

Bisphenol-A, an Environmental Contaminant that Acts as a Thyroid Hormone Receptor Antagonist *in Vitro*, Increases Serum Thyroxine, and Alters RC3/Neurogranin Expression in the Developing Rat Brain

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Considering the importance of thyroid hormone (TH) in brain development, it is of potential concern that a wide variety of environmental chemicals can interfere with thyroid function or, perhaps of greater concern, with TH action at its receptor (TR). Recently bisphenol-A (BPA, 4,4' isopropylidenediphenol) was reported to bind to the rat TR and act as an antagonist *in vitro*. BPA is a high production volume chemical, with more than 800 million kg of BPA produced annually in the United States alone. It is detectable in serum of pregnant women and cord serum taken at birth; is 5-fold higher in amniotic fluid at 15–18 wk gestation, compared with maternal serum; and was found in concentrations of up to 100 ng/g in placenta. Thus, the human population is widely exposed to BPA and it appears to

accumulate in the fetus. We now report that dietary exposure to BPA of Sprague Dawley rats during pregnancy and lactation causes an increase in serum total T_4 in pups on postnatal d 15, but serum TSH was not different from controls. The expression of the TH-responsive gene RC3/neurogranin, measured by *in situ* hybridization, was significantly up-regulated by BPA in the dentate gyrus. These findings suggest that BPA acts as a TH antagonist on the β -TR, which mediates the negative feedback effect of TH on the pituitary gland, but that BPA is less effective at antagonizing TH on the α -TR, leaving TR α -mediated events to respond to elevated T_4 . (*Endocrinology* 146: 607–612, 2005)

THYROID HORMONE (TH) is essential for normal brain development in both humans (1, 2) and animals (3–5). Moreover, it is becoming increasingly clear that mild and transient TH insufficiency can affect cognitive outcome in humans (6), and that the developmental timing of transient TH insufficiency produces different cognitive deficits (7). An important implication of these studies is that environmental chemicals that produce TH insufficiency or interfere with TH signaling during development may alter important developmental events. Moreover, if an environmental chemical alters TH signaling by selectively interfering with subset(s) of TH receptors (TRs), the consequences to brain development may be a mosaic of effects on the nervous system because different TRs mediate different actions of TH during development (8–11).

Although several authors have speculated that specific environmental chemicals might bind to TRs and alter TH signaling (12–16), we (17) and others (18) have failed to find convincing evidence that suspected thyroid toxicants can bind with high affinity to the TR. Therefore, it was surprising that Moriyama *et al.* (19) recently reported that the estrogenic compound bisphenol-A (BPA, 4,4' isopropylidenediphenol) binds to the TR. Best characterized as a weak estrogen (20),

binding to the estrogen receptor with an inhibitory constant of approximately 10^{-5} M (21, 22), BPA binds to and antagonizes T_3 activation of the TR (19, 23). The inhibitory constant for BPA inhibition of T_3 binding to rat TR is approximately 10^{-4} M, but as little as 10^{-6} M BPA significantly inhibits TR-mediated gene activation (19). Moreover, Moriyama *et al.* found that BPA reduced T_3 -mediated gene expression in culture by enhancing the interaction with the nuclear receptor corepressor (N-CoR) (19).

Thus, BPA is the first environmental chemical known to bind to the TR and affect TH signaling *in vitro*. BPA is produced at a rate of more than 800 million kg annually in the United States alone (24) and is used primarily in the manufacture of plastics including polycarbonate plastics and epoxy resins that coat food cans and in dental sealants (25, 26). Howe *et al.* (25) estimated human consumption of BPA from food cans alone to be about 6.6 μ g/person·d. BPA has been reported in concentrations of 1–10 ng/ml in serum of pregnant women and cord serum taken at birth (27, 28). Importantly, BPA was found to be 5-fold higher in amniotic fluid at 15–18 wk gestation, compared with maternal serum (29), and was found in concentrations of up to 100 ng/g in placenta (27). Thus, the human population is widely exposed to BPA and concentrations appear to accumulate in the fetus.

