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Developmental PCB exposure induces hypothyroxinemia and sex-specific effects on cerebellum glial protein levels in rats

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ABSTRACT

Polychlorinated biphenyls (PCBs) are persistent lipophilic environmental contaminants which are found in fatty tissues of humans and wild-life alike. Maternal transfer of PCBs to offspring is easily achieved across the placenta and via lactation. In male rats, perinatal PCB exposure induces behavioral abnormalities, in addition to hypothyroxinemia and white matter changes. There are sex differences in white matter volume synthesis and density in adult and aged rodents. Yet whether PCB exposure effects on white matter are sex-specific is unclear, because the previous studies were conducted in male offspring. Furthermore, although hypothyroxinemia induced by PCB exposure is thought to trigger white matter changes, PCBs also affect interleukin-6 (IL-6) expression, and IL-6 regulates white matter growth. We hypothesized that perinatal PCB exposure would have sex-specific effects on white matter development associated with altered IL-6 levels. We found that female offspring had higher levels of myelin basic protein (MBP) than males did, at postnatal day (PND) 7, 18 and 21. PCB exposure induced hypothyroxinemia in males and females at PND7, 14, 21, and 42. PCB exposure also increased MBP and reduced glial fibrillary acidic protein (GFAP) levels in males at PND21, but had the opposite effect in females. In addition, at PND14 and 21, PCB exposure elevated IL-6 levels in male offspring only. The induction of sex-specific changes in white matter proteins, in the absence of sex differences in thyroxine levels after PCB exposure, suggests that serum thyroxine levels do not directly contribute to the white matter alterations. Instead, IL-6 may contribute to increased MBP levels in males, whereas in females estromimetic and thyromimetic PCB metabolites may affect white matter development. This data adds to an increasing body of literature showing that perinatal insults induce sex-specific effects in offspring.

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1. Introduction

Polychlorinated biphenyls (PCBs) are lipophilic environmental contaminants, which were banned in the 1970s, but persist in fatty tissues of wild-life and humans alike (Johnson-Restrepo et al., 2005). Maternal transfer of PCBs is readily achieved via the placenta and also during lactation (Jacobson et al., 1984). In male rats, perinatal PCB exposure induces behavioral hyperactivity, hypothyroxinemia and white matter changes (Holene et al., 1998; Sharlin et al., 2006). Abnormalities in cerebellar white matter tracts are associated with behavioral disorders, and there are sex differences in the activity and density of cerebellar white matter tissues (Berquin et al., 1998; Li et al., 2006; Marin-Husstege et al., 2004; Yang et al., 2008; Volkow et al., 1997; Miller et al., 2010). However, whether PCB exposure induces sex-specific effects on white matter tracts is unclear, because the previous studies were conducted using tissues derived from male offspring (Sharlin et al., 2006). In addition, PCB induced white matter alterations may be attributed to hypothyroxinemia (Sharlin et al., 2008). However, PCBs also affect interleukin-6 synthesis, and IL-6 is a cytokine which not only regulates white matter myelin expression, but is also associated with behavioral disorders (Fujimaki et al., 1997; Valerio et al., 2002; Zhang et al., 2006; Lasky-Su et al., 2008; Neale et al., 2008).

We hypothesized there would be sex-specific effects of PCB exposure on hypothyroxinemia, white matter development and IL-6 levels. To address our hypothesis we exposed developing rats to the Fox River mix of PCBs, which is an environmentally relevant mixture of PCBs similar to those found in fish from the Fox River in Michigan (Wickizer et al., 1981; Kostyniak et al., 2005). We used doses of PCBs similar to those used to induce hypothyroxinemia by other groups (Sharlin et al., 2006), and quantified sera levels of thyroxine, pituitary levels of thyroid stimulating hormone (TSH),

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cerebellum MBP, GFAP density, and sera IL-6 levels in offspring at PND7, 14, 21 and 42. We chose the aforementioned ages in particular, to determine the effects of PCB exposure prior to and after the reported peaks of myelination (PND10–12) in rodents (Sarlieve et al., 2004; Akiyama et al., 2002). In summary, we describe hypothyroxinemia and sex-specific changes in MBP, GFAP and IL-6 after PCB exposure.

2. Experimental procedures

2.1. Animals and choice of doses

We first determined the ontogeny of MBP and GFAP in the cerebellum using a cohort of unexposed offspring from timed pregnant Long-Evans (LE) rats (Taconic Farms). Six male and female pups were sacrificed at PND7, 10, 14, 18, 21 and 42. The effects of PCB exposure were next determined using a second cohort of timed pregnant LE rats. Dams were exposed to one of three dosages of Fox River PCBs: 3, 6 or 18 mg/kg or corn oil, as a control from gestational day (GD) 6 through weaning. The doses we selected were based on those used by Sharlin et al. (2006) to induce hypothyroxinemia and white matter alterations in gestationally exposed LE rats, because we wished to determine if PCB effects on white matter and thyroid hormones was sex-specific. We administered the Fox River PCB mixture by placing a measured amount of PCBs dissolved in corn oil onto a cookie, which was fed to each dam. The volume of the PCB/corn oil mixture was adjusted three times per week based on changes in the dam's weight. 6 dams were used per treatment, yielding an $n = 24$ dams in total. Litters comprised of 8–14 pups: who were cross fostered on PND2 within treatments to maintain equal numbers of male and female pups ($n = 5$ of each) per dam, and to ensure equal lactational exposure to the PCB or corn oil as appropriate. Control and dosed dams were housed in clear Plexiglas cages with stainless steel wire lids in a temperature (21–23 °C) controlled room and maintained in a sterile pathogen free environment, on a 12:12 h light:dark cycle (lights on at 7:00am). Individual male and female rats were sacrificed at PND7, 14, 21, 42, and 90. All procedures were IACUC approved and all experiments were conducted in a blinded fashion.

