena[a], Matías Rosa[a], Carlos Libertun[a,b], Victoria Lux-Lantos[a]

[a] Consejo Nacional de Investigaciones Cientificas y Técnicas, Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, CABA, Argentina
[b] Universidad de Buenos Aires, School of Medicine, Dept of Physiology and Biophysics, CABA, Argentina

A R T I C L E I N F O

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A B S T R A C T

Bisphenol A (BPA) is a component of polycarbonate plastics, epoxy resins and polystyrene found in many common products. Several reports revealed potent *in vivo* and *in vitro* effects. In this study we analyzed the effects of the exposure to BPA in the hypothalamic-pituitary-thyroid axis in female rats, both *in vivo* and *in vitro*.

Female Sprague-Dawley rats were injected sc from postnatal day 1 (PND1) to PND10 with BPA: 500 μg 50 μl^{-1} oil (B500), or 50 μg 50 μl^{-1} (B50), or 5 μg 50 μl^{-1} (B5). Controls were injected with 50 μl vehicle during the same period.

Neonatal exposure to BPA did not modify TSH levels in PND13 females, but it increased them in adults in estrus. Serum T4 was lower in B5 and B500 with regards to Control, whereas no difference was seen in T3. No significant differences were observed in TRH, TSHβ and TRH receptor expression between groups. TSH release from PPC obtained from adults in estrus was also higher in B50 with regard to Control.

In vitro 24 h pre-treatment with BPA or E_2 increased basal TSH as well as prolactin release. On the other hand, both BPA and E_2 lowered the response to TRH.

The results presented here show that the neonatal exposure to BPA alters the hypothalamic pituitary-thyroid axis in adult rats in estrus, possibly with effects on the pituitary and thyroid. They also show that BPA alters TSH release from rat PPC through direct actions on the pituitary.

1. Introduction

According to the Environmental Protection Agency (EPA) of the United States, an endocrine disruptor is “an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior” (Kavlock et al., 1996). Bisphenol A (BPA), a chemical found in many consumer products, is an endocrine disruptor (Nagel and Bromfield, 2013). BPA is a constituent of polycarbonate plastics and epoxy resins used in food industry and dentistry. The polymer bonds hydrolyze at high temperature and release BPA. BPA can be ingested by humans, as detectable amounts were found in food cans, microwave containers, human saliva after treatment with dental sealants, and polycarbonate bottles (Vandenberg et al., 2007). Neonates and children are among the groups where more elevated levels of BPA were found. Infants can be exposed to BPA through different sources: some studies have found detectable levels of BPA in breast milk with levels of 0.28–0.97 ng ml^{-1} and 1.1 ng ml^{-1} according to two different studies, in human colostrum with levels of 1–7 ng ml^{-1}, in polycarbonate baby bottles (Vandenberg et al., 2007) and medical devices (Calafat et al., 2009). Edginton and col. (Edginton and Ritter, 2009) estimated the daily exposure for newborns and children at 3 and 6 months of age, under different feeding scenarios. They showed that they could be exposed to BPA at doses that range from 0.25 μg kg^{-1}/day (in newborn breast-fed) to 8.3–13 μg kg^{-1}/day (in 6 month-old infants fed commercial formula or other beverages using polycarbonate bottles).

Although *in vitro* assays have suggested that BPA is a weak environmental estrogen receptor alpha agonist, it has also been shown to antagonize the effects of estrogens, androgens, and thyroid hormones; act through non-genomic pathways; and influence enzyme activity or receptor expression (Wetherill et al., 2007). *In vivo* effects also have been reported based on studies using a wide range of doses, animal models, and end-points. The current EPA reference dose for BPA

* Corresponding author at: Laboratory of Neuroendocrinology, Instituto de Biología y Medicina Experimental (IByME-CONICET), Vuelta de Obligado 2490, Ciudad Autónoma de Buenos Aires, C1428ADN, Argentina.
E-mail address: mfernandez@dna.uba.ar (M.O. Fernandez).

