CLARITY-BPA: Bisphenol A or Propylthiouracil on Thyroid Function and Effects in the Developing Male and Female Rat Brain

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The CLARITY-BPA experiment, a large collaboration between the National Institute of Environmental Health Sciences, the National Toxicology Program, and the US Food and Drug Administration, is designed to test the effects of bisphenol A (BPA) on a variety of endocrine systems and end points. The specific aim of this subproject was to test the effect of BPA exposure on thyroid functions and thyroid hormone action in the developing brain. Timed-pregnant National Center for Toxicological Research Sprague-Dawley rats (strain code 23) were dosed by gavage with vehicle control (0.3% carboxymethylcellulose) or one of five doses of BPA [2.5, 25, 250, 2500, or 25,000 μg/kg body weight (bw) per day] or ethinyl estradiol (EE) at 0.05 or 0.50 μg/kg bw/d (n = 8 for each group) beginning on gestational day 6. Beginning on postnatal day (PND) 1 (day of birth is PND 0), the pups were directly gavaged with the same dose of vehicle, BPA, or EE. We also obtained a group of animals treated with 3 ppm propylthiouracil in the drinking water and an equal number of concordant controls. Neither BPA nor EE affected serum thyroid hormones or thyroid hormone–sensitive end points in the developing brain at PND 15. In contrast, propylthiouracil (PTU) reduced serum T4 to the expected degree (80% reduction) and elevated serum TSH. Few effects of PTU were observed in the male brain and none in the female brain. As a result, it is difficult to interpret the negative effects of BPA on the thyroid in this rat strain because the thyroid system appears to respond differently from that of other rat strains. **(*Endocrinology* 160: 1771–1785, 2019)**

Several authoritative bodies have documented an increase in the prevalence of neurobehavioral disabilities globally, including the National Center on Birth Defects and Developmental Disabilities (1), the United Nations Environment Program, and the World Health Organization (2), as well as a large group of experts in the field engaged in Project TENDR (Targeting Environmental Neuro-Development Risks (3). Bennett *et al.* (3) emphasized that neurobehavioral disorders are increasing, with one in 10 children now estimated to have an attention deficit and one in 40 children with an autism spectrum disorder. Of note, the increase in these neurobehavioral disorders is likely due in part to environmental factors, including chemical exposures (4). Moreover, disturbances in thyroid hormones during development have been implicated in some neurodevelopmental and cognitive disorders (5–10). Considering the large number of chemicals in the environment that can affect the thyroid system (11, 12), it is important to consider that a proportion of these public health trends are related to chemical exposures acting on the thyroid system. Moreover, these public health trends come with an economic burden (13, 14).

Within this context, it is important that some studies have indicated that bisphenol A (BPA) may interfere with thyroid hormone action. Moriyama *et al.* (15) reported that BPA could displace T3 from isolated nuclei and can act as an indirect antagonist in tsa201 cells, a human SV40-transformed embryonal cell line, on the two major

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Abbreviations: BPA, bisphenol A; bw, body weight; CLARITY-BPA, Consortium Linking Academic and Regulatory Insights on BPA Toxicity; EE, ethinyl estradiol; G, gestational day; MAG, myelin-associated glycoprotein; ME, malic enzyme; MHC, myosin heavy chain; NCTR, National Center for Toxicological Research; NIEHS, National Institute of Environmental Health Sciences; PND, postnatal day; PTU, propylthiouracil; PVN, paraventricular nucleus; SD, Sprague-Dawley; SERCA2α, sarcolemmal calcium ATPase; TR, thyroid hormone receptor.

forms of human thyroid hormone receptors (TRs; TRα1 and TRβ1). Others have shown that BPA can interfere with thyroid hormone–dependent processes in frogs (16) and in zebrafish (17). We reported that BPA exposure increased serum total T4 levels in 2-week-old male and female Zivic-Miller Sprague-Dawley (SD) rat pups (18), consistent with an inhibitory effect of BPA on TRβ_2 in the pituitary that mediates negative feedback of T4 on TSH (19). In contrast, BPA-treated male pups exhibited a substantial increase in hippocampal RC3/neurogranin mRNA, a gene that is directly regulated by thyroid hormones (20, 21). This observation was consistent with elevated serum T4 level (21). These data indicated that in this experiment in rats, BPA may selectively antagonize TRβ_2 compared with TRα, producing a hormonal profile similar to that of thyroid resistance syndrome in which the T4 level is elevated by a lack of negative feedback, which in turn increases the expression of thyroid hormone–regulated genes on the αTR (22).

However, the literature informing us about the potential action(s) of BPA on thyroid hormone signaling is complex. Lee *et al.* (23) reported that BPA could reduce the expression of a number of genes in rat GH3 cells related to controlling thyroid hormone levels, but only at 10 μM. No effects of BPA were observed in FRTL-5 cells. Sheng *et al.* (24) reported that 10^{-9} M of BPA could suppress T3-induced gene expression in CV-1 cells, but through a nongenomic mechanism. Kitamura *et al.* (25) reported that BPA essentially does not bind to the mammalian TR. Likewise in rodents, Xu *et al.* (26) reported in SD rats (from Nippon Clea, Inc., Tokyo, Japan) that BPA did not affect thyroid hormone signaling in perinatal rats, and Kobayashi *et al.* (27) reported that BPA exposure to Crj:CD(SD) rat dams from gestational day (G) 6 to postnatal day (PND) 20 did not affect serum T4 levels in offspring at 9 weeks of age. In humans, Park *et al.* (28) reported that BPA is negatively associated with serum TSH, an observation similar to that of Aung *et al.* (29).

Although these studies provided provocative information on the potential effects of BPA on thyroid hormone action, they often did not include a wide range of doses of BPA or control for potential unintended exposure to other compounds with goitrogenic properties, such as dietary phytoestrogens (30). Thus, the purpose of the current study was to test the hypothesis that perinatal BPA exposure can interfere with thyroid hormone action in the developing rat brain within the context of a tightly controlled good laboratory practice–compliant study. This hypothesis was tested as part of the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) program. The CLARITY-BPA study is a collaboration between academic and federal government scientists that was organized by the National Toxicology Program, the National Institute of Environmental Health Sciences (NIEHS), the US Food and Drug Administration, and its National Center for Toxicological Research (NCTR) (31, 32). The goal of this research consortium is to combine the strengths of both independent and guideline-compliant studies to obtain better translational research (31, 32).

Methods

This study was conducted as part of the CLARITY-BPA Consortium. The methods for this consortium have been published in detail (31), but are briefly described here.