Despite these observations, there is no direct information about its effects on TH signaling *in vivo*, especially in brain development. Iwamuro *et al.* (29) reported that BPA has an antimetamorphic effect on *Xenopus laevis*, blocking T_3 -induced tail resorption and T_3 -induction of TR in tail tissue. In addition, Seiwa *et al.* (30) showed that BPA antagonizes the

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Abbreviations: BPA, Bisphenol-A; G, gestational day; N-CoR, nuclear receptor corepressor; P, postnatal day; TH, thyroid hormone; TR, TH receptor; UTP, uridine 5-triphosphate.

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ability of TH to affect oligodendrocyte differentiation *in vitro*. We now report that maternal exposure to BPA in rats can elevate serum T_4 in nursing rat pups but that the effect on TH signaling in the developing brain is consistent with the elevated levels of T_4 , producing a profile that is reminiscent of thyroid resistance syndrome (31).

Materials and Methods

Animals

All animal procedures followed the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and were approved by the University of Massachusetts-Amherst Institutional Animal Care and Use Committee. Timed-pregnant female Sprague Dawley rats ($n = 36$, 314 ± 53 g; Zivic Miller Laboratories, Inc., Pittsburgh, PA) arrived in our facility on gestational day (G) 2. Animals were individually housed in plastic cages with food and water provided continuously and maintained on a 12-h light, 12-h dark cycle (0600–1800 h). Beginning on the day of arrival, each dam was weighed in the morning and provided with a single untreated wafer (Keebler miniwafers; Keebler, Elmhurst, IL) 1 h before lights off. This initial period (G2–G6) trained the animals to consume the wafer quickly during the experimental procedure. Beginning on G6 and continuing throughout the experiment, dams were weighed in the morning and provided daily with a wafer dosed with 1 μ g/g body weight of a solution calibrated to deliver specific doses of BPA. To accomplish this, BPA was dissolved in contaminant-free methanol at one of four concentrations: 0, 1, 10, and 50 μ g/ μ l and pipetted (1 μ l/g body weight of the dam; final dose of 0, 1, 10, and 50 mg/kg) onto a wafer and allowed to dry under a fume hood throughout the day before feeding. Administration of BPA on a wafer that the animals voluntarily consume has the advantage of mimicking the route of exposure in humans (oral) and avoiding the variability in exposure by mixing BPA in the feed or water or causing the stress associated with gavage.

Control wafers (0 mg/kg) were dosed with methanol alone and allowed to dry. The dose range of BPA was chosen to overlap with that of Tyl et al. (32), who chose these nominal doses to bracket the effects of BPA on mouse prostate and testis weight (33–35). Pups were weighed and killed on postnatal day (P) 4, P8, P15, and P35. Trunk blood was collected for serum at all time points; the brain was collected, labeled, and stored at -80°C .

In situ hybridization

Frozen brain tissues were sectioned in coronal plane at 12 μ m in a cryostat (Reichert-Jung Frigocut 2800N, Leica Corp., Deerfield, IL). Coronal sections were made through the rostral hippocampus of P15 males approximately corresponding to Figs. 29–33 of Paxinos and Watson (36). Two adjacent sections were thaw mounted onto each twice gelatin-coated microscope slides and stored at -80°C until hybridization. Prehybridization treatments, hybridization, and posthybridization washes were carried out for RC3/neurogranin as described earlier (17, 37). Two slides from each brain (four sections) were hybridized, arranged in x-ray cassettes with ^{14}C standards (American Radiolabeled Chemicals, St. Louis, MO), and apposed to BioMax film (Eastman Kodak Co., Rochester, NY).

