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

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ABSTRACT

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

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(50 μg 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 μg 50 μl^{-1}, (B500; 62.5–25.0 mg kg^{-1} on PND1-PND10) 50 μg 50 μl^{-1} (B50; dose range: 6.2-2.5 mg kg^{-1} from PND1-PND10), 5 μg 50 μl^{-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: CCTCCACCTTTTGTGCTGTT, *Trhr*-for: GATGTACGTGGACAGGAGAGAGTGT, *Trhr*-rev: GACATCCTGAGAGAGTGCGTACTTG, *Trh*-for: TTCTGGATTCCTGGTTCTCAGATG, *Trh*-rev: GGATGTTGCCTCTTGGTGACA, *Ppib*-for: GACCCTCCGTGGCCAACGAT, *Ppib*-rev: GTCACTCGTCCTACAGGTTCGTCTC

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

plated (50,000 cells/well) in DMEM low glucose, supplemented with 10% horse serum (PAA Laboratories, Austria), 2.5% fetal calf serum (PAA), 1% MEM nonessential amino acids (GIBCO), fungizone and gentamicin (medium 1). After 5 days in culture, cells were washed and incubated with serum-free DMEM-F12 (GIBCO) containing 2.2 g l^{-1} NaCO3H and 0.1% BSA (SIGMA-Aldrich, MO) (medium 2) for 1 h. Media were then stored at −20 °C until TSH analysis by RIA.

To assess the *in vitro* exposure to BPA, PPC were performed from PND13 normal Sprague-Dawley females as before. Cultures were incubated for 24 hs in medium 2 in the presence or absence of BPA 1.10^{-7} mol L^{-1} or E_2 1.10^{-7} mol L^{-1}. After changing the media, cells were further incubated for an extra hour with the same stimuli alone or in presence of TRH 1.10^{-7} mol L^{-1}. TSH and prolactin (PRL) in the media were determined by RIA.

2.4. Hormone dosage

TSH and PRL were determined by RIA using kits obtained through NHPP, NIDDK and Dr. A. Parlow. Results were expressed in terms of RP-3 rat TSH and RP-3 rat PRL standards. Assay sensitivity was 0.031 ng ml^{-1} for TSH and 1.6 ng ml^{-1} for PRL. Intra and inter assay coefficients of variation were 8.7% y 13.4%, respectively for TSH and 8.1% y 11.4% for PRL. Hormones were measured in more than one assay with all groups represented in each assay. T3 and T4 were determined by ELISA using the Microparticle Enzyme Immunoassay (MEIA) technology (Abbot), according to manufacturer's instructions.

2.5. Statistical analyses

Data was expressed as Mean +/− Standard Error. Results were analyzed by one-way or two-way analysis of variance (ANOVA) (Statistica v8, StatSoft, OK, USA) on the mean with a Fisher posttest and transformed when the test for homogeneity of variances so required. Results obtained from the primary pituitary cultures were analyzed by Repeated Measures Two-Way ANOVA with a Fisher posttest. Normality and homogeneity of variances were tested using Statistica v8. Results were considered significant when $p < 0.05$.

3. Results

3.1. Neonatal exposure to bisphenol a alters TSH serum levels in adult female rats but not in infantile rats

TSH was determined in serum from infantile and adult female rats. There was no difference in serum TSH levels in PND13 animals (Fig. 1A). On the other hand, neonatal exposure to 50 µg of BPA increased serum TSH levels in adults in estrus (Fig. 1B). Interestingly, 5 and 500 µg did not significantly change TSH serum levels when compared to controls. Endocrine disruptors, and especially BPA, often act in non-monotonic dose-response curves, so although this result is interesting, it is not surprising for this compound (Vandenberg, 2014).

Anterior pituitary cells from controls and from females treated neonatally with 50 and 500 µg of BPA were obtained as described (Mongiat et al., 2006). Cultures from females treated with 50 µg of BPA released more TSH to the media than Controls, resembling *in vivo* results (Fig. 1C).