Reagents

BPA [CAS no. 80-05-7; catalog no. B0494, lot no. 111909/AOHOK (air-milled), 0.99% pure; TCI America, Portland, OR] and propylthiouracil (PTU; 6-propyl-2-thiouracil, CAS no. 51-52-5, product no. P3755, lot no. BCBF0745V; Sigma-Aldrich, Allentown, PA) were used in these studies. The purity of BPA was verified at 6-month intervals during the study and again at the end of the study to confirm test article stability. BPA was dissolved in 0.3% aqueous carboxymethylcellulose (catalog no. C5013, lot no. 041M0105V; Sigma-Aldrich) and delivered by gavage with a modified Microlab ML511C programmable 115V pump (Hamilton Co., Reno, NV). PTU was provided in drinking water at a concentration of 3 ppm.

BPA experiment

All animal use and procedures for the core study were approved by the NCTR Laboratory Animal Care and Use Committee and conducted in an American Association for Laboratory Animal Care–accredited facility. NCTR SD rats (strain code 23) from the NCTR rodent breeding colony were used in all experiments. Breeders were housed in polysulfone cages with hard chip bedding and glass water bottles in rooms at 23 ± 3°C with a relative humidity of 50% ± 20% and were provided food (soy- and alfalfa-free verified casein diet 10 IF, 5K96; catalog no. 1810069; Purina Mills, Richmond, IN) and water for *ad libitum* consumption from weaning (approximately PND 21). All animal rooms were under 12-hour light/12-hour dark cycles, with lights on at 6:00 AM.

Timed-pregnant rats were dosed by gavage with vehicle control (0.3% carboxymethylcellulose) or one of five different doses of BPA (2.5, 25, 250, 2500, or 25,000 μg/kg body weight (bw) per day; n = 8 each] or ethinyl estradiol [0.05 or 0.50 μg/kg bw/d; n = 8) beginning on G 6. Starting on PND 1 (day of birth is PND 0), the pups were directly gavaged with the same dose level of vehicle or BPA. To focus on a dose range of regulatory concern, the doses of BPA were based on results from a 90-day BPA study conducted by the NCTR before the CLARITY BPA program (33), estimates of human exposure levels (34, 35), and agreement among NIEHS-funded university-based researchers and National Toxicology Program and US Food and Drug Administration scientists.

Eight male and eight female pups were euthanized on PND 15 (one male and one female from each dam). Trunk blood was collected after decapitation, and serum was collected from each sample. Brains were dissected from the cranium and

frozen on a flat surface of pulverized dry ice, taking care to preserve morphology. The liver, heart, and pituitary were also dissected and frozen on pulverized dry ice. Serum and tissue samples were stored frozen at −80°C before being shipped on dry ice to the University of Massachusetts Amherst laboratory for analysis.

PTU experiment

Timed-pregnant SD rats as described previously were assigned to two treatment groups: control and PTU-treated (n = 8 each). PTU was delivered to the animals in their drinking water (3 ppm) as described by Bansal *et al.* (36). This PTU dose was designed to reduce serum total T4 level by ~80% to serve as a positive control for the BPA experiment. Both the control and PTU-treated animals were also gavaged daily with 0.3% carboxymethylcellulose from G 6. One male and one female pup were euthanized on PND 15 from each dam. Blood was collected to harvest serum, and brains were dissected and stored as described previously. The brain, liver, and heart were also collected as described previously for the BPA experiment.

Serum hormone measurements

Total T4 level was measured in 25 μL of pup serum in duplicate using a commercial radioimmunoassay kit (T4 MAb; ICN Orangeburg, NY) according to the manufacturer's instructions. The assay was validated for rat serum by demonstrating parallelism between the standard curve and a dilution series of rat serum. The two slopes did not vary significantly as evaluated by *t* test for two slopes (data not shown). All experimental samples were evaluated in a single assay. The lowest standard was 2 μg/dL, and the sensitivity of the assay was 0.76 μg/dL.

TSH was measured in 25 μL of serum using an ELISA kit for rat TSH (Alpco, Salem, NH). Samples were analyzed in duplicate according to the manufacturer's instructions. The sensitivity of the assay was 0.1 ng/mL.

In situ hybridization

Because thyroid hormone interacts with a nuclear receptor that regulates gene expression, we evaluated the effect of PTU or BPA treatment on the expression of several genes known to be directly regulated by thyroid hormone in the brain. The gene coding for RC3/neurogranin is expressed widely throughout the brain but is regulated by thyroid hormone specifically in the dentate gyrus of the hippocampus (20) on PND 15. Thus, we measured the relative expression of RC3/neurogranin mRNA in the hippocampus. Likewise, oligodendrocyte number is very sensitive to changes in serum T4 level (37), and oligodendrocyte numbers in areas of white matter are correlated with the expression of myelin-associated glycoprotein (MAG) (37); therefore, we measured the relative level of MAG mRNA in the corpus callosum and anterior commissure as an index of oligodendrocyte number. Low thyroid hormone level leads to an upregulation of thyrotropin-releasing hormone (TRH) mRNA in the hypophysiotropic TRH neurons in the hypothalamic paraventricular nucleus (PVN) (38, 39); therefore, we also measured the expression of this mRNA specifically in the PVN. Finally, as an index of chronic stress, we measured the mRNA coding for CRH in the hypophysiotropic region of the PVN.

Coronal sections of frozen brain tissue were taken at 12 μm in a cryostat (Reichert-Jung Frigocut 2800N; Leica Corp., Deerfield, IL). Two adjacent sections were thaw-mounted onto each microscope slide twice coated with gelatin and stored at −80°C until hybridization. The rostrocaudal placement of the section was matched using internal landmarks when slides were chosen for the *in situ* hybridization. DNA sequences for these probes are shown in Table 1. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). End labeling was carried out using terminal transferase (Roche Applied Sciences, Indianapolis, IN) and ^{33}P-labeled deoxyadenosine triphosphate according to the manufacturer's instructions. Two slides from each brain, four sections total, were thawed at room temperature and hybridized as previously described (37). After *in situ* hybridization, slides were exposed to BioMax film (Eastman Kodak, Rochester, NY) in x-ray cassettes along with ^{14}C-labeled standards (American Radiolabeled Chemicals Inc., St. Louis, MO) to control for overexposure. Film density was measured in the dentate gyrus of PND 15 brains for RC3/neurogranin mRNA, as previously described (44), except that we used a SPOT Insight 2 camera and a Macintosh G5 computer. Film density values of the dentate gyrus and CA1/3 of Ammon's horn were averaged over the four sections for each brain, with one brain representing each litter. Film density of the anterior commissure was measured similarly.