The RC3 probes (complementary or sense-strand) were generated *in vitro* from an RC3 cDNA [kindly provided by Dr. Juan Bernal (plasmid PRC/cytomegalovirus-RC3) (38), Madrid, Spain]. The transcription reaction was performed in a final volume of 10 μ l. RNA was synthesized in the presence of 1 μ g DNA template (linearized plasmid); 500 μ M each of GTP, ATP, and CTP; and 12 μ M uridine 5-triphosphate (UTP) (UTP to ^{33}P -UTP at a molar ratio of 1:1). For cRNA production, pRC/CMV-RC3 was linearized with *Hind*III and transcribed in the presence of SP6 RNA polymerase. The DNA template was then removed by deoxyribonuclease digestion, and the probe was purified by phenol/chloroform extraction followed by two ethanol precipitations.

Autoradiography and signal quantitation

To analyze the hybridization signal, a 5-fold magnified image of the signal over the hippocampus was captured using a Scion AG-5 capture

board interfaced with the public domain NIH-Image 1.61/ppc (W. Rasband, National Institute of Mental Health, Bethesda, MD) run on a Macintosh (Cupertino, CA) G4. The optical system consisted of a Dage-72 (Michigan City, IN) series video camera equipped with a Nikon (Melville, NY) macrolens mounted onto a bellows system over a light box. Film density was measured over the dentate gyrus (upper and lower leaflet) or cortex of P15 brains as an index of the relative levels of mRNA expression. Resulting values were average over the four sections for each brain, with one brain per litter and nine litters per treatment. Differences in sample sizes reflect both sample loss during processing and differences in litter sizes among individual dams.

RIA

Total T_4 was measured in 5 μ l of rat serum as we have described previously (17). Briefly, each assay tube contained 100 μ l barbital buffer [0.11 M barbital (pH 8.6), 0.1% wt/vol 8-anilino-1-naphthalene-sulfonic acid ammonium salt, 15% bovine γ -globulin Cohn fraction II, and 0.1% gelatin], 100 μ l anti- T_4 (rabbit, Sigma, St. Louis, MO) diluted to provide a final concentration of 1:30,000, and 100 μ l ^{125}I -labeled T_4 (Perkin-Elmer/NEN Life Science Products, Boston, MA). Standards were prepared from T_4 (Sigma) measured using a Cahn electrobalance; standards were run in triplicate and calibrated to measure serum T_4 from 0.4 to 25.6 μ g/dl. Tubes were incubated at 37°C for 30 min and then chilled on wet ice for 30 min. Bound counts were precipitated by adding 300 μ l ice-cold polyethylene glycol 8000 (20% wt/vol; Sigma). Tubes were centrifuged at $1800 \times g$ for 20 min at 4°C , and the supernatant was aspirated and counted in a γ -counter (CobraII, Packard, Meriden, CT).

Statistical analysis

A two-way ANOVA was performed on body weight of the dams, followed by two single one-way ANOVAs on body weight gain (pregnancy vs. lactation). Serum total T_4 was initially analyzed using a three-way ANOVA (age $\times$ treatment $\times$ gender as main factors); because there was no interaction between treatment and gender, two-way ANOVAs were then performed separately on data derived from males and females. One-way ANOVAs were used to analyze all other data. *Post hoc* tests, where appropriate, were performed by Bonferroni's *t* test, where the mean square error term in the ANOVA table was used as the point estimate of the pooled variance (SuperAnova Software, Abacus Concepts, Inc., Berkeley, CA).

Results

Body weight

Two-way ANOVA of maternal body weight revealed a significant effect of treatment ($F_{3,396} = 9.005$; $P = 0.0001$), day (of pregnancy/lactation) ($F_{12,396} = 32.607$; $P = 0.0001$), and a significant interaction ($F_{36,396} = 1.458$; $P = 0.0464$) (Fig. 1A). However, further evaluation indicated that maternal body weight gain during pregnancy was significantly lower in dams treated with 50 mg/kg BPA, compared with controls (Fig. 1B; $F_{3,27} = 2.908$; $P = 0.0089$) but that body weight gain during lactation was not affected by BPA treatment. Despite these effects on maternal body weight during pregnancy, there were no observed effects of BPA exposure on litter size (data not shown) or pup weights taken at P4, P8, or P15 ($F_{3,121} = 2.033$; $P = 0.113$) (Fig. 2).