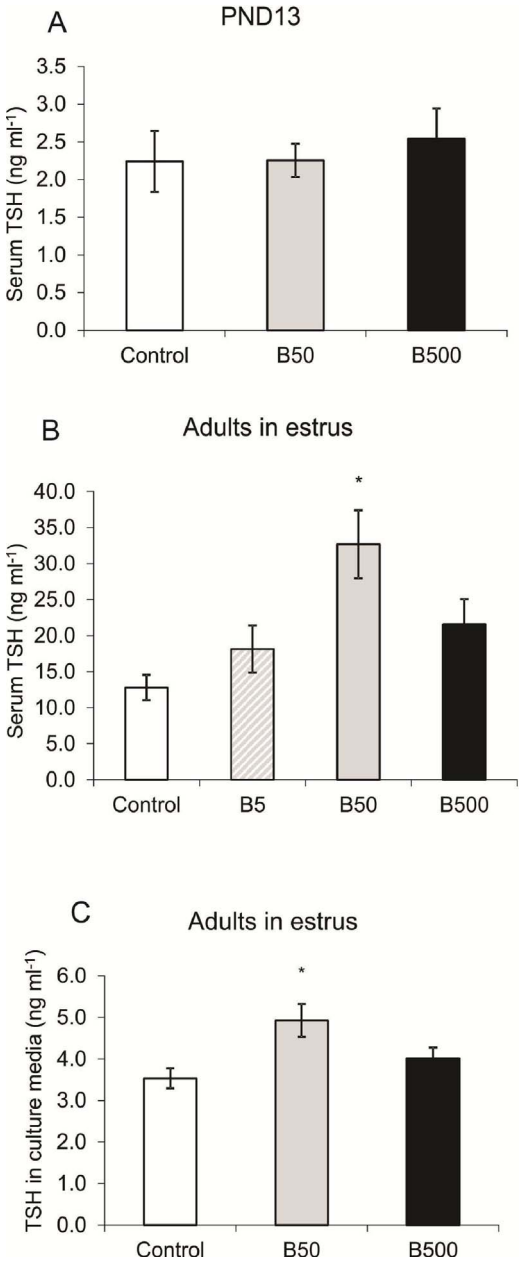

Fig. 1. Neonatal exposure to BPA alters TSH in adult females.
A) Serum TSH levels in PND13 rats neonatally exposed to B50 (n = 4), B500 (n = 5) or controls (n = 6). ANOVA [F = 0.2; p = 0.8]: not significant (ns). B) Serum TSH levels in adult rats in estrus, neonatally exposed B5 (n = 8), B50 (n = 11), B500 (n = 11) or controls (n = 11). ANOVA [F = 5.98; p = 0.002]; * = different from Control, $p < 0.005$. C) PPC were obtained from adult females exposed neonatally to BPA or from controls. TSH was measured in serum free media. ANOVA [F = 5.27; p = 0.02]; * = different from Control, $p < 0.01$, n = 6.

3.2. Neonatal exposure to BPA alters T4 but not T3 serum levels

Females exposed neonatally to 5 and 500 µg of BPA, but not 50 µg, had lower serum T4 levels than Controls (Fig. 2). T3 levels were not significantly different between groups when analyzed by ANOVA. However, B500 was different from Control by *t*-test (Control = 0.69 ± 0.03, B500 = 0.56 ± 0.05; $p < 0.05$, n = 10). This result suggests that the exposure to BPA alters the physiology of the pituitary thyrotrope and also the thyroid. The decrease in thyroid hormone does not translate into an increase in TSH, showing that the negative feedback loop could be disrupted.