Table 1. DNA Probe Sequences

	Target mRNA	Reference
Probes for *in situ* hybridization		
TRH	5′-GTC TTT TTC CTC CTC CTC CCT TTT GCC TGG ATG CTG GCG TTT TGT GAT-3′	(40)
RC3	5′-ACC TGT CCA CGC GCC CAG CAT GCA GCT CTG CCT CCG CAG CCT CGG-3′	(41)
MAG	5′-CAG GAT GGA GAC TGT CTC CCC CTC TAC CGC CAC CAC CGT CCC ATT CAC-3′	(42)
CRH	5′-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3′	(43)
TRβ	5′-CTG GTG TCT GTA TGG AAC CAA ATC CCT GTC TTC TCG TCT CTG GTG TGA GA-3′	
TRα	5′-CAG TTA GGA TGA CTA CCA TTT TTA CCT CCA GGG GAG GAG CTA AGC CAA GC-3′	
	5′-GGC CAA GGA ACT TGG CAG GGC TCT CCT GTG TGT GTG TAG GGG TGA GTA AG-3′	
Primers for quantitative real-time PCR		
TRα	Forward 5′-GTCAACCACCGCAAACACAA-3′; reverse 5′-CGATCATGCGGAGGTCAGTC-3′	
TRβ	Forward 5′-TGTTGTCCTCAAGGCAGTGG-3′; reverse 5′-ATTCCTGGCACTGGTTACGG-3′	

DNA sequences for the probes used for *in situ* hybridization are presented from 5′ to 3′, as are the probes used for quantitative real-time PCR.

Abbreviations: RC3, RC3/neurogranin; TRα, thyroid hormone receptor α 1 isoform; TRβ , thyroid hormone receptor β isoform.

Cerebellar histogenesis

Histological analysis of sagittal sections of frozen PND 15 cerebellum were taken at 12 μm in a cryostat (Reichert-Jung Frigocut 2800N; Leica). Two adjacent sections were thaw-mounted onto microscope slides twice coated with gelatin and stored at −80°C. Two slides per animal were thawed, fixed with 4% formaldehyde, and stained with hematoxylin and eosin (Sigma), dehydrated in ethanol, and cover-slipped using Permount. Images were magnified using a SPOT Insight 2 camera equipped with a Nikon macrolens mounted on a bellows and captured using a Scion AG-5 capture board interfaced with Image version 1.61 [W. Rashband, National Institute of Mental Health, Bethesda, MD] run on Macintosh G5. For each cerebellum, the deepest sulcus was located, and a 1-mm grid was placed over the image. The area of each layer was measured over a 1-mm length using a digitized image calibrated with a stage micrometer (Fig. 1). Four sections were measured from each brain, with a single measurement made for each layer taken in a single section.

mRNA isolation and real-time PCR

Total RNA was extracted from fresh frozen tissue (pituitary, liver, and heart) using Trizol Reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. Small samples of tissue were isolated on dry ice and homogenized with a Bullet Blender (NextAdvance Inc, Averill Park, NY). After extraction and precipitation, RNA was resuspended in RNAse-free water. RNA abundance was measured using a Thermo Fisher Scientific NanoDrop 1000 (Waltham, MA), and RNA integrity was confirmed using an Agilent Bioanalyzer (Santa Clara, CA) and the RNA Nano 6000 Analysis Kit.

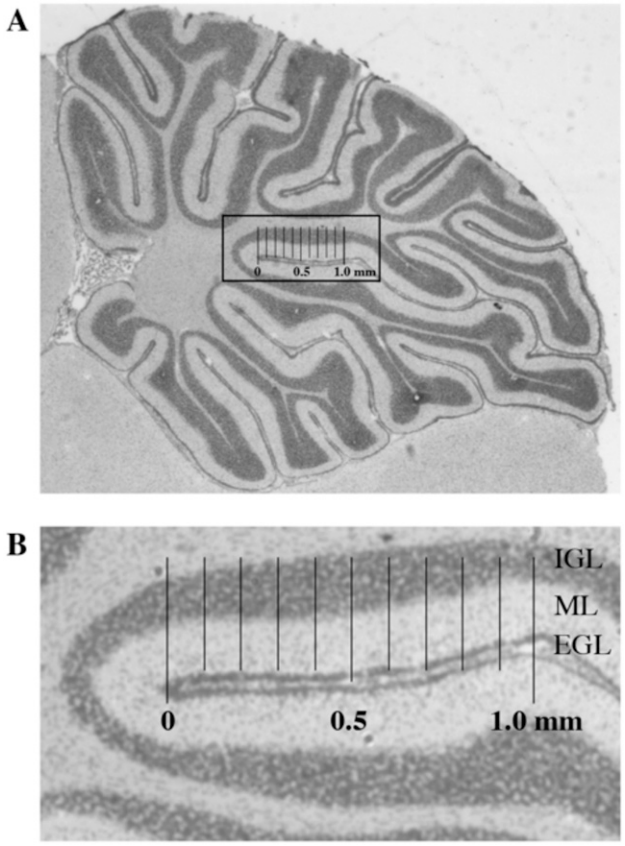

Figure 1. Measurement of cerebellar layers. Midsagittal sections of PND 15 rat cerebellum were fixed, stained with hematoxylin and eosin, and cover-slipped. (A) The deepest sulcus for each individual brain was identified, and (B) a 1-mm grid was placed over the magnified image. The area of each layer was measured over a 1-mm length using a digitized image calibrated with a stage micrometer. EGL, external germinal layer; IGL, internal granule layer; ML, mitral layer.

Quantitative real-time PCR

One microgram of RNA was reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a final volume of 20 μL. Relative mRNA levels were measured in 10-μL reactions using the FastStart SYBR Green Master (Rox) Kit (Roche Diagnostics Corp, Indianapolis, IN), 300 nM each of forward and reverse primer (Table 1), and cDNA. Duplicate wells were run for each target gene, and β-actin was used as the reference gene for $\Delta\Delta$CT calculations. Quantitative PCR was carried out on a Stratagene MX3000P (Agilent Technologies, Santa Clara, CA). All samples were subject to melting curve analysis to verify the production of a single product.

Statistical analysis

The raw, blinded data were uploaded to the National Institutes of Health Chemical Effects in Biological Systems database and locked. These data were then inspected by NCTR and decoded. After this process, we performed unpaired *t* tests on data derived from the PTU experiment and a one-way or two-way ANOVA for the BPA data. The data were analyzed using Prism 6.0 for Macintosh and *post hoc* analysis (when necessary) using the Dunnett comparison test for significance from controls. Follow-up studies were not technically part of CLARITY and were not uploaded to the National Institutes of Health Chemical Effects in Biological Systems database. Although these data were generated after decoding, we took normal precautions to control the risk of bias as described in the next section.

Controlling the risk of bias

Several types of bias—or systematic error—can occur in these kinds of studies, and we followed several standard protocols to limit the risk of systematic error. First, all samples were blinded before they arrived in the laboratory; thus, bias derived from the knowledge of group identity was controlled for in the CLARITY data. Second, all serum samples were evaluated in the same assay; thus, interassay variability was not a factor. This is especially important with blinded samples to control for the possibility that some groups were randomly analyzed in one assay and other groups in a different assay. Likewise, the PTU and BPA experiments were analyzed separately so that we could better manage the logistics of multiple samples being analyzed in a single assay. For *in situ* hybridization, all samples to be compared statistically were placed against the same film, eliminating potential error due to film differences. For quantitative real-time PCR, we had to identify the control group so that all control samples could be included on each 96-well plate to serve as a fiducial in the calculation of $\Delta\Delta$CT. All analyses performed after decoding remained blind to the operator handling the samples, maintaining the same sample code to retain functional blinding.

