



Anxiety-like behaviors in adulthood are altered in male but not female rats exposed to low dosages of polychlorinated biphenyls *in utero*☆



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ABSTRACT

Exposure to polychlorinated biphenyls (PCBs), a class of endocrine-disrupting chemicals, can result in altered reproductive behavior in adulthood, especially when exposure occurs during critical periods of brain sexual differentiation in the fetus. Whether PCBs alter other sexually dimorphic behaviors such as those involved in anxiety is poorly understood. To address this, pregnant rat dams were injected twice, on gestational days 16 and 18, with the weakly estrogenic PCB mixture Aroclor 1221 (A1221) at one of two low dosages (0.5 mg/kg or 1.0 mg/kg, hereafter 1.0 and 0.5), estradiol benzoate (EB; 50 µg/kg) as a positive estrogenic control, or the vehicle (3% DMSO in sesame oil). We also conducted a comprehensive assessment of developmental milestones of the F1 male and female offspring. There were no effects of treatment on sex ratio at birth and age at eye opening. Puberty, assessed by vaginal opening in females and preputial separation in males, was not affected in females but was advanced in males treated with A1221 (1.0). Males and females treated with A1221 (both dosages) were heavier in early adulthood relative to controls. The earliest manifestation of this effect developed in males prior to puberty and in females slightly later, during puberty. Anxiety-like behaviors were tested using the light:dark box and elevated plus maze tests in adulthood. In females, anxiety behaviors were unaffected by treatment. Males treated with A1221 (1.0) showed reduced indices of anxiety and increased activity in the light:dark box but not the elevated plus maze. EB failed to replicate the phenotype produced by A1221 for any of the developmental and behavioral endpoints. Collectively, these results indicate that PCBs increase body weight in both sexes, but their effects on anxiety-like behaviors are specific to males. Furthermore, differences between the results of A1221 and EB suggest that the PCBs are likely acting through mechanisms distinct from their estrogenic activity.

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1. Introduction

Endocrine disrupting chemicals (EDCs) are environmental contaminants that interfere with reproductive, endocrine, and metabolic functions. Fetal exposures to EDCs, even at low dosages, can affect the developmental trajectory of an individual due to the high sensitivity of the developing organism to both natural and synthetic hormones. In the embryonic brain, endogenous gonadal hormones play important roles in neuronal birth, apoptosis, migration, and synaptic connectivity in a sex-specific manner that dictates sex-typical behaviors and functions later in life (Crews et al., 2014; Gore et al., 2015).

Polychlorinated biphenyls (PCB), a class of EDCs, are widespread synthetic organic chlorinated compounds that were used for decades

in industry. When improperly stored or disposed of, PCBs entered the environment in soil, water, and air, and were subsequently incorporated into the food chain. Although banned in 1979 (McFarland and Clarke, 1989), nearly all humans and wildlife have measurable amounts of PCBs in their bodies (Meeker et al., 2011; Quinn et al., 2011).

PCB exposure late in fetal development altered developmental milestones (Dickerson et al., 2011a) and affected adult sexual (Chung et al., 2001; Colciago et al., 2009; Steinberg et al., 2007a, 2008) and social (Reilly et al., 2015) behaviors. Moreover, PCBs changed levels of neurotransmitters and their receptors in the brain, including those involved in anxiety and affective states. In Wistar rats, serotonin metabolites in the prefrontal cortex and hippocampus were increased by exposure to PCBs in late gestation (Morse et al., 1996). Work conducted both *in vivo* and *in vitro* brain slices showed that dopamine synthesis, release, and reuptake were perturbed by PCB treatments (Bemis and Seegal, 1999; Chishti et al., 1996).

Although the literature on links between PCBs and affective behaviors is limited, in male rats, exposure to PCBs during fetal development

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resulted in increased anxiety behavior and hypothalamic-pituitary-adrenal reactivity to stressful events during adolescence (PND 28–35) (Orito et al., 2007). These results are likely translatable to humans, as epidemiological studies associated increased PCB blood concentrations in aged adults with deficits in learning and memory, and increased depressive symptoms (Fitzgerald et al., 2008). Here, we hypothesized that exposure during sensitive periods of fetal development to low dosages of PCBs would alter anxiety-like behaviors in adulthood in a sex-specific manner. We selected a lightly-chlorinated, weakly estrogenic industrial PCB mixture, Aroclor 1221 (A1221), comparing it to estradiol benzoate (EB), and thereby enabling us to determine whether A1221's effects, if any, were similar to those of estradiol. In addition, we sought to determine whether these treatments had more general physiological effects on postnatal developmental landmarks influenced by prenatal hormones and EDCs.