3.3. Neonatal exposure to BPA does not alter pituitary TSHβ, TRHR or hypothalamic TRH mRNA expression

Expression of TSHβ and TRHR from pituitaries and TRH from hypothalamus of females exposed neonatally to BPA and controls were examined (Fig. 3A–C). There was no significant difference in any of the evaluated genes between the groups, although TSHβ tended to be higher in BPA treated groups, which is consistent with the observed increase in TSH.

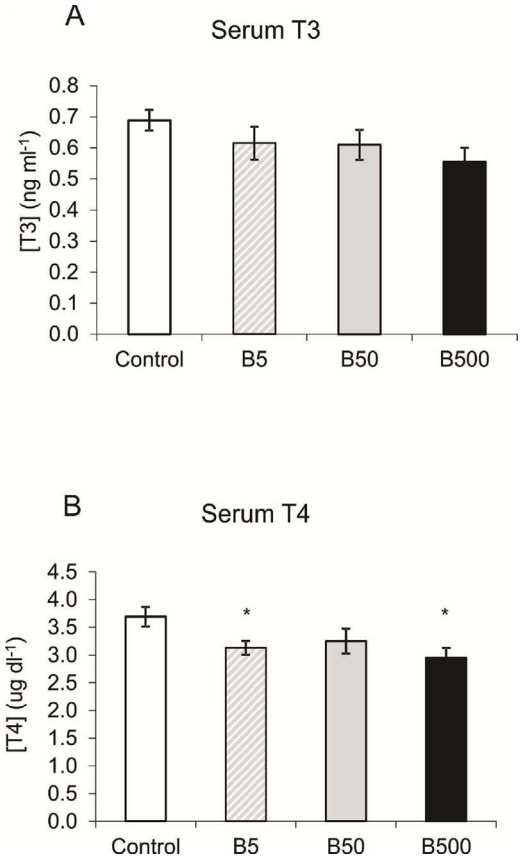

Fig. 2. Neonatal exposure to BPA alters thyroid hormone in adult females. Serum T3 (A) and T4 (B) levels in adult rats neonatally exposed to B5, B50, B500 or controls. (A) ANOVA [F = 1.4; p = 0.2]: ns. B) ANOVA [F = 2.9; p = 0.047]: significant; * = different from Control, $p < 0.05$, n = 10 per group.

3.4. In vitro exposure to BPA alters the pituitary response to TRH

To assess the direct effects of BPA in PPC from naïve rats, PPCs from PND13 females were obtained. Cultures were pretreated for 24 h with BPA, E_2 or vehicle as Control. *In vitro* exposure to BPA and E_2 increased TSH as well as PRL release to culture media with a similar potency (Figs. 4A and 5A respectively) and decreased the response to TRH (Figs. 4B and 5B respectively). This result suggests that BPA is able to act directly on the pituitary thyrotropes to increase TSH (and on lactotropes to increase PRL) and that the exposure also desensitizes the pituitary response to TRH action.

4. Discussion

In this paper we report that the neonatal exposure to BPA alters the hypothalamic-pituitary-thyroid axis in adult female rats. Serum TSH and T4 are altered in BPA-exposed adult females, although the results are intriguing. Females exposed to the medium dose of BPA exhibit the highest TSH levels, whereas the high and low doses show slight increases that are not statistically significant. As noted before, endocrine disruptors act through non monotonic dose response curves (Vandenberg, 2014). Release of TSH from primary pituitary cultures resembles the result obtained *in vivo*, suggesting that the neonatal exposure to 50 µg BPA reprogrammed the thyrotrope to release higher TSH independently from the action of the thyroid and other organs of the body. On the other hand, PND13 females did not exhibit an increase in TSH. This result is consistent with the report from Zoeller et al. that show that the exposure to BPA, from gestational day 6 through lactation does not alter TSH on PND15 in males (Zoeller et al., 2005).