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Table 2. Effect of PTU on Thyroid Hormone and End Points of Thyroid Hormone Action in the Developing Brain

	Serum T4 (μg/dL)	RC3-DG (% Control)	RC3-CA1 (% Control)	RC3-CA3 (% Control)	TRH (% Control)	EGL Thickness (μm)	ML Thickness (μm)	IGL Thickness (μm)	MAG (% Control)
Male									
Control	5.79 ± 0.25 n = 8	100 ± 3.04 n = 8	100 ± 2.26 n = 8	100 ± 3.30 n = 8	100 ± 4.99 n = 8	30.8 ± 1.63 n = 7	173.6 ± 5.5 n = 7	199.4 ± 5.7 n = 7	100 ± 3.38 n = 7
PTU	1.40 ± 0.14 n = 8 *t* = 15.37; df = 14 ***P* < 0.0001**	87.14 ± 1.83 n = 8 *t* = 3.62; df = 14 ***P* = 0.0028**	94.19 ± 1.26 n = 8 *t* = 2.25; df =14 ***P* = 0.0414**	94.54 ± 1.17 n = 8 *t* = 1.56; df = 14 *P* = 0.1415	105.9 ± 4.46 n = 8 *t* = 0.884; df = 14 *P* = 0.3915	44.1 ± 4.57 n = 8 *t* = 2.579; df = 13 ***P* = 0.0229**	161.3 ± 4.5 n = 8 *t* = 1.76; df = 13 *P* = 0.1026	214.5 ± 11.8 n = 8 *t* = 1.097; df = 13 *P* = 0.2927	105.8 ± 2.48 n = 7 *t* = 1.389; df = 12 *P* = 0.1901
Female									
Control	6.13 ± 0.23 n = 8	100 ± 5.75 n = 8	100 ± 5.22 n = 8	100 ± 6.12 n = 8	100 ± 9.64 n = 8	35.4 ± 4.76 n = 8	176.8 ± 5.6 n = 8	197.5 ± 5.58 n = 8	100 ± 3.91 n = 8
PTU	1.43 ± 0.12 n = 8 *t* = 18.14; df = 14 ***P* < 0.0001**	91.87 ± 1.84 n = 8 *t* = 1.35; df = 14 *P* = 0.199	94.49 ± 2.33 n = 8 *t* = 0.964; df = 14 *P* = 0.3514	94.98 ± 2.61 n = 8 *t* = 0.7549; df = 14 *P* = 0.4628	112.6 ± 8.78 n = 8 *t* = 0.968; df = 14 *P* = 0.3494	32.3 ± 1.71 n = 8 *t* = 0.619; df = 14 *P* = 0.5460	172.0 ± 4.83 n =8 *t* = 0.6523; df = 14 *P* = 0.5248	194.7 ± 7.25 n = 8 *t* = 0.2977; df = 14 *P* = 0.7703	87.91 ± 8.54 n = 8 *t* = 1.287; df = 14 *P* = 0.2190

PTU (3 ppm) was provided in drinking water to NCTR SD dams from G 6 to PND 15 (termination of experiment). One male and one female pup from each dam (n = 8) were euthanized on PND 15, trunk blood was collected to harvest serum, and brains were collected and frozen as described in the "Methods." Both controls and PTU-treated dams were also gavaged daily with carboxymethylcellulose. Values represent mean ± SEM. Significant *P* values are present in bold type for emphasis.

Abbreviations: EGL, external germinal layer; IGL, internal granule layer; MAG, myelin associated glycoprotein; ML, mitral layer; RC3, RC3/neurogranin.

Table 3. Effects of BPA on Measures of Thyroid Hormone Action in Male Rats

Treatment Group	Serum T4 (μg/dL)	RC3-DG (% Control)	RC3-CA1 (% Control)	RC3-CA (% Control)	TRH (% Control)	EGL Thickness (μm)	ML Thickness (μm)	IGL Thickness (μm)	MAG (% Control)
Control	6.3 ± 0.39 n = 8	100 ± 5.02 n = 8	100 ± 5.61 n = 8	100 ± 6.59 n = 8	100 ± 4.48 n = 8	32.5 ± 2.78 n = 8	119.2 ± 5.08 n = 8	177.4 ± 5.10 n = 8	100 ± 3.94 n = 8
2.5, μg/kg bw/d	6.5 ± 0.44 N = 8	84.8 ± 5.05 n = 8	88.0 ± 4.33 n = 8	86.0 ± 4.60 n = 8	106.0 ± 2.49 n = 8	27.2 ± 2.02 n = 7	125.2 ± 3.84 n = 7	182.1 ± 2.10 n = 7	105.8 ± 2.36 n = 8
25, μg/kg bw/d	6.9 ± 0.33 N = 8	90.9 ± 3.12 n = 7	92.6 ± 3.71 n = 7	92.5 ± 3.46 n = 7	92.3 ± 13.88 n = 7	34.9 ± 3.21 n = 8	125.9 ± 3.49 n = 8	176.0 ± 6.97 n = 8	100.3 ± 3.74 n = 8
250, μg/kg bw/d	6.4 ± 0.28 N = 8	88.2 ± 6.56 n = 8	88.9 ± 5.88 n = 8	89.5 ± 5.71 n = 8	103.1 ± 4.23 n = 8	29.7 ± 2.28 n = 8	130.7 ± 3.16 n = 8	176.5 ± 8.65 n = 8	100.3 ± 3.98 n = 8
2500, μg/kg bw/d	6.3 ± 0.28 N = 8	94.5 ± 4.09 n = 8	94.4 ± 4.62 n = 8	94.3 ± 3.84 n = 8	108.1 ± 3.16 n = 8	30.3 ± 3.46 n = 8	126.9 ± 4.07 n = 8	171.7 ± 3.93 n = 8	105.6 ± 3.81 n = 8
25,000, μg/kg bw/d	6.6 ± 0.33 N = 8	95.8 ± 3.87 n = 8	97.0 ± 3.46 n = 8	94.8 ± 3.23 n = 8	102.2 ± 5.02 n = 8	28.6 ± 2.23 n = 8	119.1 ± 4.22 n = 8	162.1 ± 7.18 n = 8	100.0 ± 1.35 n = 8
EE 0.05, μg/kg bw/d	6.2 ± 0.27 N = 8	95.6 ± 3.89 n = 8	98.4 ± 3.23 n = 8	95.0 ± 4.43 n = 8	104.1 ± 3.73 n = 8	28.2 ± 1.73 n = 8	129.9 ± 3.85 n = 8	170.4 ± 3.96 n = 8	106.8 ± 3.36 n = 8
EE 0.5, μg/kg bw/d	5.9 ± 0.22 N = 8	89.1 ± 5.85 n = 8	85.2 ± 3.3 n = 8	84. 9 ± 4.21 n = 8	98.4 ± 2.50 n = 8	27.9 ± 1.59 n = 8	129.4 ± 4.83 n = 8	181.1 ± 6.80 n = 8	102.5 ± 2.17 n = 8
	$F(7,56) = 0.77$ $P = 0.6136$	$F(7, 55) = 1.058$ $P = 0.4026$	$F(7, 55) = 1.488$ $P = 0.1908$	$F(7, 55) = 1.187$ $P = 0.3255$	$F(7, 54) = 0.7330$ $P = 0.6448$	$F(7, 55) = 1.095$ $P = 0.3795$	$F(7, 55) = 1.230$ $P = 0.3023$	$F(7, 55) = 1.150$ $P = 0.3467$	$F(7, 56) = 0.8387$ $P = 0.5601$