Serum hormone levels

A three-way ANOVA (age $\times$ treatment $\times$ gender) of T_4 levels in pups revealed significant effects of age ($F_{3,213} = 217.97$; $P = 0.0001$) and treatment ($F_{3,213} = 3.75$; $P = 0.0118$) but not gender ($F_{1,213} = 0.003$; $P = 0.953$). In addition, there was a significant interaction between age and treatment ($F_{9,213} = 2.43$; $P = 0.012$), but no other significant interactions

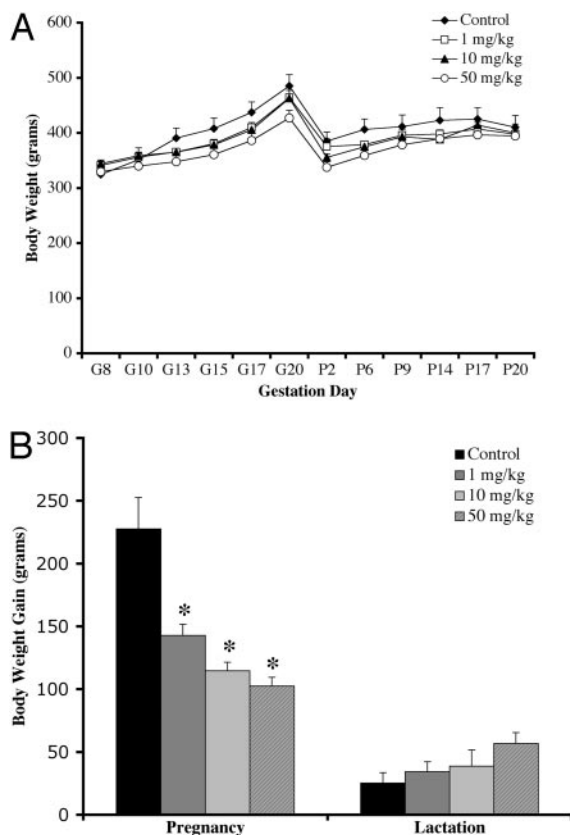


FIG. 1. Effect of BPA treatment on maternal body weight (A) and body weight gain (B) in Sprague Dawley dams ($n = 9/\text{group}$). Although ANOVA revealed that, overall, BPA caused a reduction in body weight ($F_{3,365} = 13.734$; $P = 0.0001$), the extent to which dams gained weight during pregnancy was significantly affected by all doses of BPA (B). Note that maternal body weight gain during lactation was not significantly affected by BPA exposure. Data points (A) or bars (B) represent mean $\pm$ SEM. *, Significantly different from body weight gain in control animals ($P > 0.001$) using Bonferroni-Dunn's *post hoc* test.

were present (age $\times$ gender, treatment $\times$ gender, or age $\times$ treatment $\times$ gender). Further inspection of these data indicated that maternal BPA exposure significantly increased serum total T_4 in both male and female pups (Fig. 3). Pups derived from BPA-treated dams exhibited significantly higher levels of T_4 on P15 ($F_{3,55} = 3.704$; $P = 0.0169$) (Fig. 3).

Because P15 is a critical time for TH action in the rat brain (39) and there appeared to be no substantive gender differences in the effects of BPA on the hypothalamic-pituitary-thyroid axis, we chose to focus on the ability of BPA to alter TH signaling in P15 males. First, we determined whether the BPA-induced increase in serum T_4 in male pups on P15 is associated with an increase in serum TSH; however, we observed no significant differences in mean TSH levels among P15 males born to dams being treated with various BPA concentrations ($F_{3,23} = 0.802$; $P = 0.505$) (Fig. 4). However, despite the finding that TSH levels were not different among groups, there were significant treatment effects of BPA on RC3/neurogranin expression in the upper ($F_{3,22} = 6.48$; $P = 0.01$) and lower leaflet of the dentate gyrus ($F_{3,22} = 6.58$; $P = 0.0024$) but not in the cortex ($F_{3,22} = 1.514$; NS) (Fig. 5). RC3/neurogranin mRNA levels in both leaflets of the