The effect on serum T3 and T4 levels is also interesting. Serum T3 is

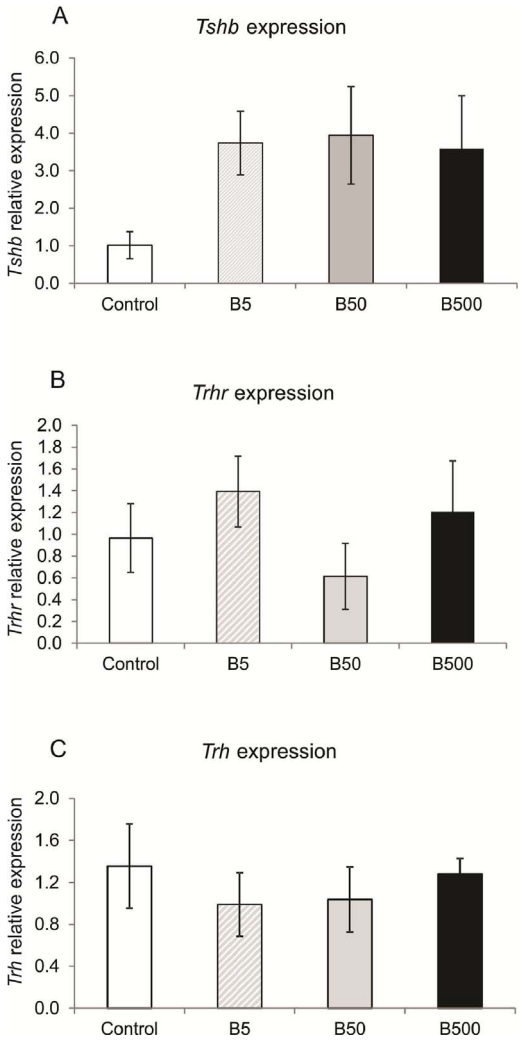

Fig. 3. Neonatal exposure to BPA does not alter pituitary *Tshb* or *Trhr* gene expression in the adult pituitary nor hypothalamic *Trh* expression.
Tshb (A) (n = 5–9) and *Trhr* (B) (n = 3k4) relative expression in pituitaries from adult females in estrus neonatally treated with vehicle or BPA were measured by qPCR. Cyclophilin B (*Ppib)* was used as housekeeping gene and results were relativized to Control. ANOVA for *Tshb* [F = 2.5; p = 0.08], ANOVA for *Trhr* [F = 0.8; p = 0.5]: ns. *Trh* (C) (n = 5) relative expression in hypothalamus from adults in estrus treated with vehicle or BPA were measured by qPCR. Cyclophilin B (*Ppib)* was used as housekeeping gene and results were relativized to Control. ANOVA [F = 0.3; p = 0.8]: ns.

not significantly different between groups, although B500 show a decrease when compared to the control group alone. Serum T4 is lower in the B5 and B500 groups, and there is no difference in the B50 group. If the hypothalamic-pituitary-thyroid axis had been functioning properly, and considering the higher TSH levels in the B50 groups, we would have expected that this group would have had the highest thyroid hormone levels. This is not the case, so this suggests that the thyroid has an impaired sensitivity to TSH levels in BPA-treated groups. The classical explanation for the phenomenon observed is that, as T4 is low in BPA-treated groups, TSH should be higher; however, the lower thyroid hormone levels in the B5 and B500 groups did not result in a significant increase in serum TSH in these animals. Although significant, these lower levels of thyroid hormone might not be sufficient to produce an increase in TSH, so it is evident that this feedback loop is also disrupted by neonatal BPA. BPA is a thyroid hormone antagonist (Moriyama et al., 2002), so exposure during the neonatal period could reprogram the pituitary and also the thyroid. It is also possible that BPA could modify TSH glycosylation and therefore bioactivity, as it was reported that the thyroidectomy and the hypothyroid state decreases TSH