NCTR SD dams were treated daily with BPA or EE by gavage at the doses shown in the left column. One male was euthanized on PND 15 from each litter, and blood and brain were collected for analysis. Values represent mean ± SEM. Significant *P* values are present in bold type for emphasis.

Abbreviations: EGL, external germinal layer; IGL, internal granule layer; MAG, myelin associated glycoprotein; ML, mitral layer; RC3, RC3/neurogranin.

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Table 4. Effects of BPA on Measures of Thyroid Hormone Action in Female Rats

Treatment Group	Serum T4 (μg/dL)	RC3-DG (% Control)	RC3-CA1 (% Control)	RC3-CA3 (% Control)	TRH (% Control)	EGL Thickness (μm)	ML Thickness (μm)	IGL Thickness (μm)	MAG (% Control)
Control	6.5 ± 0.32 n = 8	100 ± 5.78 n = 8	100 ± 5.20 n = 8	100 ± 5.36 n = 8	100 ± 2.91 n = 8	30.8 ± 4.01 n = 8	131.8 ± 6.16 n = 8	183.6 ± 8.00 n = 8	100 ± 12.98 n = 8
2.5, μg/kg bw/d	6.3 ± 0.44 n = 8	103.8 ± 3.31 n = 7	104.8 ± 5.31 n = 6	104.4 ± 3.62 n = 7	102.3 ± 5.35 n = 7	27.6 ± 3.87 n = 8	129.6 ± 6.18 n = 8	165.1 ± 6.01 n = 8	89.6 ± 12.54 n = 8
25, μg/kg bw/d	6.4 ± 0.29 n = 8	89.3 ± 3.24 n = 8	92.2 ± 3.78 n = 8	88.7 ± 4.61 n = 8	110.3 ± 5.03 n = 8	31.1 ± 2.51 n = 8	124.6 ± 4.38 n = 8	174.3 ± 5.37 n = 8	100.9 ± 8.12 n = 8
250, μg/kg bw/d	7.3 ± 0.69 n = 8	108.7 ± 1.77 n = 8	108.9 ± 2.45 n = 8	107.0 ± 2.40 n = 8	107.0 ± 4.08 n = 8	25.6 ± 3.66 n = 7	118.8 ± 7.75 n = 7	156.8 ± 6.76 n = 7	83.3 ± 11.87 n = 7
2500, μg/kg bw/d	6.6 ± 0.19 n = 8	92.6 ± 5.16 n = 8	87.6 ± 6.82 n = 8	93.0 ± 4.66 n = 8	109.2 ± 3.86 n = 8	23.3 ± 1.45 n = 8	129.2 ± 4.62 n = 8	164.6 ± 6.39 n = 8	75.8 ± 4.68 n = 8
25,000, μg/kg bw/d	7.1 ± 0.43 n = 8	100.8 ± 4.70 n = 8	101.5 ± 4.86 n = 8	99.6 ± 2.74 n = 8	103.8 ± 3.54 n = 8	32.4 ± 2.42 n = 8	136.8 ± 7.31 n = 8	174.9 ± 8.10 n = 8	105.2 ± 7.85 n = 8
EE 0.05, μg/kg bw/d	6.6 ± 0.54 n = 8	98.1 ± 5.16 n = 8	100.2 ± 5.26 n = 8	97.3 ± 5.76 n = 8	100.3 ± 4.56 n = 7	27.5 ± 3.64 n = 8	122.3 ± 8.55 n = 8	165.3 ± 3.06 n = 8	89.3 ± 11.80 n = 8
EE 0.5, μg/kg bw/d	6.6 ± 0.24 n = 8	107.6 ± 2.46 n = 8	101.9 ± 6.36 n = 8	107.8 ± 2.74 n = 8	100.6 ± 2.83 n = 8	28.3 ± 2.57 n = 8	126.2 ± 4.69 n = 8	167.1 ± 5.44 n = 8	91.7 ± 8.33 n = 8
	$F(7, 56) = 0.7007$ $P = 0.6713$	$F(7, 55) = 2.634$ **$P = 0.0202$**	$F(7, 54) = 1.735$ $P = 0.1203$	$F(7, 55) = 2.567$ **$P = 0.0231$**	$F(7, 54) = 1.043$ $P = 0.4128$	$F(7, 55) = 0.9385$ $P = 0.4848$	$F(7, 55) = 0.7761$ $P = 0.6099$	$F(7, 55) = 1.665$ $P = 0.1370$	$F(7, 55) = 0.3566$ $P = 0.9233$

NCTR SD dams were treated daily with BPA or EE by gavage at the doses shown in the left column. One female was euthanized on PND 15 from each litter, and blood and brain were collected for analysis. Values represent mean ± SEM. Significant *P* values are present in bold type for emphasis.

Abbreviations: EGL, external germinal layer; IGL, internal granule layer; MAG, myelin associated glycoprotein; ML, mitral layer; RC3, RC3/neurogranin.

Results

The primary findings are provided in Tables 2–4. PTU treatment produced a significant reduction in serum total T4 levels in both males and females (Table 2). In fact, PTU reduced serum total T4 level by about 75%, as we (36) and others (45) reported previously for 3 ppm PTU in drinking water. In male pups, the PTU-reduction in serum T4 level resulted in predicted effects in some, but not all, thyroid hormone–sensitive end points in the brain (Table 2). For example, PTU-treated male pups exhibited a relatively small but significant reduction in RC3/neurogranin mRNA expression in the dentate gyrus [13% reduction in film density compared with controls (t = 15.37; df = 14; P = 0.0028) compared with ~20% reduction in our previous report (46). There was also a slight but significant reduction in RC3 mRNA expression in CA1 of the hippocampus, although this was not always seen [*e.g.*, Sharlin *et al.* (46) vs Zoeller *et al.* (44)], and a slight increase in external germinal layer thickness of the cerebellar cortex. However, TRH mRNA was not affected in the hypothalamic PVN, nor were other measures of thyroid hormone action, including known effects on cerebellar histogenesis or MAG expression (a reflection of oligodendrocyte number) in the anterior commissure. These measures in the female brain were unaffected by PTU-induced reduction in serum T4 level (Table 2).