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($50\,\mu g\,kg^{-1}$/day) was calculated by dividing the lowest observed adverse effect level (LOAEL = $50\,mg\,kg^{-1}$/day), based on an uterotrophic assay performed in rats and mice (Morrissey et al., 1987) by 1000 (Welshons et al., 2003). In some reports, low doses are considered those below the LOAEL. Effects of BPA can vary depending on species, strain, dose, time of exposure and end-point studied, and adult animals exposed to BPA show effects that are reversible when the exposure ceases (Richter et al., 2007). In contrast, perinatal/neonatal exposures produce "organizational" effects [effects resulting from exposure during organ development and continuing through puberty that may result in persistent alterations of the affected systems (Richter et al., 2007)], in different strains of rats (Fernandez et al., 2009, 2010; Adewale et al., 2009; Rubin et al., 2001; Moral et al., 2008; Patisaul et al., 2006; Kato et al., 2003; Ramos et al., 2003; Durando et al., 2007; Khurana et al., 2000).

Thyroid hormone is essential for normal development, and for maintenance of normal physiological functions. The regulation of thyroid hormone delivery to tissues and cells during development and in the adult represents a very complex and unique (among endocrine systems) web of feedback systems. Environmental factors, such as iodine deficiency or the presence of specific toxicants, such as BPA, can perturb this web at various points of regulation, inducing a variety of responses that are captured in toxicological assays. The thyroid axis is controlled by the thyrotropin-releasing hormone (TRH) synthesized in the hypothalamus. TRH is released into the pituitary-portal circulation and it targets the anterior pituitary, where it stimulates the synthesis and release of thyrotropin (TSH) from specific cells, the thyrotropes; TRH also affects the post-translational glycosylation of TSH, which affects its biological activity. TSH, on the other hand, binds to receptors on the surface of thyroid follicle cells in the thyroid gland, stimulating adenylate cyclase. The effect of the increased cyclic AMP is to increase the production of thyroxine (T4) and triiodothyronine (T3). T4 is the major product released from the thyroid gland. Thyroid hormones (T4 and T3) exert a negative feedback effect on the release of pituitary TSH and on the activity of hypothalamic TRH neurons (Zoeller et al., 2007).

Previous studies showed that, among its various mechanisms of action, BPA is able to act as a thyroid hormone antagonist *in vitro* (Moriyama et al., 2002) and that exposure to BPA from gestational day 6 through lactation altered serum T4 on PND15 pups and increased RC3/neurogranin in males without changes in TSH (Zoeller et al., 2005). On the other hand, exposure to male rats in the peripubertal period showed increased thyroid weight (Tan et al., 2003). All these studies suggest that BPA affects the hypothalamic-pituitary-thyroid axis in vivo. In this study, we analyzed the effects of the exposure to BPA in another sensitive period of development, the neonatal period, on the hypothalamic-pituitary-thyroid axis in infantile and adult female rats. We hypothesized that exposure to BPA during this period of time could have long lasting effects the hypothalamic-pituitary-thyroid axis in females.

2. Materials and methods

2.1. Animals

Studies on animals were performed according to protocols for animal use, approved by the Institutional Animal Care and Use Committee (IBYME-CONICET), in accordance with the Division of Animal Welfare, Office for Protection from Research Risks, National Institutes of Health, Animal Welfare Assurance for the Institute of Biology and Experimental Medicine A#5072-01. Sprague-Dawley rats (200–250 g) from the IBYME colony were maintained under a controlled 12-h light/dark cycle and temperature conditions. They were treated humanely, housed in steel cages with bedding material and given free access to laboratory chow and tap water in glass bottles.