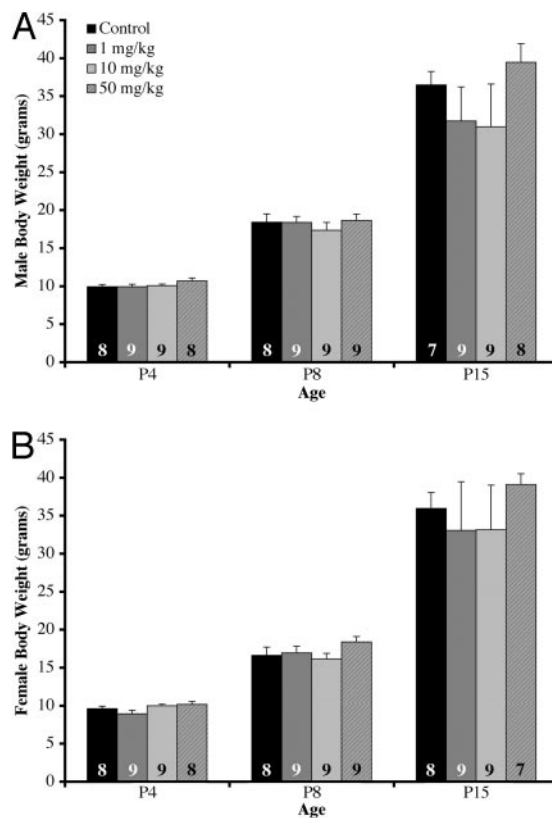


FIG. 2. Effect of BPA treatment on body weight of male (A) and female (B) pups on P4, P8, and P15. No effects of treatment were observed. Bars represent mean $\pm$ SEM. Group size is listed in the bar.

dentate gyrus were significantly higher in all BPA-treated animals, compared with controls.

Discussion

The present findings strongly suggest that BPA acts as a thyroid hormone antagonist *in vivo*. Maternal exposure to BPA caused an increase in serum T_4 of both male and female pups but simultaneously increased the expression of RC3/neurogranin in the hippocampus. The simplest explanation for these findings is that BPA acts as an antagonist of TH action on the β -TR, inhibiting TH-negative feedback but leaving the α -TR unopposed in responding to elevated T_4 in the hippocampus and presumably elsewhere. This is the first report of the *in vivo* effects of an environmental chemical that

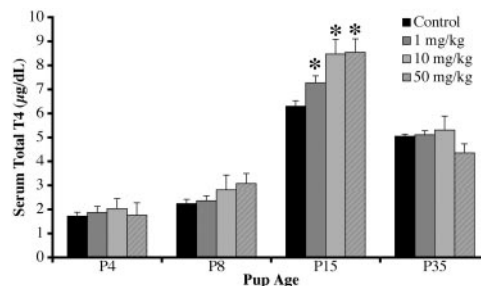


FIG. 3. Effect of BPA treatment on serum total T_4 in pups on P4, P8, P15 and P35. Bars represent mean $\pm$ SEM (n). *, Significantly different from control ($P > 0.01$).

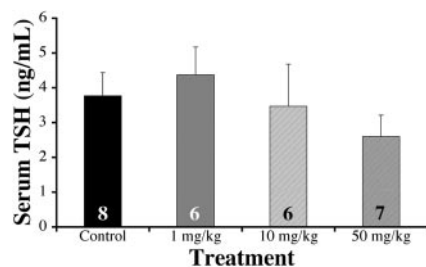


FIG. 4. Effect of BPA on serum TSH in male pups at P15. No significant differences were observed among the groups. Bars represent mean $\pm$ SEM (n).

can exert direct effects on TH signaling *in vitro*, and the findings suggest the possibility that incidental exposure to BPA could influence TH signaling in the developing brain.