Neither BPA nor ethinyl estradiol (EE) treatment affected serum total T4 levels in male or female pups (Tables 3 and 4). Consistent with this finding in male pups, no end points of thyroid hormone action responded to BPA or EE treatment (Table 3). In contrast, there was a significant difference among means for RC3/neurogranin expression in the dentate gyrus and in CA3 in female pups (Table 4). However, the *post hoc* analysis could not identify individual means that were significantly different from controls.

Because the PTU-induced reduction in serum T4 level did not produce the effects in the brain that we expected in the CLARITY experiment, we explored several additional measurements in these animals to further inform us about this model.

1. *Serum TSH and pituitary TSHβ mRNA abundance.* A reduction in serum T4 level should cause an increase in serum TSH concentration, pituitary TSHβ mRNA abundance, and TRH mRNA abundance [*e.g.*, Koller *et al.* (38)]. The observed failure of TRH mRNA to exhibit increased abundance in the hypothalamic PVN of PTU-treated animals suggested the possibility that the TRH-TSH axis was not functional in these animals. However, we determined that serum TSH level was increased in PTU-treated males and females by fourfold to fivefold, and pituitary TSHβ mRNA was increased by more than sixfold (Table 5), as was shown previously for this degree of thyroid hormone insufficiency (36, 46). Thus, the pituitary-thyroid axis was intact and functional in both male and female pups in these NCTR PTU-treated animals. In addition, we compared the abundance of TSHβ mRNA in the NCTR animals with that of SD controls from our laboratory and found no differences, indicating that there were no strain differences in this measure (Table 5).
2. *Liver.* There are two often-used markers of thyroid hormone action in the liver that are directly regulated by the TR. These are "Spot 14" (also called Thyroid Hormone Responsive Protein) (47) and malic enzyme (ME) (48). Of note, we found that

Table 5. Serum TSH Concentration and TSHβ mRNA Level in the Pituitary

	NCTR Control	NCTR PTU	Zoeller Laboratory Control
Serum TSH, ng/mL			
Male	2.2 ± 0.27 n = 8	13.8 ± 1.50 n = 8 t = 7.669; df = 7.45 $P < 0.0001$	
Female	1.9 ± 0.15 n = 8	13.2 ± 2.49 N = 8 t = 4.54; df = 7.-5 $P < 0.0026$	
TSHβ mRNA[a] in pituitary			
Male	100 ± 14.22 n = 7 $F(2,17)$ = 16.92	371.9 ± 55.82 n = 8 $P < 0.0001$	84.40 ± 14.69 n = 5
Female	100 ± 13.63 n = 8 $F(2,18)$ = 19.37	528.6 ± 84.58 N = 8 $P < 0.0001$	104.2 ± 20.92 n = 5

[a]Values are percentage of NCTR control.

Table 6. Spot 14 and Malic Enzyme mRNA in the Liver

	Control	PTU
Spot 14	100 ± 14.77 n = 16 t = 2.942; df = 25.38	48.56 ± 17.49 n = 16 P = 0.0069
Malic enzyme	100 ± 64.86 n = 16 t = 0.3929; df = 28.41	95.81 ± 8.26 n = 16 NS

Values represent percentage of control.

Abbreviation: NS, not significant.

both Spot 14 and ME mRNAs exhibited the same response to 3 ppm of PTU, as we showed earlier (49); specifically, Spot 14 mRNA was decreased and ME mRNA was not (Table 6). Both males and females exhibited the same results as demonstrated by two-way ANOVA (data not shown).

3. *Heart*. As in the liver, there are several well-known thyroid hormone–responsive genes in the heart. myosin heavy chain (MHC)β expression is a direct target of thyroid hormone action in the heart, and its abundance is increased perinatally in hypothyroid animals (50). In contrast, MHCα expression is also a direct target of thyroid hormone action in the heart, and its abundance is decreased perinatally in hypothyroid animals (51). Finally, sarcolemmal calcium ATPase (SERCA2α) is upregulated by the thyroid hormone (52). Interestingly, in the current experiment, PTU-induced T4 suppression caused a significant reduction in SERCA2α mRNA but did not significantly affect the expression of either MHCα or MHCβ (Table 7). The SE of the mean of MHCα and MHCβ was considerably higher than that of SERCA2α, which may account for the lack of significance for the MHCs. There was no sex difference in these effects, as revealed by two-way ANOVA (data not shown).

Table 7. SERCA2 and MHC mRNA abundance in the Heart

	Control	PTU
SERCA2α	100 ± 3.20 n = 16 t = 3.029; df = 29.94	86.13 ± 3.34 n = 16 P = 0.005
MHCα	100 ± 10.37 n = 16 t = 0.9742; df = 26.31	115.8 ± 12.45 n = 14 NS
MHCβ	100 ± 8.10 t = 0.7958; df = 20.41	116.4 ± 20.42 NS

Values represent mean ± SEM mRNA level expressed as percent of the control.

Abbreviations: NS, not significant; SERCA2α, sarcolemmal calcium ATPase.

4. *TR expression*. A key finding in the PTU study was that the pituitary-thyroid axis responded normally to low T4 level (*i.e.*, increased serum TSH and TSHβ mRNA values in the pituitary), and there was no sex difference in these responses. In contrast, however, the brain of females did not respond to low serum thyroid hormone levels, despite a response similar to that of males in the liver and heart. Because the negative feedback within the hypothalamic-pituitary-thyroid axis is driven by TRβ_2 (53) and the lack of TRα expression can protect the brain from low T4 levels (54), we considered the possibility that TRα expression may be sexually dimorphic in these NCTR rats, accounting for some of the differences in response to low T4 levels. In the hippocampus, we found that TRα mRNA expression trended higher in NCTR females than in SD rats in our laboratory or the NCTR males (Table 8). As such, this finding does not help explain the overall insensitivity of the female brain to low T4 levels, though it is possible that TRα protein level is low despite elevated TRα mRNA. In the liver, TRα mRNA was much higher in NCTR animals than in SD rats from our laboratory, though this was not sexually dimorphic.
5. *CRH* mRNA *in the hypothalamic PVN*. Because the animals in this study were exposed by gavage on a daily basis, we postulated that the stress of this procedure may have altered the response to low T4 levels induced by PTU. Although there is little evidence for this, none of our previous measures provided insight into this enigma. Therefore, we compared relative levels of CRH mRNA in the PVN of 15-day-old laboratory control SD rats (Zivic Miller) to control 15-day-old male and female CLARITY pups (Table 9). We found no significant differences in CRH mRNA in the PVN among these groups.