2.2. Tissue preparation

Rats were sacrificed by CO_2 asphyxiation, followed by decapitation. Brains from exposed and control rats were immediately removed and frozen at −80 °C for use in HPLC or Western blot assays. The pituitary gland was extracted at the time of sacrifice, then homogenized in a lysis buffer, containing PBS buffer (pH 7.2) and NP-40 (Sigma–Aldrich) and a protease inhibitor cocktail (Sigma–Aldrich) and then stored at −80 °C until use in ELISA assays. Serum was extracted from trunk blood collected after decapitation, and stored at −80 °C until use in ELISA assays. Frozen brains were cryo-microdissected on a rostral-caudal gradient into 350 μm thick sections, and micro-punches were taken from the cerebellum at Bregma −6 mm (Paxinos and Watson, 2007). Micro-punches were suspended in 100 μL of lysis buffer and sonicated on ice, prior to use in ELISA and western blot assays. The bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) was used to calculate protein concentration in cerebellar and pituitary tissues. Brains for immunohistochemistry (IHC) were rapidly immersed in 4% paraformaldehyde (PFA) for 24 h at 4 °C followed by a second dissection and immersion step as previously described (Miller et al., 2010). Brain tissue blocks were then embedded in paraffin using a Tissue Tek™ embedder and tissue sections (8 μm) were cut using a rotary microtome prior to being mounted on charged pre-coated slides.

2.3. ELISAs to determine T4, TSH and IL-6 levels

Commercially available thyroxine (T4) (Calbiotech), IL-6 (R&D Biosystems) and TSH (Shibayagi) ELISAs determined serum T4 and IL-6 levels, and pituitary TSH levels. Sample quantities were as follows, for T4 25 μL of sera, for IL-6 75 μL of sera and for TSH 25 μl of pituitary homogenates containing 1 μg/μL of protein were run in triplicate per rat. Samples were run according to kit instructions. To determine the quantity of T4, TSH or IL-6 per sample, plates were loaded with a HRP-conjugated T4, TSH or IL-6 standard, at low to high concentrations supplied with the kit for 1 h RT. The ELISA plates were then incubated with a HRP-TMZ reagent for 20 min. After the substrate color was developed an acidic stopping solution provided with the kit was added to each well, and the mean absorbance of the solution per well was read using a spectrophotometric plate reader at 450 nm within 15 min of stopping the reaction. The concentration of T4, TSH or IL-6 per well was generated by comparing values per sample with the appropriate standard curve. In addition, control samples spiked with T4, IL-6 or TSH standards were used as positive controls in the assay. Data were entered into SPSS v 17 for statistical analysis. For ELISA assays an $n = 3$ animals per group were run in triplicate.

2.4. Western blotting

Cerebellar brain homogenates containing 40 μg of protein were diluted with a 1:1 volume of sample buffer containing 10% (w/v) sodium dodecyl sulfate, 30% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.25% (w/v) bromophenol blue, and Tris Buffered Saline with Tween (TBS-T) (pH 7.2). SDS-PAGE and gel transfer onto Immobilon-P transfer membranes were completed as previously described. Blots were blocked in a 5% fish gelatin solution for 24 h, prior to incubation with antibodies to GFAP (Mouse, Calchiochem 1:250) or MBP (Mouse, Millipore 1:250) in the blocking buffer at 4 °C for 24 h. Gels were also probed with an anti-β actin antibody (Mouse, Sigma 1:10,000) as a loading control. Gels were then incubated with an anti-mouse secondary antibody, prior to incubation in streptavidin coupled to HRP as previously described. The blot was developed by incubating it with ECL (Pierce Biotechnology) for 5–10s and then photographed with a LAS-3000plus gel imaging camera (Fuji). Photomicrographs of blots were analyzed using Image J software to calculate the mean optical density (OD) of the bands in arbitrary units per well per gel (Miller et al., 2010). Individual gels were run per antibody, per age, i.e. PND7 or 14 and per sex, i.e. male or female. For gel analysis an $n = 3$ animals were run per group, and gels were run in duplicates.

2.5. IHC and histology

Tissue sections were cleared in xylene and rehydrated through graded alcohols prior to use. For optimum antigen retrieval, sections were pretreated by boiling in a pH 6.0 citrate buffer using a microwave for 10 min full power. To quench endogenous peroxidase activity, tissue sections were immersed in a 0.3% H_2O_2 solution for 15 min. To block non-specific antigen binding, tissue sections were pre-incubated for 30 min with the appropriate blocking serum (horse/goat) from the Vector ABC kit (Vector Labs, Burlingame, CA). Antibodies (polyclonal or monoclonal) were suspended in PBS and 0.1% BSA, incubated for 1 h RT. Primary antibodies were: mouse anti-GFAP (Calbiochem, San Diego, CA; 1:500) and mouse anti-MBP for myelin (Millipore 1:500). The specificity of each antibody was demonstrated by using serial sections from the same rats incubated with either ascites/serum alone, antibody in the absence of secondary antibody, or a secondary antibody without the primary antibody. DAB was used to visualize immunostained sections. Sections were dehydrated through graded alcohols