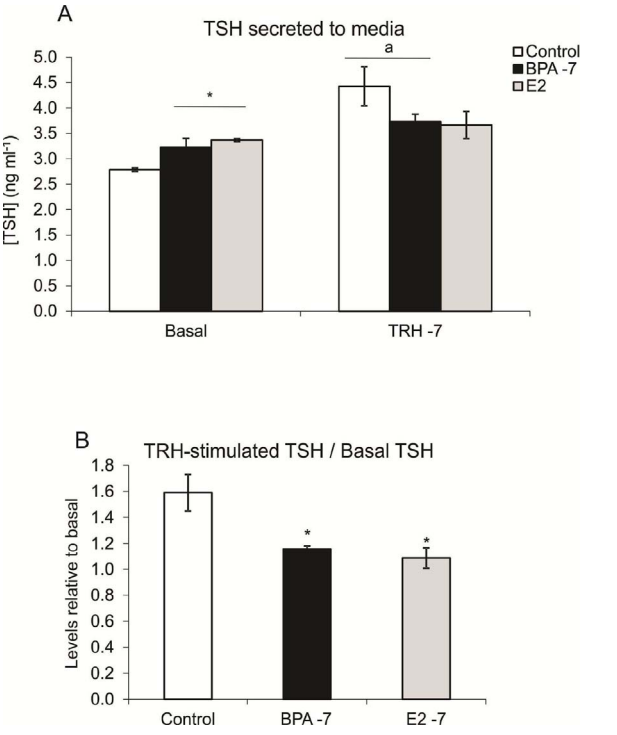

Fig. 4. *In vitro* exposure to BPA alters thyrotrope response to TRH.
A) TSH secreted to media from normal PND13 PPC (n = 3). Cultures were pretreated with BPA or E_2 (1.10^{-7} mol L^{-1}) or media alone and response to TRH (1.10^{-7} mol L^{-1}) was evaluated. Repeated measures two-way ANOVA: Interaction [F = 7.1; p = 0.03]: significant. * = different from Control, $p < 0.05$; a = different from basal, $p < 0.05$. B) TRH-stimulated TSH/basal TSH. Repeated measures ANOVA [F = 7.1; p = 0.048]: significant, *: different from control, $p < 0.05$.

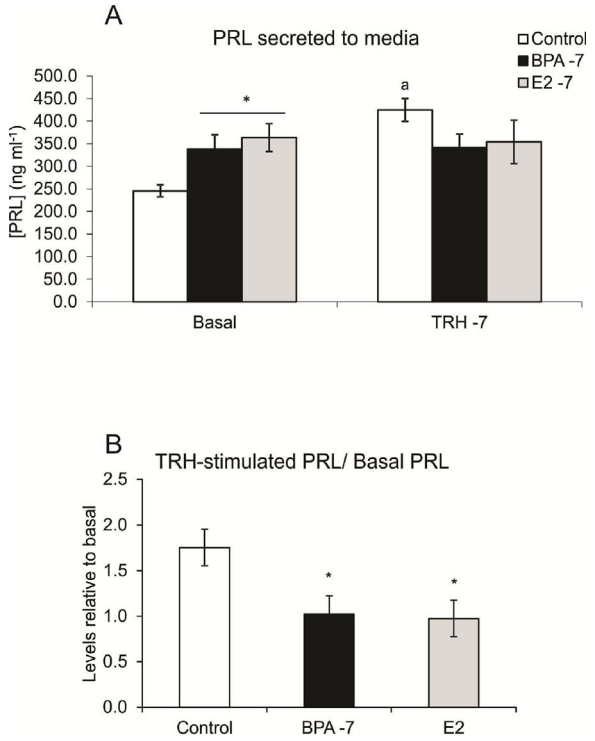

Fig. 5. *In vitro* exposure to BPA alters lactotrope response to TRH.
A) PRL secreted to media from normal PND13 PPC (n = 3). Cultures were pretreated with BPA or E_2 (1.10^{-7} mol L^{-1}) or media alone and response to TRH (1.10^{-7} mol L^{-1}) was evaluated. Repeated measures two-way ANOVA: Interaction [F = 8.8; p = 0.02]: significant. * = different from Control, $p < 0.05$; a = different from basal, $p < 0.05$. B) TRH-stimulated PRL/basal PRL. Repeated measures ANOVA [F = 14.3; p = 0.02]: significant. *: different from control, $p < 0.02$.

bioactivity (Oliveira et al., 2007; Menezes-Ferreira et al., 1986).