Discussion

Although it has not been systematically tested in side-by-side experiments, the premise of our approach was that thyroid hormone affects the same fundamental neurodevelopmental processes (*e.g.*, oligodendrocyte differentiation and cerebellar development) in different mammals, including rodents and humans. Therefore, we predicted that PTU-induced suppression of serum T4 would affect the same end points in the neonatal brain as BPA-induced changes in serum T4 and that this would be true for NCTR SD rats as well as for other strains of rats and mice published in the open literature and that it would be predictive of human responses.

Table 8. TR mRNA in the Hippocampus and Liver

	Laboratory Controls	CLARITY Control Female	CLARITY Control Male
Long-Evans			
Hippocampus TRα	100 ± 1.31 n = 7	108.5 ± 3.25 n = 8 $F(2,19) = 3.652$; $P = 0.046$ Dunnett $P < 0.05$	101.5 ± 1.90 n = 7
	Sprague-Dawley	**CLARITY PTU Control**	**CLARITY PTU**
Liver			
TRβ	100 ± 9.56 n = 5	139.3 ± 15.5 n = 7 $F(2,16) = 2.67$ $P = 0.100$	122.0 ± 5.99 n = 7
TRα	100 ± 8.00 n = 5	252.3 ± 37.56 n = 7 $F(2,16) = 6.272$; $P = 0.0097$	266.0 ± 37.59 n = 7

Values represent mean ± SEM mRNA level expressed as percent of the control (Long-Evans or SD).

Abbreviations: TRα, thyroid hormone receptor α 1 isoform; TRβ, thyroid hormone receptor β isoform.

In the current experiment, however, neither BPA nor EE affected serum T4 levels on PND 15 in either male or female NCTR SD pups. Moreover, consistent with this finding, neither BPA nor EE affected measured downstream end points previously shown to be sensitive to thyroid hormone insufficiency in other rat strains, with the exception of RC3 in the female hippocampus. This finding remains enigmatic. These results are generally consistent with previous studies of BPA in NCTR SD rats (Table 10). Specifically, Delclos *et al.* (33) reported that BPA, given by gavage at a dose of up to 3 mg/kg bw/d, did not affect serum T4 concentrations on PND 15. In their study, BPA treatment was initiated prenatally and continued after birth until euthanasia, similar to the current CLARITY study. Ferguson *et al.* (55), who also used NCTR SD rats on PND 21, reported that BPA did not affect serum T4 levels. In this study, oral gavage of BPA at doses up to 25 μg/kg bw/d was continued through to the day of euthanasia on PND 21.

These findings contrast with several studies performed with other strains of rats. Zoeller *et al.* (18) reported that BPA given orally on a wafer caused an increase in serum total T4 levels in male SD rats from Zivic-Miller on PND 15, but not on PND 4, PND 8, or PND 30 (BPA exposure had been discontinued on PND 21). Xu *et al.* (26) reported that for male SD rats from Nippon Clea, Inc., BPA increased or reduced serum free T4 level depending on the PND of measurement. There was no effect of BPA in female pups. Interestingly, this study measured serum "free" T4 in dried whole blood spotted on filter paper using a kit optimized to measure T4 in human neonates from a heel prick. Ahmed *et al.* (56) also reported that BPA reduced serum free T4 level on PND 30 in male Wistar rats treated postnatally, and Fernandez *et al.* (57) reported that BPA reduced serum total T4 level in adult female SD rats. Kobayashi *et al.* (27) found that BPA had no effect on serum T4 level in male or female Charles River rats at 1, 3, or 9 weeks of age, nor did it affect TSH-induced T4 increase. However, T4 levels in control rats were low (3 μg/dL) for animals of this age and may reflect the soy-based diet they used. Howdeshell *et al.* (59) reported that for Long-Evans rats treated with BPA perinatally only, T4 levels were unaffected on PND 150. Finally, Viguié *et al.* (58) found that BPA administration to pregnant ewes caused a significant decrease in cord blood and jugular blood T4 levels in newborn lambs.

Although the number of studies investigating the ability of BPA to affect serum T4 in animals is relatively low and the timing and route of BPA exposure and timing of analysis are variable among these studies, the pattern that appears to emerge is that BPA does not affect serum T4 in the NCTR SD strain at either high or low doses (Table 10), but it does affect serum T4 perinatally during the period of treatment in most other rat strains. Finally, these studies may indicate a sex difference in the ability of BPA to influence serum T4, although the mechanism by which this occurs is unclear. It does not appear to be related to the estrogenic properties of BPA because EE also did not affect serum T4 in the current study.

Table 9. CRH mRNA in Hypothalamic PVN[a]

Laboratory Controls	CLARITY Control Female	CLARITY Control Male
153.8 ± 9.63	118.5 ± 6.8	127.5 ± 11.48

[a]Values represent mean ± SEM of the integrated corrected density of the film over the PVN after *in situ* hybridization. Values were not significantly different as measured by one-way ANOVA.

Table 10. Animal Studies of BPA Effects on Serum T4

Author	Animal Strain	Exposure Period	Route of Exposure and Dose	Analyte	Time of Assay	Sex	Finding
Zoeller *et al.* (18)	SD (Zivic-Miller)	G 6–PND 21 Maternal only	Wafer 0, 1, 10, and 50 mg/kg daily	Total T4	PND 4, PND 8, PND 15, PND 35	No sex differences	↑Total T4 on PND 15 only
Xu *et al.* (26)	SD Nippon Clea	G 11–PND 21 Maternal only	Drinking water (in 0.01% EtOH) 0, 0.1, and 50 mg/L	Free T4	PND 0, PND 7, PND 11, PND 21	No sex differences	↔T4 on PND 0,↑T4 on PND 7,↓T4 on PND 21
Ahmed *et al.* (52)	Wistar VACSERA	PND 15–PND 30 Pups	Gavage 0, 20, and 40 μg/kg	Free T4	PND 30	Not reported	↓T4 on PND 30
Fernandez *et al.* (53)	SD IBYME colony	PND 1–PND 10 Pups only	Subcutaneous injection nominal dose 0, 5, 50, and 500 μg	Total T4	PND 90–PND 120	Females only	↓T4 in estrus females
Kobayashi *et al.* (27)	Crj:CD(SD) Charles River Japan	G 6–PND 20 Maternal only	Gavage 0, 4, 40, and 400 mg/kg	Total T4	3 and 9 weeks	No sex difference	↔T4
Delclos *et al.* (33)	NCTR Sprague-Dawley rats	G 6–PND 15 or PND 21	Gavage 0; 2.5; 8; 25; 80; 260; 840; 2700; 100,000; and 300,000 μg/kg/d	T3, T4, TSH	PND 15	Male	↑T3, ↑TSH
Ferguson *et al.* (51)	NCTR Sprague-Dawley rats	G 6–PND 21	Gavage 0, 2.5, and 5.0 μg/kg/d	T4, T3	PND 21	No sex difference	↔T4
Viguié *et al.* (58)	2- to 5-y-old Lacaune ewes	G 28–G 145 Maternal only	Subcutaneous injection 5 mg/kg	Total and free T4	Newborn cord blood	No sex difference	↓Total T4, ↓free T4

↑ Increase; ↓ decrease; ↔ no change.