Males and females were mated. On the day of birth (postnatal day 1, PND1), eight neonates were left with the dam. Twelve dams (twelve litters) were used for the study, each litter was adjusted to eight pups. In a first pilot study, only Control (C), B50 and B500 groups were included and later a more extensive study including all doses was performed (C, B5, B50 and B500). One or two female pups from each dam were randomly assigned to the different experimental groups to avoid the litter effect and were injected subcutaneously (sc) from PND1 to PND10 (ten consecutive doses, one per day during the first ten days of life) with BPA (Aldrich, WI, USA) in castor oil, as indicated: $500\,\mu g$ $50\,\mu l^{-1}$, (B500; 62.5–25.0 $mg\,kg^{-1}$ on PND1-PND10) $50\,\mu g$ $50\,\mu l^{-1}$ (B50; dose range: 6.2-2.5 $mg\,kg^{-1}$ from PND1-PND10), $5\,\mu g$ $50\,\mu l^{-1}$ (B5; 0.625–0.25 $mg\,kg^{-1}$ on PND1-PND10) or with vehicle (50 µl castor oil, also called ricinus oil, Control). The selection of castor oil for vehicle treatment as well as BPA diluent was based on its reported weak estrogenicity (Hughes, 1988) and was previously used in our laboratory as a vehicle for BPA (10;11). If a litter contained two animals from the same experimental group, those animals were assigned to different end points. Blood samples were obtained by decapitation from 9 to 11 AM, collected in tubes and serum was prepared. Briefly, tubes were left at room temperature for 2–3 h until clots formed, sera were collected in microcentrifuge tubes, centrifuged at 1000 rpm at room temperature in a Sorvall Legend microcentrifuge to pellet the remaining cells, supernatant collected in new tubes and kept at −20 °C until used. Serum TSH was measured by RIA at PND13 (n = 4–6) and in adults in estrus PND90-PND120 (n = 8–11). Serum T3 and T4 were measured in adults in estrus PND90-PND120 by ELISA (n = 10). Animals were sacrificed in the morning of estrus (9–11 am) as B500 showed marked estrus persistence (Fernandez et al., 2009). Estrous cycles were determined from PND60-PND120 by examining vaginal smears obtained with a pipette under a light microscope (Fernandez et al., 2009). Smears were classified as diestrus, proestrus or estrus based on cellular morphology.

Primary pituitary cell cultures (PPC) were obtained from adults in estrus exposed neonatally to BPA and controls. TSH released to the media was measured by RIA.

2.2. Gene expression determination

Anterior pituitary (n = 3–9) and hypothalamic mRNAs (n = 5) were extracted from adult females in estrus neonatally exposed to BPA and controls. Animals were sacrificed in the morning of estrus as before. The hypothalami (POA-MBH) were delimited as follows: anteriorly by a plane at the height of the anterior commissure, laterally by the hypothalamic fissures, posteriorly by the mammilary bodies, and in-depth by the subthalamic sulcus. 1 µg of RNA was retrotranscribed and quantitative PCR was performed using HOT FIREPol EvaGreen qPCR Mix Plus (ROX) (Solis Biodyne) in a Bio Rad CFX96 Real-Time System. Cyclophilin B (*Ppib*) as housekeeping gene and results were analyzed using the mathematical model of Pfaffl (Pfaffl, 2001). QPCR primers were (5′- > 3‘): *Tshb*-for: ATCTTCCTGCCAGAGGGACT, *Tshb*-rev: CCT CCACCTTTTGTGCTGTT, *Trhr*-for: GATGTACGTGGACAGGAGAGAG TGT, *Trhr*-rev: GACATCCTGAGAGAGTGCGTACTTG, *Trh*-for: TTCTGG ATTCCTGGTTCTCAGATG, *Trh*-rev: GGATGTTGCCTCTTGGTGACA, *Ppib*-for: GACCCTCCGTGGCCAACGAT, *Ppib*-rev: GTCACTCGTCCTACA GGTTCGTCTC

2.3. Primary pituitary cell cultures

PPC were obtained from C, B50 and B500 adults in estrus as described (Mongiat et al., 2006). Briefly, pituitaries were rapidly removed and placed in Dulbecco's modified Eagle medium low glucose (DMEM, GIBCO, Invitrogen Corporation, Grand Island, NY) supplemented with 2 $mmol\,l^{-1}$ glutamine (AppliChem, Darmstadt, Germany), 25 $ng\,ml^{-1}$ fungizone (GIBCO) and 25 $ng\,ml^{-1}$ gentamicin (GIBCO). Pituitaries were cut into small pieces and incubated in 0.2% trypsin for 30 min. After addition of DNase and fetal bovine serum, fragments were washed in Krebs-Ringer bicarbonate buffer without Ca^{2+} and Mg^{2+}, dispersed gently into individual cells and filtered through Nytex mesh. Cells were

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