2. Materials and methods

2.1. Animals, husbandry, and PCB treatment

Male and female Sprague Dawley rats were purchased from Harlan (Houston, TX) at 2–3 months of age. All animals were housed in standard rat polycarbonate cages (46 cm × 24 cm × 20.5 cm) with *ad libitum* access to low phytoestrogen rat chow (Harlan, #2019) and tap water, on a 12L:12D cycle (lights off at 1200). Eleven DMSO and EB and ten A1221 (1.0 and 0.5) litters distributed equally across seven cohorts were bred for analysis. All animal work was conducted using humane procedures that were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Following acclimation to the housing facility for 1 week, females (virgins) were tested for receptivity on proestrus. If receptive they were left with a sexually experienced male overnight. The following morning the male was removed and presence of sperm was verified *via* vaginal smear. If sperm was present, embryonic day (E)1 was noted. Pregnant dams were weighed weekly and checked for health but otherwise left undisturbed until E16. On that date, and again on E18, dams were injected *i.p.* with the same treatment given on both days: vehicle (3% dimethyl sulfoxide [DMSO] in sesame oil; Sigma), 0.5 mg/kg A1221 (Accustandard), 1.0 mg/kg A1221, or 50 µg/kg estradiol benzoate (EB), within 2 h of lights off. All treatments used 3% DMSO in sesame oil as the vehicle. EB served as a positive estrogenic control for the weakly estrogenic effects of A1221. PCB concentrations in fetal tissue was not measured, but it has previously been predicted that the dosages used in the current study result in a fetal exposure of approximately 2 µg/kg (Dickerson et al., 2011b; Steinberg et al., 2007b; Takagi et al., 1986), an exposure that is relevant to circulating PCBs in humans (Fitzgerald et al., 2008; Centers for Disease Control, 2009). Nesting material was provided to pregnant dams beginning on E18.

On postnatal day (PND) 1, the day after birth, pups were weighed and their anogenital distance (AGD) was measured using a digital microcaliper. The anogenital index (AGI) was calculated (AGI = AGD divided by the cube root of body weight) as it enables proper sexing of neonates and also serves as an indicator of masculinization or feminization (Vandenbergh and Huggett, 1995). Litters were culled based on AGI. The four males and females with AGIs closest to the median of the same sex and litter were kept to achieve equal litter size and sex ratio. All litters, including controls, were culled to a total of 8 pups (4 male and 4 female) to ensure that sex ratio was equal, because a biased sex ratio changes behavioral outcomes (de Medeiros et al., 2010) and to allow us to attain adequate statistical power based on a power analysis. We note that litters of 8 pups, compared to litters of 10 raised similarly in our lab, are about 10% heavier prior to weaning, but that this levels off after weaning, and treatment effects on body weight are similar in litters of 8 or 10 (Unpublished data). AGD was measured once per week until PND 14. Pups were weaned at PND 21 and were housed with same-sex siblings four per cage until PND 49 and then subdivided to

two per cage. Beginning on PND 28 and PND 35 individuals were checked daily for vaginal opening and preputial separation, respectively. All animals were weighed and handled for 5 min once per week after PND 21. Two males and two females from each litter were randomly selected for behavioral analysis. Dams were euthanized between 1 and 2 weeks after pups were weaned in order to examine the uterus for the number of implantation sites.

2.2. Testosterone radioimmunoassay in males

Trunk blood was collected from males when they were later euthanized at PND 90. Serum was used to determine circulating testosterone according to the manufacturer's protocols (MP Biomedical; Testosterone ¹²⁵I RIA, Catalog #07189102). Littermates that were not behaviorally characterized were included in testosterone analysis. The assay sensitivity was 0.03 ng/ml and the average intra-assay variability was 1.56%. This assay is not sensitive enough to measure serum testosterone in females.

2.3. Behavioral testing

Beginning on PND 60, each animal randomly selected for behavioral testing (2 males and 2 females per litter) was subjected to a battery of 5 behavioral tasks in a randomized order in attempt to minimize the effect of previous tests. Results on tests of sociability and social novelty have been published (Reilly et al., 2015). Tests of sexuality (mate preference and ultrasonic vocalizations) are still under analysis (manuscript under preparation). The tests of anxiety-like behaviors (elevated plus maze and light:dark box) are presented here. We made the decision to publish the 3 categories of behaviors separately as we found that each one told a unique story, and overlapping those stories became unwieldy. Each behavioral task was performed at least 48 h apart and there was no effect of testing order. Estrous cycles of females were monitored daily, but females were tested in the elevated plus maze and light:dark box on a random cycle day. Cycle status was included as a covariate during statistical analysis and confirmed not to affect behavioral outcomes. All behavioral tasks were conducted 1–3 h after lights off, using apparatus and protocols modified from work previously published by our lab (Gillette et al., 2014). All work was done with the experimenter blind to treatment, and data uncoded only after analysis was complete.

2.3.1. Light:dark box

The rectangular light:dark apparatus (Stoelting) was bisected into two chambers, one clear and the other opaque black Plexiglas (each 50 × 50 cm), with a passageway between them. The light field was diffusely illuminated with a 60 w incandescent light bulb ~3 m from the top of the enclosure. The dark field was covered with a black Plexiglas transparent lid that permitted red light to pass, allowing observation and video recording of the animal in the dark field. At the beginning of the light:dark box test, the animal was placed at the entrance to the dark field facing the light field and tracked for 5 min.

2.3.2. Elevated-plus maze

The elevated plus apparatus (Stoelting) was raised 40 cm above the ground. The arms of the plus maze were 10 cm wide and 50 cm long. The two closed arms of the elevated plus maze were surrounded by 40 cm walls. Elevated plus testing was conducted under diffuse red light. Animals were allowed 5 min to acclimate to the testing room and then placed at the center of the cross facing an open arm, and recorded for 5 min.

Behavioral tasks were video recorded and subsequently analyzed with Any-Maze (Stoelting). Between tests, the Plexiglas enclosures were wiped down with 70% ethanol and allowed to air dry for 10 min. Any-Maze recorded the position of each animal 30 times per second. The barrier between a closed arm and open arm or the light field and

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