The flat dose response of BPA on serum T_4 levels may be attributable to the mechanism by which BPA antagonizes T_3 -induced TR activation. Specifically, BPA causes the TR to recruit a corepressor (N-CoR) (19), which is likely to be in limited abundance. Thus, the antagonism will saturate in direct proportion to N-CoR abundance. This type of dose response may be characteristic of this type of antagonism. For example, RU 486, an indirect antagonist of the progesterone receptor, exerts its antiprogesterone activity (40, 41) in the same way that BPA affects the TR. Like the effect of BPA on serum T_4 , RU 486 produces a flat dose response on progesterone receptor-regulated gene expression (40, 41). Thus, the shape of this dose response (*i.e.* flat) does not abrogate its therapeutic efficacy. Likewise, the flat dose response of BPA on serum T_4 may be no less important.

Control pups exhibited a postnatal rise in serum T_4 levels from P4 (~ 1.5 μ g/dl) to P15 (~ 6 μ g/dl); this postnatal rise

is well documented (*e.g.* Refs. 37, 42, 43) and is not related to gender. However, BPA had no effect on serum T_4 on P4 (Fig. 2), perhaps because the negative feedback action of TH on the hypothalamic-pituitary axis does not mature until around P7 in the rat (44). Therefore, failure of BPA to elevate serum T_4 on P4 is consistent with the hypothesis that BPA inhibits TH-negative feedback. In addition, BPA did not affect serum T_4 in animals on P35, perhaps because these animals had not received BPA since they were weaned on P21 and BPA body burden would have diminished due to metabolic clearance. BPA is rapidly metabolized in rats and humans; the predominant metabolite of BPA is BPA-monoglucuronide (45–47), which is devoid of estrogenic activity (48). However, BPA-glucuronide has not been tested for its ability to bind to the TR.

BPA bioavailability is dependent on the route of administration. BPA is rapidly metabolized to BPA-glucuronide within an hour after oral administration in adult rats, but injection (ip or sc) results in a prolonged (8–12 h) presence of parent BPA in rat serum (48). In contrast, the serum half-life of parent BPA in pups after direct oral administration is about 7–8 h (48). However, the transfer of BPA to pups from the dam is quite low. Snyder *et al.* (45) reported that milk contains only 0.0008% of the maternal dose (100 mg/kg) 24 h after oral administration and that P14 pups receive about 0.006% of the dose given orally to the dam. Therefore, we can estimate that pups in this experiment received about 0.06, 0.6, and 3.0 μ g BPA when the dams were treated with 1, 10, or 50 mg/kg, respectively. However, our findings indicate that as low as 10 mg/kg oral dose to the dam (0.6 μ g/pup) caused a significant increase in serum T_4 .

We focused our additional work on P15 males because P15 is a time during postnatal development that RC3/neurogranin is particularly sensitive to TH (49) and there appear to be no gender differences in TH regulation of RC3/neurogranin on P15. Serum TSH levels were not significantly different in BPA-treated pups on P15, despite elevated levels of T_4 . Because of the duration of BPA treatment (from G6), the TSH levels likely represent a balance between the antagonistic effect of BPA on the pituitary, which would tend to increase TSH release by inhibiting negative feedback, and the elevated T_4 levels in serum, which would tend to suppress TSH release. Thus, in the presence of BPA, T_4 levels increase to maintain serum TSH levels similar to those of control animals.

Considering the effect of BPA on serum T_4 and TSH, we expected that measures of TH action in the developing brain would follow the same pattern as that of TSH (*i.e.* balanced antagonism of BPA and elevated T_4 , producing no difference among treatment groups). In contrast, BPA significantly increased RC3/neurogranin expression in the dentate gyrus but not the somatosensory cortex. This spatial pattern of RC3 regulation by BPA is identical with the spatial pattern of RC3 regulation by TH (37, 38), suggesting that BPA is not affecting RC3 expression independently of the TR. Therefore, the parsimonious interpretation is that the observed increase in RC3 expression after BPA exposure is due to increased circulating levels of TH. Because RC3 expression is likely to be regulated by the TR α in the dentate (38, 50), these data indicate that BPA may not interfere with TH action on TR α as it does on