Studies of BPA effects on thyroid hormone action *in vitro* and in model systems provide a glimpse at the potential complexity of BPA effects on thyroid hormone action. For example, Moriyama *et al.* (15) found that BPA displaced ^{125}T3 from isolated MtT/E-2 rat pituitary cell nuclei at relatively high micromolar concentrations (100 μM), but it inhibited TR-mediated transcription in luciferase assays in the low micromolar range. Likewise, Seiwa *et al.* (60) found that 10^{-5} M of BPA could inhibit T3-induced differentiation of primary oligodendrocyte precursor cells derived from embryonic mouse cerebral cortex. Iwamuro *et al.* (61) found that 10^{-7} M of BPA could inhibit T3-induced tail resorption in a *Xenopus* assay and that the expression of several T3-dependent genes was suppressed by BPA. These findings may reflect a nongenomic effect of BPA on thyroid hormone action, as suggested by Sheng *et al.* (24), who reported that low concentrations of BPA (*e.g.*, 10^{-9} M) could inhibit T3-induced luciferase expression by suppressing the integrin-dependent pathway leading to recruitment of cofactors to the TR.

In dispersed cells from frog pituitary, BPA significantly inhibited the CRH-induced TSH release (62) at a concentration of 10^{-4} M, but Heimeier *et al.* (16) found that nanomolar concentrations of BPA could inhibit T3-induced transcription in frog oocytes and could inhibit T3-induced metamorphosis. Using zebrafish, Gentilcore *et al.* (63) found complex effects of BPA, increasing the expression of thyroid responsive genes and decreasing the expression of others, indicating that the effects of BPA are dependent upon many factors, including perhaps tissue type and age of the embryo. Taken together, these findings support the concept that BPA can interfere with thyroid hormone action, but the mechanism(s) by which this occurs is not clear.

Although these data support the hypothesis that BPA can interfere with thyroid hormone action, perhaps at low, environmentally relevant concentrations, it is not currently clear why there is such variability in the literature.

Studies of the effects of BPA on serum thyroid hormone levels in humans also generally support this conclusion. In men using an infertility clinic, multiple spot urine measurements of BPA were inversely correlated to serum TSH level, perhaps suggesting a thyroid hormone–like effect on negative feedback (64). Using National Health and Nutrition Examination Survey data, this study later showed that urinary BPA was inversely related to serum T4, consistent with a suppressive effect on serum TSH. Likewise, Chevrier *et al.* (65) reported that BPA was inversely related to serum T4 in pregnant women and was inversely related to serum TSH in newborn boys. Wang *et al.* (66) reported in a large Chinese study that urinary BPA was positively associated with serum free T3 but negatively associated with serum TSH. Geens *et al.* (67) found that BPA was positively associated with serum TSH in a population of lean individuals (in a study comparing subjects who were lean and subjects who were obese). This is consistent with findings from the HOME study, in which maternal BPA was inversely correlated with

cord blood TSH level in girls, but not in boys (68). In contrast, Andrianou *et al.* (69) found that BPA was positively associated with serum TSH level in adult women. However, Aung *et al.* (29) found in a repeated measures study that BPA was inversely related to serum TSH throughout pregnancy. This was also observed in Korea by Park *et al.* (28).

In principle, these data indicate that BPA can interfere with thyroid hormone action, though perhaps not by interacting directly with the TR. We must be somewhat cautious in this conclusion because this has not been systematically and extensively evaluated. In addition, the degree to which the effects of BPA on human health are mediated by interfering with thyroid hormone action is also difficult to estimate, but it cannot be discounted. We can also conclude that NCTR SD rats appear to be particularly insensitive to the thyroidal effects of BPA exposure. Thus, they do not appear to be a useful system in which to study BPA effects on thyroid endocrinology or physiology, and it is not clear the degree to which this is generalizable to chemical effects on other endocrine systems.

The finding that PTU-induced T4 suppression in NCTR SD rats only weakly affected a subset of well-known thyroid-sensitive end points in male pups and no end points in the female brain emphasizes the possibility that this particular strain is uniquely insensitive to thyroid hormone insufficiency. To further explore the sensitivity of this NCTR SD strain to low thyroid hormone levels during brain development, we first confirmed that the pituitary-thyroid axis functioned normally in both sexes. We found that there was a sixfold increase in serum TSH level in both sexes and a fourfold to fivefold increase in TSHβ mRNA in the pituitary at PND 15. The magnitude of these effects on serum TSH and TSHβ mRNA in the pituitary is similar to that in our previous reports in Long-Evans rats and in SD rats from Charles River (36, 49). Thus, the pituitary-thyroid axis in NCTR SD rats responded in a quantitatively predictable manner to PTU.

In the liver, PTU-induced T4 reduction was associated with a reduction in Spot 14 mRNA but not in ME mRNA. Interestingly, in PND 15 SD rats, 3 ppm of PTU produced the same results for Spot 14 and ME (49), but in Long-Evans rats, 3 ppm of PTU reduced both S14 and ME mRNA in the liver (36). Thus, there may be strain (or age) differences in the sensitivity or responsiveness to thyroid hormone in ME.

The PTU arm of this work was surprising inasmuch as the female brain exhibited no effects of PTU-induced serum T4 suppression. We had shown previously in Long-Evans female pups that a similar PTU-induced reduction in serum T4 level produced ~60% reduction in MAG expression in the anterior commissure (37), conflicting with the current findings.

Lasley and Gilbert (70) presented data suggesting that brain-derived neurotrophic factor expression in the hippocampus is less sensitive to low thyroid hormone levels in females than in males, but this experiment differed in that brain-derived neurotrophic factor was measured in the adult offspring of dams treated with PTU.

In conclusion, BPA did not affect serum T4 or any downstream marker of thyroid hormone action in this CLARITY-BPA project. These findings are consistent with those of other studies using NCTR SD rats, but they conflict with studies using other strains (even SD) of rats. It is important to emphasize that PTU-induced reduction in serum T4 level exerted an appropriate increase in serum TSH but that almost no other effects were observed in male or female pups. This finding would appear to render the NCTR SD rat inappropriate for studying the adverse effects of thyroid toxicants; however, it may be an important model to understand how thyroid hormone action in the brain is controlled independent of serum T4 levels. The degree to which gavage affected these findings is also unclear. The dams were restrained and treated with gavage on a daily basis, and the pups were directly treated with gavage after birth. This was true for the BPA as well as the PTU arms of the study. It is important to recognize from a design point of view that vehicle gavage does not control for the potential interaction between gavage and chemical exposure.

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