

Zoeller Laboratory - Material and Methods

Description of the test articles used, study material evaluations (diet, drinking water, cage and bedding leachates), general study design, animal treatments, and animal allocations to the 2 year toxicology study conducted at NCTR and the grantee studies can be found in Heindel et al. (2015).

Pregnant rats were dosed by gavage with vehicle control (0.3% carboxymethylcellulose, CMC) or one of five different doses of BPA (2.5, 25, 250, 2500, or 25,000 µg/kg bw/d; n=8 each) beginning on gestational day (GD) 6. Starting on postnatal day (PND) 1 (day of birth is PND 0), the pups were directly gavaged with the same dose level of vehicle or BPA. The doses of BPA were based on the results from a 90-day BPA study conducted by the NCTR prior to the CLARITY BPA program (Delclos et al. 2014), and were designed to estimate human exposure levels (Peretz et al. 2014; Vandenberg et al. 2007) as agreed by the different members of the consortium to focus on a dose range of regulatory concern.

Eight male and 8 female pups were euthanized on PND 15 (one male and one female from each dam; set 3). 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. Liver, heart, and pituitary were also dissected and frozen on pulverized dry ice. Serum and tissue samples were stored frozen at -80 °C prior to being shipped on dry ice to the University of Massachusetts Amherst laboratory for analysis.

PTU Experiment

Pregnant Sprague Dawley rats from set 16 were assigned to two treatment groups: control and PTU-treated (n=8 each). PTU (6-propyl-2-thiouracil, CAS # 51-52-5, product # P3755, lot # BCBF0745V, Sigma-Aldrich, Allentown, PA) was delivered to the animals in their drinking water (3 ppm) from GD 6, as described by Bansal et al. (Bansal et al. 2014). This PTU dose was designed to reduce serum total T₄ by about 80% to serve as a positive control for the BPA experiment. Both control and PTU-treated animals

were gavaged daily with 0.3% CMC from GD 6. One male and one female pup were sacrificed on PND 15 from each dam. Blood was collected to harvest serum and tissues were dissected and stored as described above.

T₄ measurement

Total T₄ was measured in 5 µL of rat serum using a barbital buffer system. Briefly, each assay tube contained 100 µL barbital buffer (0.11 M barbital, pH 8.6; 0.1% wt/vol 8-anilino-1-naphthalenesulfonic acid ammonium salt; 15% bovine γ-globulin Cohn fraction II; 0.1% gelatin), 100 µL anti-T₄ (rabbit, diluted to provide a final concentration of 1:30,000; Sigma, St. Louis, MO), and 100 µL ¹²⁵I-labeled T₄ (Perkin-Elmer/NEN; Boston, MA). Standards were prepared from T₄ (Sigma) measured using a Cahn electrobalance; standards were run in triplicate, whereas samples were run in duplicate. Standards were calibrated to measure serum T₄ levels from 0.2 µg/dL to 25.6 µg/dL. Tubes were incubated at 37°C for 30 min and then chilled on ice for 30 min. Bound counts were precipitated by adding 300 µL ice-cold polyethylene glycol 8000 (20% wt/wt; Sigma). Tubes were centrifuged at 1800 x g for 20 min at 4°C; the supernatant was then aspirated and the pellet counted in a gamma counter (Packard Cobra II; Global Medical Instrumentation, Inc., Albertville, MN). The assay was run at 40–50% binding; nonspecific binding was generally < 8%. 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). The variability within the assay was determined by running 10 replicates of three different standards that represent a low, medium, and high value on the standard curve. The coefficients of variance were 0.9% for 0 ng/mL; 4.7% for 3.2 µg/dL, and 3.8% for 25.6 µg/dL. All experimental samples were evaluated in a single assay.

In Situ Hybridization

We evaluated the effect of PTU or BPA treatment on several endpoints in the developing brain known to be thyroid hormone-dependent. 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 (Guadano-Ferraz et al. 1997) on PND 15. Thus, we measured the relative expression of RC3/Neurogranin mRNA in hippocampus. Likewise, oligodendrocyte number is very sensitive to changes in serum T₄ (Sharlin et al. 2008) and oligodendrocyte number in areas of white matter are correlated with the expression of myelin associated glycoprotein (MAG) (Sharlin et al. 2008); therefore, we measured the relative level of MAG mRNA in corpus callosum and rostral anterior commissure. Finally, low thyroid hormone leads to an upregulation of thyrotropin releasing hormone (TRH) mRNA in the hypophysiotropic TRH neurons in the hypothalamic paraventricular nucleus (Kadar et al. 2010; Koller et al. 1987); therefore, we also measured the expression of this mRNA.

Coronal sections of frozen brain tissues 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 rostro-caudal placement of the section was matched using internal landmarks when slides were chosen for the *in situ* hybridization. RC3/neurogranin in the rostral hippocampus was measured using a 3'-end-labeled 45-base oligonucleotide directed against bp 830–786 of the RC3/neurogranin mRNA (5'-ACC TGT CCA CGCGCCAGCATGCAGCT CTG CCTCCGCAGCCT CGG-3'). The mRNA coding for MAG was detected using a 48-base oligonucleotide directed toward exon 8 of the rat MAG gene (5'-CAG GAT GGA GAC TGT CTC CCC CTC TAC CGC CAC CAC CGT CCC ATT CAC-3'; accession no. X05301, bp 1163–1116). The TRH mRNA was detected using a 48-base oligonucleotide directed toward bases 319-366 (Lechan et al. 1986) (5'-GTCTTTTCTCCTCCTCCCTTTTGCCTGGATGCTGGCGTTTTGTGAT-3'). Oligonucleotides were purchased from IDT DNA (Coralville, IA). End labeling was carried out using terminal transferase (Roche Applied Sciences, Indianapolis, IN) and ³³P-dATP according to the manufacturer's instructions. Two slides from

each brain, four sections total, were thawed at room temperature and hybridized as previously described (Sharlin et al. 2008). 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 as previously described (Zoeller et al. 2000), except that we used a SPOT Insight 2 camera and a Macintosh G5 computer. Film density values of the dentate gyrus and CA1/3 or 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.

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 $-80\text{ }^{\circ}\text{C}$. Two slides per animal were thawed, fixed with 4% formaldehyde, and stained with hematoxylin and eosin (Sigma), dehydrated in ethanol, and coverslipped using Permount. Images were magnified using a SPOT Insight 2 camera equipped with a Nikon macro lens mounted on a bellows and captured using a Scion AG-5 capture board interfaced with NIH 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 NIH image calibrated with a stage micrometer. Four sections were measured from each brain, with a single measurement made for each layer taken in a single section.

Statistical Analysis

The raw, blinded data were uploaded to the CEBS database and locked. These data were then inspected by NCTR and de-coded. After this process, we performed unpaired t-tests on data derived from the PTU experiment, and a One-Way ANOVA for the BPA data. The data were analyzed using Prism 6.0 for Macintosh and post-hoc analysis (when necessary) using Dunnett's multiple comparison to test for significance from controls.

Controlling the Risk of Bias

There are several types of bias – or systematic error – that 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 when they arrived in the laboratory; thus, bias derived from the knowledge of group identity was reduced. Second, all serum samples were evaluated in the same assay; thus, inter-assay 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 another. Likewise, samples from set 3 and set 16 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 were placed against the same film, eliminating the potential error due to film differences.

References:

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