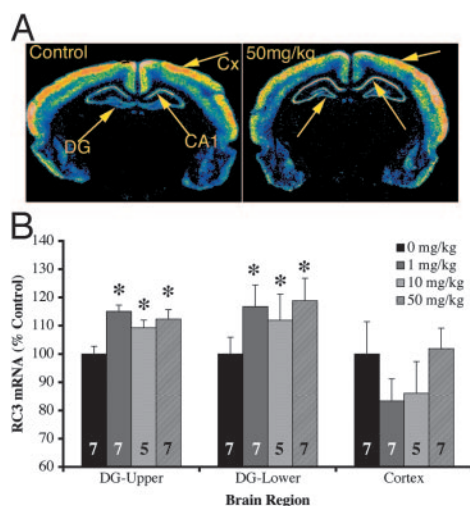


FIG. 5. Effect of BPA on RC3/neurogranin expression in the P15 male brain. A, Pseudocolor image of a film autoradiogram of the signal resulting from *in situ* hybridization RC3 in coronal sections containing the dentate gyrus. Note the difference in intensity of the signal in the image of the dentate taken from a BPA-treated animal (50 mg/kg). B, Graphical representation of the results of the RC3/neurogranin densitometry. Bars represent mean $\pm$ SEM (n). DG, Dentate gyrus; CA1, subfield of Ammon's horn; Cx, somatosensory cortex; upper, upper leaflet of the dentate gyrus; lower, lower leaflet of the dentate gyrus. *, Significantly different from controls ($P > 0.01$).

the TR β , leaving the TR α unopposed to respond to elevated levels of T $_4$.

The BPA-induced reduction in maternal body weight and body weight gain during pregnancy is similar to that observed by others (32, 51). In contrast, BPA exposure did not affect body weight gain during lactation in the current study (Fig. 1). The effect of BPA on maternal body weight may well be related to the estrogenic action of BPA inasmuch as ethinyl estradiol exposure causes a similar decrease in maternal body weight gain during pregnancy (52). In contrast, the increased serum T $_4$ in the offspring of BPA-treated dams cannot be explained by an estrogenic action of BPA because, unlike humans in which serum T $_4$ is elevated by estrogen (53, 54), estrogen treatment in rats decreases serum T $_4$ (55). Although it is theoretically possible that the BPA-induced decrease in maternal weight gain during pregnancy may have affected pup RC3 expression independent of TH, this seems unlikely considering that the effect of BPA on RC3 expression was observed only 2 wk after birth and at a time when RC3 expression is known to be most sensitive to TH. Thus, at the developmental time that RC3 expression is known to be sensitive to TH, it was associated with BPA exposure.

The results of this study indicate that BPA can exert a selective TH antagonism independent of estrogenic effects. Specifically, BPA appears to exert a selective antagonism on the TR β , causing serum levels of T $_4$ to rise, which in turn produces a local hyperthyroidism on the TR α , causing RC3 mRNA to rise. Considering that BPA is an indirect antagonist, its effects on TH action *in vivo* will likely be dependent on the composition and relative abundance of cofactors available in the cell. This could lead to a mosaic of effects in the developing brain, producing effects on different brain regions that reflect TH antagonism or potentially TH agonism. Likewise in humans, BPA could produce effects that are not consistent with global TH insufficiency or excess. In addition, BPA is commercially halogenated (brominated or chlorinated) for use as flame retardants; tetrabromobisphenol-A is the most commonly used with more than 60,000 tons produced annually (56, 57). Thomsen *et al.* (58) recently reported that brominated flame retardants, including tetrabromobisphenol-A, have increased in human serum from 1977 to 1999, with concentrations in adults ranging from 0.4 to 3.3 ng/g serum lipids. However, infants (0–4 yr) exhibited serum concentrations that ranged from 1.6 to 3.5 times higher (58). Considering that the halogenated BPAs exhibit a much higher affinity than parent BPA for the TR (23), this class of environmental chemicals will be important to evaluate for their ability to interfere with TH action in development.

Acknowledgments

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