When pituitary and hypothalamic gene expression was analyzed, no significant difference was observed between groups, but TSHβ expression seemed higher in the BPA-treated groups. This result is consistent with the observed increase in TSH serum levels, as there may be other factors involved in the observed TSH serum increase, like an increase in the rate of translation or secretion of TSH. Further experiments would be needed in order to explain the phenomenon observed. It is important to note that the fact that the whole hypothalamus was used for the qPCR analysis could have influenced these findings. Hypophysiotropic TRH neurons are located in the PVN (Zoeller et al., 2007), and these are the neurons that respond to thyroid hormone, and, as a large area of the hypothalamus was employed in our study, effects on those neurons were possibly swamped by non-responsive TRH neurons. A detailed study analyzing expression in the specific nucleus would have been more accurate and would have given extra information about the effects of BPA, and this will be addressed in future studies.

Literature shows conflicting data regarding effects of estrogenic compounds on the thyroid axis. Depending on the animal model and strain, route of application and duration of treatment various results have been reported. Lisboa et al. show that treatment with estradiol benzoate increases serum TSH in rats (Lisboa et al., 1997); Seidlova-Wuttke et al. show a tendency for increased TSH levels in E_2-treated ovariectomized (ovx) rats. In this paper, serum T4 levels were unaffected by E_2 and other compounds like BPA. In contrast, a significant reduction of T3 serum levels was measured in the E_2-treated ovx rats (Seidlova-Wuttke et al., 2005). Klammer et al. showed a slight but not significant increase in TSH-serum levels after E_2 treatment, and a slight (also not significant) decrease in T3 with no difference in T4 (Klammer et al., 2007). Thomas et al. (Thomas et al., 1986) reported lower T3 levels in ovx compared to intact rats with no change in T4, while Chen and Walfish (Chen and Walfish, 1978) reported an E_2-induced elevation of total T3-levels and a decrease in total T4. Another report showed no effects of a long-term treatment with E_2 on either TSH-, T4- or T3-levels in young ovx rats while in middle-aged ovx T3 did not change and T4 was decreased by E_2 (Bottner and Wuttke, 2005). Some of these reports suggest that there could be interplay between the hypothalamic-pituitary-gonadal and thyroid axes that could be responsible for the effects observed in our report *in vivo*, as an increase in estradiol was previously published in the B50 and B500 animal groups (Fernandez et al., 2010). No positive control, like 17β-estradiol or estradiol benzoate, was included in our study.

Lastly, the direct effect of BPA on normal pituitary cell function was assessed. *In vitro* exposure to BPA as well as E_2 increased basal TSH and PRL release to the media when compared to controls, and lowered the release in response to TRH. The reduced response to TRH may be due to increased basal secretion that would deplete the cells from TSH or PRL vesicles ready to be released upon secretagogue addition. Interestingly, the potency to exert these effects is similar between E_2 and BPA, contrary to the concept that BPA is a mild estrogen agonist. These results suggest that BPA could be acting as a thyroid hormone antagonist or an estrogen receptor agonist, reinforcing the notion of the multiple mechanisms of action proposed for this compound. To our knowledge this is the first report that shows that in vitro exposure to E_2 and BPA alters basal pituitary release of TSH and also the pituitary to response to TRH.

The results presented here show that exposure to BPA alters the hypothalamic pituitary thyroid axis both *in vivo* and *in vitro*, and suggest that it could be acting both at the pituitary and thyroid gland levels.

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